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Original Research Article

A Hospital Based Cross-Sectional Study Assessing Role of PCR in Diagnosing Tb in Children

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Abstract

Aim: The aim of the present study was to assess the role of PCR in diagnosing TB in children.

Methods: This cross-sectional study was carried out in the Department of Pediatrics, SRIMS and Sanaka Hospital, Durgapur, West Bengal, India and included 100 patients aged <15 years who were clinically diagnosed with TB.

Results: The median (IQR) age of enrolled TB patients (N = 100) was 1.7 (0.75, 3.0) years. Most of them (88%, 88/100) belonged to the <5 years age group; only five (5%) were 10 years old. Fifty-eight percent of participants were male (58/100). The majority (90%, 90/100) had a TBS of 7 according to the TBS chart, and 45% (45/100) had a score of 5 according to the MKJ scoring system. Gastric lavage was collected from 96% of the participants, including all of the children <5 years of age and seven of the 11 children >5 years of age who were unable to produce a spontaneous sputum specimen. Pathogen detection in the children with clinically diagnosed TB, according to the age group and type of respiratory sample. Smear for AFB microscopy and conventional (solid) culture were performed on all respiratory samples collected (N = 100). MGIT culture was performed on 100 samples. Smear microscopy was positive only in three cases and all of them were from gastric lavage. All smear-positive cases were positive by both culture methods. The newer sensitive PCR technique using IS6110 primers was positive for the children and similar positivity was found among younger children.

Conclusion: In conclusion, this study demonstrated that the PCR method using IS6110 primers might have greater importance when compared to the performance of smear microscopy in the detection of MTB among younger and nutritionally compromised children, for whom bacteriological confirmation can rarely be achieved. It might also be beneficial in detecting more pathogens within a shorter period of time when compared to the gold standard culture method.

Keywords: PCR, TB, children, diagnosis

Introduction

Paediatric tuberculosis (PaeTB) diagnosis presents a major challenge. [1] Tuberculosis (TB), an infectious disease caused by the bacillus Mycobacterium tuberculosis (MTB), is spread from person to person predominantly through an airborne route and remains a major global health problem as it causes ill-health among millions of people. [2] TB is the leading cause of death from a single infectious agent (ranking above HIV/AIDS) and about a quarter of the world's population is infected with MTB. [3] According to the World Health Organization, globally, an estimated 10.0 million (range, 8.9–11.0 million) people fell ill with TB in 2019, a number that has been declining very slowly in recent years. However, World Health Organization (WHO) estimates that annually, 1.2 million children have TB disease and many more harbor a latent form of infection. [3] The disease typically affects the lungs (pulmonary TB) but can also affect other sites (extra-pulmonary TB).

Microbiological confirmation of pulmonary tuberculosis (TB) in children remains challenging despite recent advances. [4] Collection of specimens

for testing, such as induced sputum (IS) or gastric aspirate, is perceived to be invasive and complex. Even when such specimens are obtained and tested, the diagnostic yield can be suboptimal, with microbiological confirmation achieved in only half of children diagnosed with pulmonary TB, even in well-resourced research studies. [5] Improved microbiological confirmation is important, not only for clinical care but also because assessment of novel diagnostic biomarkers for TB in children is severely constrained by the lack of a sensitive reference standard test.

Pediatric tuberculosis diagnosis is impeded by difficulty obtaining sputum samples in children and the paucibacillary nature of their disease that often necessitates invasive procedures such as gastric aspiration or bronchoscopy [6] or reliance on unreliable clinical scoring systems. [7] The important issues and challenges in pediatric tuberculosis differ markedly between developed and developing countries. In more developed countries such as the United States, rates of tuberculin skin test reactivity in the general population are low, so the tuberculin skin test is a useful diagnostic test for tuberculosis. In contrast, in many developing countries interpretation of the tuberculin skin test for diagnosis of pulmonary tuberculosis is less reliable. [8,9] The diagnostic yield of gastric aspiration ranges from 20% to 40%. [6,10] Although children and infants may be unable to expectorate sputum samples, most sputum is swallowed, [10] and tuberculosis DNA may remain intact after intestinal transit.

The aim of the present study was to assess the role of PCR in diagnosing TB in children.

Materials and Methods

This cross-sectional study was carried out in the Department of Pediatrics, SRIMS and Sanaka Hospital, Durgapur, West Bengal, India for one year and included 100 patients aged <15 years who were clinically diagnosed with TB. These patients were admitted for acute illnesses such as diarrhoea, dysentery, pneumonia, sepsis, or malnutrition, and had one or more of the criteria suggestive of TB: prolonged cough (for >2 weeks), prolonged low-grade fever (for >2 weeks), persistent loss of appetite, weight loss and failure to thrive, pneumonia not responding to conventional therapy with broad-spectrum anti- biotics, abnormal findings on CXR not commensurate with clinical condition and therapy, weight gain <5 g/kg/day, and history of contact with TB patients in the family.

