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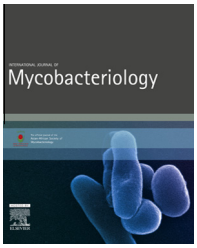

Diagnosis of pediatric pulmonary tuberculosis with special reference to polymerase chain reaction based nucleic acid amplification test

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ABSTRACT

Objective: To determine the utility of polymerase chain reaction (PCR) for diagnosing pediatric pulmonary tuberculosis (PPTB).

Method: A prospective cross-sectional study was carried out on 100 children less than 14 years of age, with strong clinical suspicion and radiological evidence suggestive of pulmonary tuberculosis (TB). Sputum samples/gastric lavage were collected. Direct smears and culture on Lowenstein Jensen (LJ) media were performed. DNA extraction and amplification was performed using Genei™ Amplification Reagent set for *Mycobacterium tuberculosis* (MTB) (by Genei, Bangalore, India). This test is based on the principle of single-tube nested PCR which amplifies the repetitive insertion sequence IS6110.

Results: When compared with culture, sensitivity and specificity of PCR was 93.55% and 92.75%, respectively. The PPV was 85.29% and the NPV was 96.97%. When intention to treat (ITT) was used as the standard, sensitivity, specificity, PPV and NPV of PCR was 47.88%, 93.1%, 94.4%, and 42.19%, respectively, and that of culture was 40.85%, 100%, 100% and 40.85%, respectively. Against response to treatment (RTT), PCR demonstrated sensitivity, specificity, PPV and NPV of 50.9%, 93.1%, 93.33% and 50%, respectively, and for culture it was 43.64%, 100%, 100% and 48.33%, respectively.

Conclusion/recommendation: The present study reinforces better case detection rate with PCR-based nucleic acid amplification test as compared with microscopy and culture in pediatric pulmonary TB. PCR showed a higher correlation with clinical diagnosis as compared with microscopy and solid culture. Hence, a molecular platform should be the test of choice for detecting PPTB.

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Introduction

Pediatric pulmonary tuberculosis (PPTB) remains a major cause of morbidity and mortality worldwide, particularly in developing countries [1]. In India, it is estimated that 5–14% of tuberculosis [TB] cases occur in children [2], and in Mumbai, the incidence was 9% in 2010 [3].

A major challenge to PPTB is establishing an accurate diagnosis. Children less than 5 years rarely expectorate sputum for evaluation, and the disease is paucibacillary with a smear positivity rate of <15% and a culture positivity rate of 30–40% [4]. Gastric lavage and induced sputum only marginally improve sensitivity [5]. Rapid culture systems with their improved yield and reduced turnaround time still require a period of 10 days in most cases and may not be cost effective [6]. Most cases are diagnosed based on clinical presentations, radiographic abnormalities, contact history and tuberculin skin test results, all of which suffer from low specificity [7].

The development of polymerase chain reaction (PCR)-based assays for the detection of mycobacterial DNA in clinical specimens has proved to be a diagnostic boon. The role of nucleic acid amplification tests (NAAT) has been well established worldwide [8]. Most NAAT assays use the mycobacterial insertion element IS6110 as the target for detecting *Mycobacterium tuberculosis* (MTB) complex organisms. The reported sensitivity in pediatric pulmonary specimens ranges from 95% to 100% in culture-positive cases and 50% to 60% in culture-negative cases [9]. For resource-constrained settings, the limitations of NAATs include the expensive equipment, the high cost per test and the need to obtain multiple specimens in smear-negative patients to improve sensitivity. Hence, there is an urgent need for a rapid, reliable and affordable diagnostic test.

This study was carried out to determine the utility of a PCR-based NAAT for diagnosing PPTB.

Materials and methods

Institutional ethics committee permission was obtained prior to commencing the study. A prospective, cross-sectional study was carried out on 100 children less than 14 years of age, with strong clinical suspicion of pulmonary tuberculosis (PTB), radiological evidence suggestive of PTB and whose parents/guardians provided written informed consent. Symptoms that were likely to suggest a diagnosis of PTB included: cough $\geqslant$2 weeks' duration and/or weight loss of at least 10% of healthy body weight or no weight gain in 3 months and/or fever for $\geqslant$2 weeks or one measured temperature above 38.5 °C [10]. Children who were on anti-TB therapy (ATT) for pulmonary/extra-pulmonary TB or those who had received ATT within the last 6 months were excluded from the study.

From children older than 5 years of age and who could expectorate, two sputum specimens (as per the Revised National Tuberculosis Control Programme, RNTCP, protocol) were collected in sterile, wide-mouthed, screw-capped containers [11]. In younger children and those who could not expectorate, gastric lavage (GL) was collected by the pediatrician on 3 consecutive days in sterile, wide-mouthed, screw-capped containers. The pH was immediately adjusted to neutral with sodium bicarbonate [12].

