

Detection of *Mycobacterium tuberculosis* on stool specimens by PCR among patients with pulmonary tuberculosis

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ABSTRACT

Background: To detect *Mycobacterium tuberculosis* on stool specimens by polymerase chain reaction (PCR) among patients with pulmonary tuberculosis. Detection of *M. tuberculosis* complex in sputum forms the basis of diagnosis of pulmonary tuberculosis. However, some patients tend to swallow sputum and some are unable to produce sputum. Based on the survival of *M. tuberculosis* in the gastric fluid, swallowed organisms may be detectable in stool samples. **Methods:** The study was carried out on 30 cases each in four groups: sputum smear-positive and sputum smear-negative adults, pediatric patients suspected of pulmonary tuberculosis along with healthy controls. The samples were processed for direct microscopy for acid-fast bacilli (AFB) and *M. tuberculosis* culture. Stool PCR was done on all 120 samples. **Results:** AFB was demonstrated in 42 and cultured in 39 out of 240 samples. PCR-targeting IS6110 gene showed positive results in 24 (20%) out of 120 stool samples. PCR in stool showed the highest positivity in sputum smear-positive samples followed by gastric aspirates and sputum smear-negative samples. **Conclusion:** Stool PCR is a potentially useful diagnostic method for pulmonary tuberculosis.

Keywords: Acid-fast bacilli, *M. tuberculosis*, polymerase chain reaction

Introduction

Mycobacterium tuberculosis is the causative agent for the infectious disease tuberculosis (TB). With 1.9 million new cases, i.e., one-fifth of the global TB incidence occurs every year in India, and out of these 0.87 million are smear-positive cases.^[1] Diagnosis of pulmonary tuberculosis currently relies upon the detection of the *M. tuberculosis* complex in sputum.^[2]

Diagnosis of pulmonary tuberculosis is difficult in patients who cannot produce sputum (pediatric, immunocompromised, and neurologically impaired patients). In the absence of adequate sputum production, respiratory tract specimens can be obtained by some invasive or inconvenient procedures, including nasopharyngeal aspirates, bronchoalveolar lavage, and gastric aspirate.^[3] As most sputum is swallowed and the mycobacterial DNA within sputum samples may survive transit through the gastrointestinal tract, therefore, stool specimens can be collected from patients in parallel with respiratory tract specimens for the presence of *M. tuberculosis* organism.^[4] Moreover, molecular testing of stool samples for the presence of mycobacterial DNA holds a diagnostic potential.

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Nucleic acid amplification techniques are very useful in the rapid diagnosis of infection by *M. tuberculosis*.^[5] It has high sensitivity and accuracy than microscopy and culture, turnaround time is within 3–6 h of receipt of the sample. A stool PCR assay for the rapid diagnosis of pulmonary tuberculosis was developed using oligonucleotide primers to amplify a fragment of IS6110, an insertion sequence repeated multiple times in the chromosome of *M. tuberculosis*.^[6] Stool PCR studies for the diagnosis of pulmonary tuberculosis are lacking in India. Therefore, the present study was aimed at the diagnosis of pulmonary tuberculosis by using stool PCR.

Material and Methods

A hospital-based descriptive and exploratory study was carried out in the Departments of Microbiology, Medicine, Paediatrics, and TB Clinic in a tertiary care hospital in eastern India. The study was carried out on 30 sputum smear-positive and 30 sputum smear-negative adult patients, 30 gastric aspirates from pediatric patients in clinically suspected pulmonary tuberculosis cases along with 30 healthy controls. Overall, 240 samples were collected that included 120 stool samples, 90 sputum samples, and 30 gastric aspirate samples. Any patient with a h/o Anti-tubercular drugs (ATT) intake during the past 3 months and any patient with isolated pleural effusion or mediastinal tubercular lymph node or intestinal tuberculosis were excluded from the study.

The inclusion criteria were smear-positive pulmonary tuberculosis cases, smear-negative clinically suspected pulmonary tuberculosis cases, and clinically suspected and/or microbiologically proven cases of pulmonary tuberculosis in the pediatric age group along with/without Mantoux test positive (i.e., >10 mm in diameter). Informed consent was taken from all the patients. Institutional ethics committee approval was obtained for the study. Approval from ethics committee was obtained on Aug 2013.