TB treatment

Anti-TB treatment was started free of charge once the TB diagnosis was made. Children with acute phase TB were treated on the general ward or in the special care unit of the hospital. After recovery from the acute phase of severe malnutrition, the children were transferred to the Nutritional Rehabilitation Unit (NRU) of this hospital for catch-up growth.

Clinical scoring systems

Modified Kenneth Jones (MKJ) scoring system

This scoring system is based upon criteria that include age and nutritional status of the child, non-response to therapy (absence of weight gain during nutritional rehabilitation and/or pneumonia not responding to conventional treatment), prior bacille Calmette– Guérin (BCG) vaccination, history of TB exposure, size of the skin induration after TST, and radiological evidence of hilar lymphade- nopathy. Each criterion carries a particular score, and a total score of 5 or more is suggestive of TB. [11]

TB score chart (TBS)

This score chart is based on the work of Dr K. Edwards in Papua New Guinea. [12] It does not rely on CXR findings or biopsy. Furthermore, it appears to be simpler than the MKJ score and is believed to be a rapid, easy, and inexpensive method. The TBS considers 11 different features and each has its own score. If the total score is 7 or more and there is no other likely diagnosis, the child is treated for TB.

Tuberculin skin test (TST)

All children in this study were given intradermal injections of 5 TU (0.1 ml) of purified protein derivative (PPD; Statens Serum Institute, Denmark) on the volar aspect of their forearm and the size of the induration was read after 72 h. The test was considered 'positive' when the induration measured was >10 mm after 72 h and 'doubtful' when it was 5–9 mm. However, in severely malnourished children, an induration >5 mm was considered positive. The test result was also used to calculate the MKJ scores for TB diagnosis.

Sample collection and laboratory investigations

Sample collection

Early morning sputum specimens were collected from the older children (5 years old) in 50-ml conical centrifuge tubes. Gastric lavage samples were collected in 15-ml conical centrifuge tubes and sent to the laboratory for further processing as soon as possible after collection.

AFB smear microscopy

The gastric lavage samples were checked for solid matter and initially centrifuged at 3000 g for 20 min to concentrate the specimens. Gastric lavage and sputum specimens were then digested and decontaminated using an equal volume of 0.5% N-acetyl-L-cysteine (NALC) with 2% sodium hydroxide (NaOH) and 1.45% sodium citrate solution, according to a procedure described previously (Petroff, 1915). [13] The contents of the

tubes were then mixed by vortexing and incubated at room temperature for 15 min. Phosphate-buffered saline (PBS, pH 6.8) was then added to make the volume up to 45 ml and the mixture centrifuged at 3000 g for 15 min. The supernatant was discarded carefully and the sediment resuspended in 1.5 ml of PBS; this suspension was then used to prepare the smear. The prepared slides were air-dried, heat-fixed, and stained using the Ziehl–Neelsen staining technique. The stained slides were examined under a light microscope using a 1000 immersion oil objective lens and scored as AFB-negative or positive following the standard procedure (Uddin et al., 2013). [14] The remaining pellets were used for culture and DNA extraction.

Culture on Solid (LJ) and Liquid (MGIT) Media

The manual MGIT detection system was used according to the manufacturer's guidelines (BD Diagnostic Systems, USA). The MGIT tube itself contained 4.0 ml of 7H9 broth medium. Under aseptic conditions, 0.5 ml of OADC (oleic acid, albumin, dextrose, and catalase) and 0.1 ml of PANTA antibiotic mixture was added. Processed samples (0.5 ml) were inoculated into the appropriately labelled MGIT tube, mixed by inverting, and then incubated at 37 ◦C for bacterial growth. At the same time, an unopened, uninoculated MGIT tube was used as the negative control and a MGIT tube containing 0.4% sodium sulphate solution was used as positive control. These MGIT tubes were monitored every morning under ultraviolet light. Positive fluorescence, indicating growth and manifested by a bright orange colour at the bottom of the tube, was detected by comparison to the growth control. The positive specimens were confirmed by AFB microscopy. All specimens inoculated into LJ medium were incubated at 37 ◦C for 6–8 weeks in a vertical position for the better development of individual colonies. The sample was considered positive when small and buff-coloured colonies grew on LJ medium. Contaminated cultures were discarded.