Microbiology workup

All processing was carried out in Biological Safety Cabinet (BSC) Type II, and level 2 biosafety practices were followed.

Microscopy

Direct smears were prepared from each of the specimens and stained by Ziehl Neelsen method. The results were read and recorded as per RNTCP protocol. Irrespective of the number of sputum or GL specimens submitted per case, detection of acid fast bacilli (AFB) in any one or all of the specimens submitted was considered as positive for that child.

Isolation and identification of MTB complex

All specimens were decontaminated with N-acetyl-L-Cysteine (NALC) NaOH method and concentrated by centrifuging at 3000*g* for 15 min [13]. Two loopsful of the sediment were inoculated on two Lowenstein Jensen (LJ) media. One was incubated aerobically at 37 °C and the other at room temperature (25 °C) [13]. All cultures were read daily for the first week for detecting contamination or rapid growers and then weekly thereafter for eight weeks or until growth was detected, whichever was later. If mycobacterial growth was detected in any one or all of the two/three specimens submitted, it was considered as positive for that child. The isolates were identified as MTBC using SD BIOLINE TB Ag MPT 64 Rapid® immunochromatography assay after confirming their acid-fast nature [14].

NAAT (PCR based)

DNA extraction and amplification was performed using Genei™ Amplification Reagent set for MTB (by Genei, Bangalore, India) as per manufacturer's instructions with minimal modifications [15]. The samples were decontaminated with NALC–NaOH instead of using modified Petroff's method, and the pellets of all the specimens of a single patient were pooled so as to get a single pellet on which NAAT was performed. 3 μl of a DNA sample was added to 9 μl of the first amplification premix. This test is based on the principle of a single-tube-nested PCR which amplifies the repetitive insertion sequence IS6110. It is a two-step sequential assay. In the first step, the IS region of the MTB complex DNA sequence, a 220 bp, is amplified by specific external primers.

The external primer sequence was as follows:

695 to 724 (5′-CGGGACCACCCGCGGCAAAGCCCGCAGGAC-3′) and
885 to 914 (5′-CATCGTGGAAGCGACCCGCCAGCCCAGGAT-3′)

The first amplification reaction profile consisted of a cycle of an initial denaturation step at 22 °C for 10 min and 94 °C for 5 min, followed by 20 cycles at 94 °C for 30 s (denaturation),

68 °C for 1 min (annealing) and 72 °C for 1 min (extension), and a final extension cycle of 70 °C for 7 min. The vials were removed from the thermal cycler when samples reached 4 °C and then centrifuged at 10,000 rpm for 30 s before opening to avoid aerosol contamination.

After the first PCR cycling was completed, 15 μl of the second amplification master-mix was added to each tube and the second NAAT was performed. The inner primers were used to further amplify a 123 bp amplification product.

The inner primer sequence was as follows:

Primer IS1 [5′-CCTGCGAGCGTAGGCGTCGG-3′] and
Primer IS2 [5′-CTCGTCCAGCGCCGCTTCGG-3′]

The cycling parameters consisted of an initial cycle at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s (denaturation), 68 °C for 30 s (annealing) and 72 °C for 30 s (extension), and a cycle of final extension at 72 °C for 7 min. The kit also includes an internal control DNA. The amplified products were analyzed using gel electrophoresis. A band of 340 bp is the amplification product of the internal control DNA. An amplification product of size 123 bp is indicative of the presence of MTBC. Results were considered as positive if there was a band both at 123 bp and 340 bp or a band only at 123 bp. The presence of only one band at 340 bp was considered negative. The DNA extraction was to be repeated if there was no band at all as it indicated either that the sample contained inhibitors or the DNA extraction had failed. Reprocessing of such samples was to be carried out by repeating the extraction procedure with 100 μl of the DNA.

Data recording

Clinical details, radiological findings and relevant laboratory investigations, where available, and the treatment initiated were recorded. Contact number of the patient's caregiver was also recorded for follow-up. Each child was followed up and the response to treatment (RTT) was noted at the end of 2 weeks for those receiving antibiotics only and at 6 months for those receiving anti-tuberculosis treatment (ATT). A probable case, confirmed case, cured, treatment completed, death and treatment failure were defined as er RNTCP guidelines [16].

Statistical analysis

100 clinically suspected PPTB cases were included. Data was entered in the SPSS version 15.0. Performance of PCR-based NAAT was reported in terms of sensitivity and specificity at 95% confidence interval. Three reference standards were used for comparison; intention to treat (ITT), response to ATT and culture positivity.

Results (Table 1)

Of 100 consecutive, clinically suspected PPTB cases included in the study, 36 were less than 5 years of age. Gender ratio was observed to be equal. GL was obtained from 41 children and expectorated sputum from 59 children.