Specimen collection

Specimen collection containers were wide-mouthed, screw-capped, rigid, watertight, and leakproof containers and were used to collect sputum, gastric aspirate, and stool specimens.

Direct demonstration of AFB in the specimen

Smears were prepared and staining of fixed smears was done by Zeihl–Neelsen staining.

(a) Sputum: Thick purulent portion of sputum was taken by using wire loops on a clean glass slide and spread over a 1 × 2 cm area. The smear was dried for 15 to 30 min and then fixed by passing the slide over the flame three to five times for 3 to 4 s.

(b) Gastric lavage: Samples were centrifuged at 3,000 g for 30 min and the supernatant was discarded and smears were prepared from sediments. The smears were air-dried and fixed.

(c) Stool samples: It was emulsified in distilled water and left for 15 min, after which smear was prepared from the supernatant. The smear was air-dried and heat-fixed.

Isolation of *M. tuberculosis*

Sputum specimens were decontaminated with 4% NaOH solution for 20 min, centrifugation was done at 3,000 g for 15 min. From the sediment, two slopes of Lowenstein–Jensen (LJ) medium were inoculated, one of the bottles was wrapped with kraft paper to look for pigment production in the dark bottle in case of nontubercular mycobacteria. Both the bottles were incubated in a slanted position at 37° C with a screw cap slightly loosened for a week to ensure even distribution of inoculums. Then, after a week, caps of bottles were tightened and they were further incubated in an upright position. The growth was examined 48–72 h after inoculation to detect gross contaminants. Thereafter, cultures were examined weekly, up to 8 weeks before declaring it as no growth.

Stool samples: Approx. 0.1 g of stool was resuspended in 6 mL of sterile distilled water, mixed, and left for 15 min to separate, after which 2 mL of the supernatant was processed like other samples.

Gastric aspirate samples exceeding 2 mL of volume were centrifuged and the supernatant was discarded. After processing of samples, sediments were subjected to a demonstration of AFB by Z-N staining procedure and mycobacterial culture on LJ medium.

M. tuberculosis showed eugonic growth. They grow as dry, rough, raised, irregular, and wrinkled-surfaced colonies. Initially, the colonies were tenacious and not easily emulsifiable. After seeing the morphology of culture, the growth was identified by the Niacin test.

PCR

Stool samples were subjected to PCR with IS6110 as the target gene. H37Rv strain of *M. tuberculosis* was taken as a positive control and the PCR grade water as a negative control.

DNA extraction was done using the HiPurA stool DNA purification kit according to the manufacturer's instructions. Extracted DNA from all the stool samples was amplified targeting the 438bp region of the IS6110 gene of *M. tuberculosis*. Samples were amplified in an automated thermocycler according to the following protocol: 95° for 15 min for 1 cycle (initial denaturation), 95° for 30 s (denaturation), 65° for 45 s (annealing), and 72° for 1 min (extension), and final extension 72° for 5 min. Denaturation, annealing, and extension steps were repeated for 49 cycles, and after the final extension was maintained at 4°C till detected by agarose gel electrophoresis. The amplicons were analyzed on 1.5% agarose gel using a UV Gel documentation system and amplicon size was compared with the appropriate DNA ladder.

Results

The study included 240 samples including 120 stool samples, 90 sputum samples, and 30 gastric aspirate samples [Figure 1].

Out of 120 study participants (90 cases and 30 controls), 87 (72.5%) were males, whereas 33 (27.5%) were females. The age ranged from 5 years to 56 years with the majority belonging to 16–30 years of age group (36.6%).

Results of Conventional Diagnostic Techniques:

1. Direct demonstration of Acid-Fast Bacilli (AFB)

AFB were demonstrated by Z-N staining in 42 (27.5%) of the 240 samples. Sputum samples showed the highest AFB positivity 30 (33.3%), followed by gastric lavage 9 (30%) and stool samples 3 (2.5%) [Table 1].

. B.

Isolation

1. tuberculosis was isolated in 39 out of 240 (16.25%) samples. The sputum sample showed the highest positivity 28 (31.1%), followed by gastric lavage 5 (16.66%) and stool sample 6 (5%). Of the 30 sputum smear-positive cases, 25 (83.3%) were culture-positive. In the smear-negative group ($n = 30$), three were culture positive. Gastric lavage specimens ($n = 30$) yielded five culture-positive results. Out of these five gastric lavage culture positives, three were also positive by smear microscopy. Among the six culture-positive stool specimens, three were also positive by smear microscopy [Table 1].

