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RESEARCH ARTICLE

USEFULLNESS OF THE MPB-64 GENE BASED STOOL PCR FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS IN PEDRIATIC PATIENTS

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ABSTRACT

Purpose: Pedriatic pulmonary tuberculosis diagnosis is challenging because young children not able to spontaneously produce sputum. Diagnosis stool for tuberculosis dna from swallowed sputum may diagnose pulmonary tuberculosis. in the present study we evaluate a the mpb-64 gene based stool pcr for the diagnosis of pulmonary tuberculosis in pedriatic patients.

Methods: 149 Pedriatic patients referred for infectious disease consultation with clinical features suggestive of tuberculosis were considered into our study. Culture based diagnosis was considered to be the gold standard against which stool PCR was evaluated.

Results: MPB64 gene based stool PCR had sensitivity of 90.91 % and specificity of 99.1% for confirmed TB cases.

Conclusion: MPB64 gene based stool PCR PCR would be a rapid, non-invasive test for children with suspected pulmonary tuberculosis and an important diagnostic tool for rapid diagnosis of TB.

INTRODUCTION:

TB occurs all over the world. In 2011, the highest number of new TB cases occurred in Asia, accounting for 60% of new cases worldwide. However, Sub-Saharan Africa carried the greatest proportion of new cases per population with over 260 cases per 100 000 population in 2011. Globally, tuberculosis (TB) continues to exact an inappropriately high mortality and morbidity among children, mainly in the wake of the HIV epidemic. About half a million children (0-14 years) suffering from tuberculosis, and 64,000 children died from the disease in 2011 [1].The laboratory diagnosis of pulmonary tuberculosis mostly relies on the detection of *Mycobacterium tuberculosis* complex (MTC) organisms in the sputum.The diagnosis of pulmonary tuberculosis in children very difficult because specimens are difficult to get and results of culture and AFB smearing of *Mycobacterium tuberculosis* is often negative. Some patients, however, are unable to produce sputum, including children, immunodeficient patients [2].

Diagnosis of pulmonary tuberculosis is the difficult for patients, who are not able to produce a sputum sample, a problem that is mainly common in young children and HIV-positive patients. In these comparatively immunocompromised patient groups, a diminished inflammatory response may obstruct sputum production. In these condition, another specimens obtained by invasive procedures include respiratory tract secretions obtained by bronchoscopy, gastric aspirates, nasopharyngeal aspirates, and intestinal fluid obtained by the string test, may all be used to retrieve pulmonary secretions from patients incapable to give a sputum sample but may cause logistical, cost, or biosafety challenges [3]. The invasive procedures required to obtain the above-mentioned specimens are not comfortable for the patient and are potentially harmful [4]. These limitations in the diagnosis of tuberculosis require the development of new method for the detection M. tuberculosis in samples that can be obtained more easily. Most sputum is swallowed, and the mycobacterial DNA within sputum samples may survive transfer through the gastrointestinal tract, likely allowed molecular diagnosis of stool samples for the presence of mycobacterial DNA indicative of hypothesized that stool samples may be useful for pulmonary tuberculosis molecular diagnosis and drug susceptibility testing. We assessed MPB64 gene based PCR diagnostic approaches for tuberculosis in children. In order to test this hypothesis, we used MPB-64 gene based PCR assay in this study. The results of PCR test of stool samples were evaluated by comparison with sputum AFB smearing, culture, and PCR.

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METHODS:

Our study was performed at Auroprobe Laboratories NH-58 Muradnagar, Delhi NCR, from July 2010 to June 2012. During the study period, 149 patients referred for infectious disease consultation with clinical features suggestive of tuberculosis were considered into our study. Culture based diagnosis was considered to be the gold standard against which stool PCR was evaluated. Ethical approval was not needed for the current study as all the samples from the subjects were received for clinical diagnosis from Different collection points and we had not disclosed any identification of the subjects.