Of the 241 specimens (123 GL and 118 sputa) tested by microscopy and culture, and 100 pooled specimens from an equal number of suspect children tested by PCR, PCR was positive in 36 children, culture was positive in 31 and microscopy was positive in 13. In clinically suspected cases of PPTB, the sensitivity of NAAT, culture and microscopy was 36%, 31% and 13%, respectively. All the isolates were identified as MTBC by "SD Bioline MPT 64 Rapid" test.

Concordant results by PCR-NAAT and culture (Table 1) were observed in 93 children with 29 testing positive both by culture and PCR and 64 testing negative by both. Discordance was observed in 7 of which 5 were PCR-positive and culture-negative and 2 were PCR-negative and culture-positive. When compared with culture, sensitivity and specificity of PCR-NAAT was 93.55% (95% CI: 78.54–99.02%) and 92.75% (95% CI: 83.88–97.58%), respectively. The PPV was 85.29% (95% CI: 68.93–94.99%) and NPV was 96.97% (95% CI: 89.46–99.54%).

Of the 100 children enrolled in the study, 29 received only antibiotics (Amoxicillin or Cloxacillin and/or Amikacin) and responded. Of these, none were acid-fast microscopy positive or culture positive. However, 2 of these were NAAT positive. 71 children received ATT; NAAT was positive in 34 of these cases, while culture was positive in 31 and microscopy in 13. When ITT was used as the standard, NAAT had a sensitivity, specificity, PPV and NPV of 47.88% (95% CI: 35.88–60.08%), 93.1% (95% CI: 77.19–98.95%), 94.4% (95% CI: 81.33–99.16%), and 42.19% (95% CI: 29.94–55.18%). All that were positive by microscopy were also positive by culture and PCR. The sensitivity, specificity, PPV and NPV of culture against ITT was 40.85% (95% CI: 29.32–53.16%), 100% (95% CI: 87.94–100%), 100% (95% CI: 87.94–100%) and 40.85% (95% CI: 29.32–53.16%), respectively.

Of the 71 children initiated on ATT, 5 were lost to follow-up and excluded from analysis. Of the remaining 66, treatment completion was achieved in 55 (83.3%). Eleven children had an adverse outcome of which 10 died during treatment and 1 was a treatment failure with culture-positive result. PCR was positive in 28 and culture in 24 of the 55 patients who

Table 1 – Comparison of Microscopy, culture and NAAT.

	Culture positive (n = 31)	Culture negative (n = 69)	Total (n = 100)	Sensitivity (%)	Specificity (%)
Microscopy					
Positive	13	0	13	41.9	100
Negative	18	69	87		
NAAT					
Positive	29	5	34	93.55	92.75
Negative	2	64	66		

completed their treatment and responded. Against response to treatment, NAAT demonstrated a sensitivity, specificity, PPV and NPV of 50.9% (95% CI: 37.07–64.64%), 93.1% (95% CI: 77.19–98.95%), 93.33% (95% CI: 77.89–98.99%) and 50% (95% CI: 36.08–63.92%), respectively. Culture demonstrated a sensitivity, specificity, PPV and NPV of 43.64% (95% CI: 30.31–57.68%), 100% (95% CI: 87.94–100%), 100% (95% CI: 85.62–100%) and 48.33% (95% CI: 35.23–61.6%), respectively.

Discussion

The conventional methods of diagnosis which include acid-fast stained microscopy and culture perform poorly in PPTB. In the present study on 100 children with clinical presentation strongly suggestive of TB, performance of PCR-based NAAT has been evaluated against culture on LJ medium, intention to treat with ATT and RTT. This is one of the few studies which included ITT and RTT as standards for defining performance of the different diagnostic tests.

The results observed in the present study reinforce previous published data with reference to sensitivity, specificity and rapidity of PCR-based assays [17–19]. In those 71 children who received ATT, the sensitivity of PCR, culture and microscopy was 47.8%, 43.6% and 18.3%, respectively. Higher sensitivities have been reported in literature [20–22]. However, these have compared the performance of PCR against culture. The higher sensitivity of any NAAT can be attributed to the technology itself which amplifies the original load of DNA more than a million times and in the present study, the use of LJ medium instead of the recommended liquid culture systems (MGIT 960). PCR was positive in all 13 cases which were smear-positive. In addition, PCR was also positive in 21 cases which were smearnegative and 3 cases which were culture-negative. In smear-negative cases, PCR is reported to have a lower sensitivity as compared with liquid culture [23–25].