PCR Amplification

A portion of the processed sputum sample (500 ml) was centrifuged at 10 621 × g for 5 min and the pellet was resuspended in 200 ml distilled water and incubated at 85 ◦C for 30 min. After cooling, the samples were centrifuged again at 10 621 g for 5 min. The supernatant was used for PCR analysis. Each DNA sample was used for PCR amplification using the IS6110 primer set. The primer sequences used were as follows: forward primer 50 - CCTGCGAGCG- TAGGCGTCGG-30 and reverse primer 50 -CTCGTCCAGCGCCG CTTCGG-30 (Eisenach et al., 1990; Noordhoek et al., 1994). [15,16] The PCR amplification reaction was performed in a total volume of 25.0 ml. The reaction mixture consisted of 5.0 ml of 5x Green PCR buffer (Promega, Madison, WI, USA), 0.2 mM of each forward and reverse primer, 0.2 mM of dNTP mixture, 2 mM MgCl2, 0.425 U of Taq polymerase, and 5.0 ml of template DNA. The PCR reaction was performed at 94 ◦C for 5 min, followed by 32 cycles at 94 ◦C for 45 s, at 68 ◦C for 45 s, at 72 ◦C for 2 min, and a final extension at 72 ◦C for 5 min. Amplified products (123 bp) were identified by agarose gel (1.5%) electrophoresis and images were taken with the Gel Doc XR System (Bio-Rad).

Data Analysis

All data were entered into IBM SPSS Statistics version 20.0 software (IBM Corp., Armonk, NY, USA). Differences in proportions were compared using the Chi-square test. The interquartile range (IQR) was reported for asymmetrically distributed data. The sensitivity, specificity, PPV, and NPV of the PCR technique were calculated in comparison to the culture methods and expressed as percentages with 95% confidence intervals (CI).

Results

Table 1: Demographic characteristics and treatment outcomes of child TB patients

Category	Subcategories	Number (%) of respondents
Demographic information Age, years, median (IQR)		1.7 (0.75, 3.0)
Sex	Male	58 (58)
	Female	42 (42)
Age, years	<5	88 (88)
	5–9	7 (7)
	≥10	5 (5)
Clinical score TB score chart (TBS)	Positive (score ≥7) Negative (score<7)	90 (90) 10 (10)
Modified Kenneth Jones score (MKJ)	Positive (score ≥5) Negative (score<5)	45 (45) 55 (55)
Patient outcomes with TB treatment	Completely cured Treatment defaulter	85 (85) 7 (7)
	Death	4 (4)
	Lost to follow up	4 (4)

The median (IQR) age of enrolled TB patients (N = 100) was 1.7 (0.75, 3.0) years. Most of them (88%, 88/100) belonged to the <5 years age group; only five (5%) were 10 years old. Fifty-eight percent of participants were male (58/100). The majority (90%, 90/100) had a TBS of 7 according to the TBS chart, and 45% (45/100) had a score of 5 according to the MKJ scoring system.

Table 2: Results of the laboratory investigations on the specimens from the children with tuberculosis, according to age group

Age group in years (*n*)	Samples			Laboratory tests	Positive in any sample	Gastric lavage positive	Sputum positive
	Total (%)	Gastric lavage	Sputum				
<5 (91)	88 (88)	88	0	Smear microscopy	3	3	–
				IS*6110* PCR	55	55	–
				Conventional culture	12	12	–
				MGIT culture	11	11	–
5–9 (6)	7 (7)	4	3	Smear microscopy	1	1	–
				IS*6110* PCR	3	2	1
				Conventional culture	2	2	–
				MGIT culture	2	2	–
≥10 (5)	5 (5)	3	2	Smear microscopy IS*6110* PCR	0 2	– 1	– 1
				Conventional culture MGIT culture	2 1	1 1	1 –

Gastric lavage was collected from 96% of the participants, including all of the children <5 years of age and seven of the 11 children >5 years of age who were unable to produce a spontaneous sputum specimen. Pathogen detection in the children with clinically diagnosed TB, according to the age group and type of respiratory sample. Smear for AFB microscopy and conventional (solid) culture were performed on all respiratory samples collected (N = 100). MGIT culture was performed on 100 samples. Smear microscopy was positive only in three cases and all of them were from gastric lavage. All smear-positive cases were positive by both culture methods. The newer sensitive PCR technique using IS6110 primers was positive for the children and similar positivity was found among younger children.