Sample collection:

149 Stool samples were collected from each patient in new plastic containers. Samples were kept at 4°C and transported to our laboratory and analyzed within 24 h of collection in the great majority of cases and within 72 h in all cases.

Specimen processing and culture by BACTEC MGIT 960:

Suspend 1g of feces in 5ml of sterile phosphate buffer (0.067 M, pH 6.8). Agitate the suspension on a vortex mixer for 5s. Decontamination was carried out by N-acetyl-L-cysteine (NALC) / NaOH method [5]. The specimen's suspensions were centrifuged in a 15 ml sterile centrifuge tubes at 3500 rpm for 20 min. Supernatant were discarded gently and the sediment was resuspended. 200 µl of the sediment was taken for PCR and equal volume of decontamination solution was added into it and was incubated at room temperature for 20 min. The specimens were neutralized with sterile phosphate buffer (0.067 M, pH 6.8) and then centrifuged at 3500 rpm for 20 min. Supernatant was discarded to get the sediment up to 2 ml and mixed with 500 µl of sterile phosphate buffer. 500 µl of the resuspended sediments were taken for culture and 50µl solution was used for AFB smear preparation.

Reconstitute a lyophilized vial of BBL MGIT PANTA Antibiotic Mixture with 15 mL of BACTEC MGIT Growth Supplement. Label the MGIT tubes with the specimen number. Unscrew the cap and aseptically add 0.8 mL of Growth Supplement/MGIT PANTA Antibiotic Mixture to each labeled MGIT tube. Add 0.5 mL of the concentrated specimen suspension to the prepared BBL™ MGIT™ Mycobacteria Growth Indicator Tube. Tightly recap the MGIT tube and mix well. Leave the inoculated tubes at room temperature for 30 minutes before loading in to the MGIT system. Load inoculated BBL™ MGIT™ Mycobacteria Growth Indicator Tube into the instrument following manufacturers' instructions for the duration of the recommended 42 day testing protocol. AFB smears were prepared from the vials with growth index (GI>25) was recorded.

Microscopy:

AFB smears were prepared with 50µl resuspended processed specimen over glass slides; after drying smears were stained by Ziehl–Neelsen staining.

PCR for Mycobacterium tuberculosis complex:

PCR was carried out for all the specimens. DNA isolation was carried out by Silica adsorption based column method and PCR was performed in BSL-2 for the amplification of gene mannose binding protein 64 (MPB-64 gene) using primers Forward Primer (P1) 5'-TCCGCTGCCAGTCGTCTTCC-3' and Reverse Primer (P2) 5'-GTCCTCGCGAGTCTAGGCCA-3' [6, 7]. DNA amplification by PCR was performed in the total reaction volume of 25 µl with 5 µl of extracted DNA, 10mM Tris-Cl (pH 8.3), 1.5 mM MgCl2, 50 mM NaCl, gelatin 0.01% (w/v), 100 µM of each dNTP, 0.5 µM of each primer & 0.5 Units of Taq polymerase. Amplification was carried out on a programmable Veriti® Thermal Cycle. Initial denaturation at 94ºC for 5 min. was proceeded by 30 cycles each of denaturation (94ºC for 30 sec.), annealing (60ºC for 1 min) and extension (72ºC for 2min) followed by a final extension at 72ºC for 7min [7]. 5 µl of the amplification products were analyzed by electrophoresis in an ethidium bromide stained 1.6% agarose gel (figure 1).

RESULTS:

149 stool specimens were considered for the diagnosis of *Mycobacterium tuberculosis* by AFB smearing, MGIT 960, and conventional PCR and the clinical performance of stool PCR was evaluated. Of the 149 specimens, 4 specimens got contaminated by other microorganisms. These were removed prior to analysis of the result. 34 of 145 (23.44 %) were MGIT 960 culture positive. The sensitivity, specificity, positive predictive value(PPV), negative predictive value (NPV) for all specimens by AFB smear were found (38.24%, 100%, 100%, 84.09%) , while by PCR method it was (90.91%, 99.11%, 96.77%, 97.37%) respectively (Table 1).