PCR was negative in two smear-negative but culture-positive cases. False negative NAAT may be due to the presence of inhibitors not detected by the control amplification or non-homogenous distribution of bacteria in the specimen so that the fraction tested does not contain mycobacteria or a low number of bacilli in the specimen. Both these reasons do not apply to the present study. The following precautions were taken to avoid false negative results. A recombinant plasmid DNA was provided as an internal control during the DNA extraction procedure to validate extraction protocol. Absence of an internal control band at 340 bp indicated either the presence of inhibitors or the failure of DNA extraction, and the process of DNA extraction was to be repeated as per manufacturer's instructions. This was not observed in any of the runs. All specimens were homogenized and concentrated before processing to improve the quality of specimen and to concentrate the bacillary load.

Five children were culture-negative and PCR-NAAT positive. Three of these were initiated on ATT and showed symptomatic improvement. Hence, these were considered as cases of TB. The remaining 2 children had a history of TB one year prior. These children, however, responded to antibiotic treatment and PCR may be false-positive. False-positive NAATs can be due to multiple reasons. Carryover of amplicons from previous reactions is considered a major limitation of PCR-based assays. To avoid false positivity arising from cross-contamination, DNA extraction and NAAT were done in two separate laboratory areas away from the culture laboratory. PCR workstation and dedicated equipment were used for reagent preparation. Disposable plastic ware and filter-blocked tips were used, and frequent changing of gloves was practiced. Uracil DNA glycosylase (UDG) was added to the master mix to minimize previous amplicon contamination and not more than five samples were processed in a day. The other reason for false positivity could be the amplification of DNA from dead bacilli in the respiratory secretions following successful ATT. Since DNA-based NAATs can also detect dead bacilli, the results of NAAT should be used in conjunction with clinical presentation and the pretest probabilities.

In the present study, culture positivity was 43.66% (31/71). Studies from India have reported culture positivity ranging from 32% to 37.7% [26–28]. A higher culture positivity has been reported by Petrovi et al. (Serbia, 2005, 43%) [29] and Jeena et al. (South Africa, 2002, 88%) [30]. Higher rates in these studies have been attributed to the use of a combination of induced sputum and gastric lavage as specimens, as well as the use of a combination of one solid and one liquid medium. Though culture is highly specific for the diagnosis of TB, it is relatively insensitive for pediatric TB when evaluated against the clinical reference standard [31,32].

The comparatively lower yield with culture may be attributed to the use of only solid medium for culture. Liquid culture systems with continuous monitoring for mycobacterial growth (such as MB/BacT) and mycobacterial growth indicator tube (MGIT) provide a significantly higher yield than solid culture and in half the time [6]. Microscopic observation drug susceptibility (MODS) is a potentially low-cost alternative to MGIT with comparable sensitivity [33].

In the present study, microscopy could detect only 18.3% (13/71) of cases. The yield of acid-fast microscopy of respiratory samples in children has been found to be low, ranging from 12% to 17.2% [26–28]. The inherent reasons for the low sensitivity of microscopy in children has been attributed to the paucibacillary nature of the disease and to the fact that often the child lacks post-tussive expulsive force to bring out a good quality sputum.

Excluding drop-outs and deaths, successful treatment outcome was achieved in a total of 55 out of 66 children initiated on ATT (83.3%). Though there is a wide margin for improving treatment outcome, this would need an early diagnosis and require the use of more accurate and rapid tests.

A mortality of 14.1% (10/71) was observed in the present study. Oeltmann et al. reported 10.5% mortality (Botswana, 2008, 10.5%) [34], and Harries et al. reported 17% (Malawi, 2002) [35]. Death in young children can be attributed to the rapid progression and dissemination of the disease.

Children usually acquire TB from adults. Thus, TB in children reflects the ongoing transmission in the community and indirectly reflects on the performance of national programs. This emphasizes the need for early and accurate diagnosis of the disease in children to benefit both the affected child and the community. For a long time, RNTCP has emphasized acid-fast microscopy for the diagnosis of PTB. The present

study, like many before, reinforces the poor sensitivity of microscopy and a better case detection rate with PCR-based NAAT. Recent standards on TB care recommend that in all children suspected of having pulmonary TB, microbiological confirmation should be sought through examination of a respiratory specimen for smear microscopy, Xpert MTB/RIF assay and/or culture [36]. The GeneXpert MTB/RIF assay with its simple procedure, rapid results, decreased turnaround time and almost no expertise needed, is expectedly a better option for detecting pediatric TB. The same is now recommended by RNTCP [37].

This study has some limitations. The collection of specimens were dependent on the pediatrician. Thus, there was no control over the quality of the specimens and the transport to the laboratory. Smear preparation after concentration of the specimen and the use of fluorescent/LED microscopy could have increased smear positivity. The use of liquid culture systems might have improved the culture yield.

All the three test modalities used in the present study yielded sensitivity lower than that observed in adults. PCR-NAAT showed a higher correlation with clinical diagnosis as compared with microscopy and solid culture. Hence, a molecular platform should be the test of choice for detecting PPTB. However, culture would still be required for effective case management.

Conflict of interest

None declared.

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