PCR for IS6110 gene among stool specimens

A total of 120 stool specimens collected from various study groups were subjected to PCR for IS6110 using primers already mentioned. The positive results for PCR were seen on 24 (20%) stool specimens, which included 12 (40%) from sputum smear-positive cases, four (13.3%) from sputum smear-negative cases, and eight (26.7%) from pediatric cases.

None of the stool specimens from healthy controls gave PCR positive results. Table 2 shows the distribution of PCR positives from stool specimens in the various study group. Figure 2 shows a comparison of various diagnostic techniques ($n = 240$). Positive bands (438 bp) of *M. tuberculosis* can be seen in PCR gel electrophoresis as depicted in Figure 3.

Discussion

The study participants were divided into four groups. Group one consisted of 30 sputum smear-positive cases, group two consisted of 30 sputum smear-negative clinically suspected cases of pulmonary tuberculosis, group three included 30 pediatric cases, and group 4 had 30 healthy controls. Out of 120 study

participants, 87 (72.5%) were males and 33 (27.5%) were females. The minimum age was 5 years who was a female and the maximum age was 56 years who was a male patient. The majority (36.6%) of cases belonged to the age group of 16–30 years.

Acid-fast microscopy by Z-N stain for the direct demonstration of bacilli was performed in the laboratory. It is the easiest method to detect the presence of AFB. However, there must be at least 5,000 bacilli per mL of sputum to be detected by microscopy.

In our study, AFB by direct microscopy was demonstrated in 42 out of 240 samples, out of which 30 (33.3%), nine (30%), and three (2.5%) were positive in sputum, gastric lavage, and stool specimens, respectively. In a study by Khechine *et al.*, AFB was found in 11.2% of the sputum specimens and 6.7% of the stool specimens. The fact that smear positives and smear negatives were segregated in our study could account for this difference and also gastric lavage samples were not included in the study by Khechine *et al.*

The culture techniques can detect 10–100 viable mycobacteria per milliliter of sample. *M. tuberculosis* was isolated in 39 out of 240 samples (16.25%). Sputum samples showed the highest

Table 1: Comparison of positivity among different samples by conventional diagnostic tests

Samples	AFB microscopy (n)/Total (%)	Culture (n)/Total (%)
Sputum	30/90 (33.3%)	28/90 (31%)
Gastric lavage	9/30 (30%)	5/30 (16.6%)
Stool	2/120 (2.5%)	6/120 (5%)

Table 2: PCR positivity in stool samples among study groups

Study Groups	Number	Positive (%)
Sputum smear-positive cases	30	12 (40)
Sputum smear-negative cases	30	4 (13.3)
Pediatric study cases	30	8 (26.7)
Healthy controls	30	0 (0)
Total	120	24 (20)

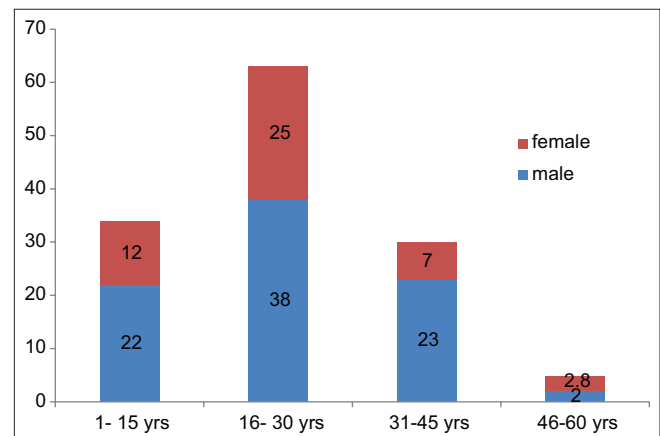


Figure 1: Frequency distribution of study groups on the basis of age and gender ($n = 120$)