Table 1. The sensitivity, specificity, positive predictive value and negative predictive values for different methods (Stool specimens, N-145)

Method	True Positive	True Negative	False Positive	False Negative	Sensitivity	Specificity	Positive predictive Value (PPV)	Negative predictive Value (PPV)
AFB	13	111	-	21	38.24 %	100%	100%	84.09 %
PCR	30	111	1	3	90.91 %	99.11%	96.77%	97.37 %

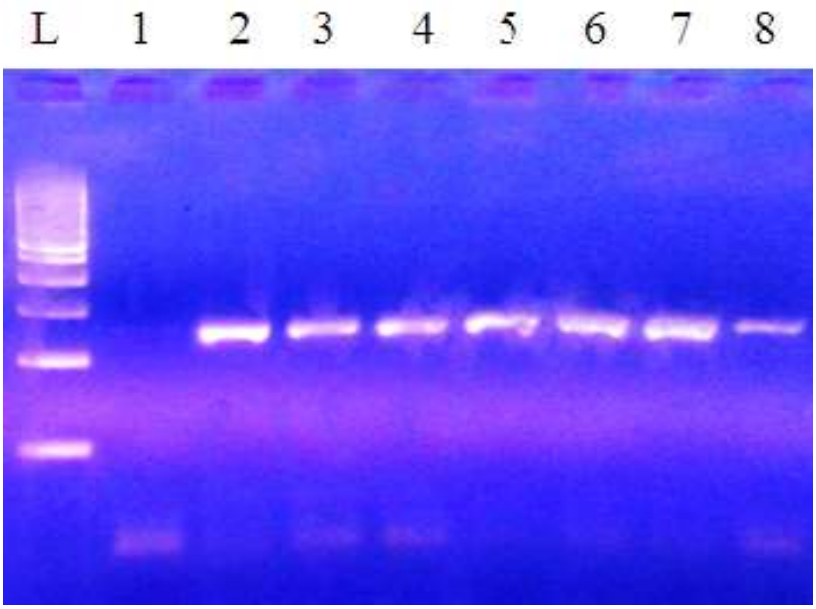

Figure 1: PCR amplification of 240 bp of MPB64 gene of M. tuberculosis on 1.5% agarose gel. Lane L: 100bp DNA marker, lane 2: Negative control 3: Positive control, lane 4, 5, 6, 7, 8: Positive stool samples.

DISCUSSION:

In spite of the availability of effective preventive method and chemotherapy, the prevalence of tuberculosis (TB) is increasing in the developing countries [8-11]. For decades, paediatric tuberculosis (TB) has been a challenge for physicians to diagnose and treat [12-14]. Most recently developed sensitive and specific diagnostic tests have not found a place in the routine evaluation of children with suspected TB, thus the need for new rapid techniques play a major role in the rapid diagnosis and disease management. Culture methods have been considered as gold standard for the diagnosis of *M. tuberculosis,* BACTEC MGIT 960 TB system has been recommended as a valuable system with its high sensitivity and shortened duration of time required for the detection of mycobacterium 10 to 14 days. The present study was designed to evaluate the utility of MPB-64 gene based stool PCR in the diagnosis of pulmonary tuberculosis in children. It was our main objective to assess the use of stool sample for better the diagnosis of pulmonary tuberculosis in those patients who are not able to produce sputum properly. We also compared different conventional techniques with PCR to assess the importance of this technology.

CONCLUSION:

For the better treatment of the tuberculosis the most important point is early diagnosis, rapid, novel and cost effectiveness of an assay, especially in developing nations. We conclude that the MPB-64 gene based stool PCR is also a good sample for the diagnosis of pulmonary tuberculosis in children and MPB-64 gene based stool also have high sensitivity and specificity. PCR improves the diagnosis of pediatric tuberculosis.

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