Discussion

In 2015, an estimated one million new TB cases (10% of the total TB incidence) occurred among children (<15 years old), however the actual percentage could be up to 15–40% in high TB burden countries. [17] Reasons for under-reporting might include the lack of reporting of TB as a cause of death in HIV and/or pneumonia-related deaths [18], lack of confirmed TB diagnosis in children due to the inadequacy of sputum in younger children [19], lack of systematic screening guidelines, and lack of referral of children with suspected TB. [20] Although alternative samples such as gastric lavage or induced sputum are available, standard smear microscopy and the gold standard culture often become negative due to the paucibacillary nature of TB disease in young children, despite this group being vulnerable to developing severe forms of TB. [21,22] Latent tuberculosis infection (LTBI) is defined as a state of persistent immune response to stimulation by MTB antigens with no evidence of clinically manifest active TB. [23] It is estimated that the lifetime risk of an individual with LTBI for progression to active TB disease is 5–10% over their lifetime. [24] This risk is particularly high among children under the age of 5 years. [25]

Tuberculin skin test (TST) or interferon-gamma release assay (IGRA) can be used to test for LTBI, as there is no 'gold standard' test for LTBI. [26] Establishing an accurate diagnosis of PaeTB in children can be more difficult than adult TB, because of the challenge children have in expectorating good-quality sputum or absence of lung parenchymal disease as in primary complex. [27] In children, culture methods have a greater, yet highly variable, sensitivity which improve diagnosis but takes between 2 and 8 weeks in most cases and the sputum sample lacks representative of lower respiratory tract. [28] Other diagnostic approaches are based on clinical presentations, imperfect tools such as radiology which is subject to inter-observer variability to detect radiographic abnormalities, contact history, and tuberculin skin test, all of which are of low specificity. [29]

The median (IQR) age of enrolled TB patients (N = 100) was 1.7 (0.75, 3.0) years. Most of them (88%, 88/100) belonged to the <5 years age group; only five (5%) were 10 years old. Fifty-eight percent of participants were male (58/100). The majority (90%, 90/100) had a TBS of 7 according to the TBS chart, and 45% (45/100) had a score of 5 according to the MKJ scoring system. Gastric lavage was collected from 96% of the participants, including all of the children <5 years of age and seven of the 11 children >5 years of age who were unable to produce a spontaneous sputum specimen. Pathogen detection in the children with clinically diagnosed TB, according to the age group and type of respiratory sample. As data regarding the diagnosis of child TB are scarce, it is very difficult to determine the reason for the lower positivity of smear microscopy found in the present study compared to those found in previous studies. However, the greater positivity in the two previous studies might be due to the collection of gastric lavage and induced sputum specimens, whereas the present study used gastric lavage and sputum: induced sputum has been reported as a safe technique with better diagnostic yield for TB compared to other respiratory specimens. [30] Moreover, both previous studies had a large sample size. On the other hand, most of the present study population were younger (<5 years old) and most were severely malnourished, and the detection of TB in severely malnourished children by smear microscopy is well known to be low due to the paucibacillary nature of MTB in this age group. [18]

Smear for AFB microscopy and conventional (solid) culture were performed on all respiratory samples collected (N = 100). MGIT culture was performed on 100 samples. Smear microscopy was positive only in three cases and all of them were from gastric lavage. All smear-positive cases were positive by both culture methods. The newer sensitive PCR technique using IS6110 primers was positive for the children and similar positivity was found among younger children. MGIT culture was positive in 14% of children, but data regarding this test among children are scarce. Different previous studies have reported culture positivity in 20–50% of children with TB. [31,32] In a study conducted by Zar et al [33], culture was positive for 23% of the children in the same age group with TB. Berggren et al [31] also found this test to be positive in 52% of child TB patients. The present study results are dissimilar to the findings of these previous studies. The reasons for the difference may be the same as those for the lower positivity rate of smear microscopy in the present study as mentioned above. This study found almost equal positivity of solid culture (recommended as the gold standard tool) and MGIT culture (16% vs. 14%) among the children with TB. Liquid culture might be helpful to isolate MTB in children, but this needs to be validated through application on a large scale.

Conclusion

In conclusion, this study demonstrated that the PCR method using IS6110 primers might have greater importance when compared to the performance of smear microscopy in the detection of MTB among younger and nutritionally compromised children, for whom bacteriological confirmation can rarely be achieved. It might also be beneficial in detecting more pathogens within a shorter period of time when compared to the gold standard culture method. This can be applied as a rapid screening test for paediatric TB. However, further evaluation of the diagnostic efficacy of this rapid and useful technique by applying it in a larger scale study is required, and this may help to improve TB detection among children, including those in the younger age groups with and without severe malnutrition.

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