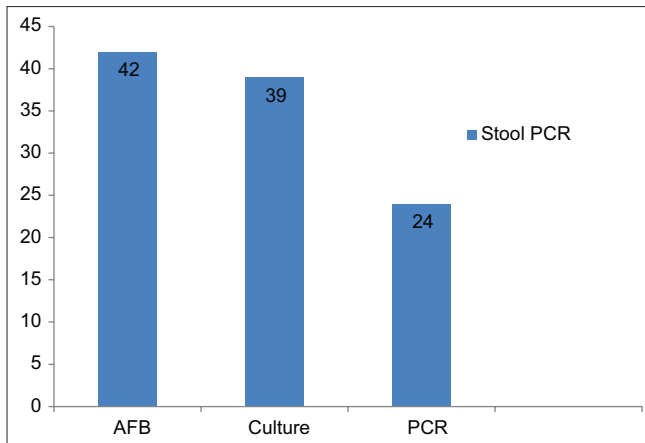


Figure 2: Comparison of various diagnostic techniques ($n = 240$)

positivity of 28 (31%) followed by gastric lavage five (16.6%) and stool six (5%). None of the healthy control samples were positive by culture. According to Khechine *et al.*, *M. tuberculosis* complex organism grew in 14.9% sputum and 9.7% stool specimens.^[3]

Oberhelman *et al.* in their study showed that recovery from gastric aspirates clearly was superior to recovery from a stool by culture.^[4] Another study from Peru also reported higher isolation rates in gastric aspirates among pediatric tuberculosis cases.^[3] Rates of *M. tuberculosis* detection from gastric aspirate specimens from children with presumptive TB range from 1% to 50% in the medical literature.^[7,8]

Only 5% of the stool specimen were culture positive in our study. Sensitivity of detection of AFB by stool culture may be increased by culturing a larger volume of the specimen and improving decontamination/concentration techniques.^[9]

In comparison of the two conventional diagnostic techniques for pulmonary TB, direct demonstration of AFB was found better than isolation in the case of sputum and gastric lavage samples, whereas for stool samples culture showed higher positivity (5%) than the direct demonstration of AFB (2.5%).

To improve stool microscopy and culture for AFB, a better decontamination method and increased volume and number of samples may be required. This may also hold true for gastric aspirates, hence more frequent sampling may be required as the organism has a greater probability of getting killed by gastric acidity.^[9]

We used primers for the IS6110 gene for evaluating all stool samples by PCR. Stool PCR had greater sensitivity when DNA was extracted with commercially available spin columns. By using the spin column-based DNA extraction technique in our study, PCR targeting the IS6110 gene showed positive results in 24 (20%) out of 120 samples. Out of the total positives, stool PCR showed the highest PCR positivity of 12 (40%) in sputum smear positives followed by eight (26.7%) in gastric aspirates and

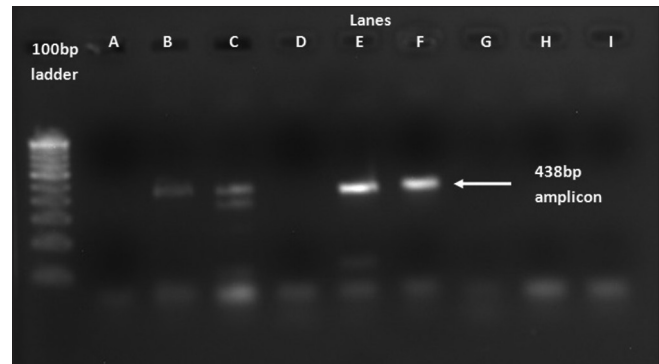


Figure 3: Stool PCR Gel picture of sputum smear-positive cases LANE A: Negative control, LANE B: Positive control, LANE C, E, F: Positive band of 438 bp amplicon of IS6110 LANE D, G, H, I: Negative for 438 bp amplicon of IS6110

four (13.3%) in sputum smear negatives. In their study, Khechine *et al.* showed positivity of 20.2% among stool specimens. They also showed that the number of IS6110 copies was higher in stool than in sputum in 11 cases.^[3] However, in our study sputum samples were not subjected to PCR and a number of IS6110 copies were not looked for in the sputum samples. PCR positivity in stool samples of pediatric patients with pulmonary tuberculosis was comparatively better than microscopy and culture of gastric aspirate in our study. There is a paucity of similar studies showing a comparison of stool PCR with gastric aspirate smear and culture positivity in pediatric patients.

Although there is a lot of work on the diagnosis of pulmonary tuberculosis, there is a paucity of literature on the diagnosis of pulmonary tuberculosis by PCR on stool specimens. To our knowledge, the same study has so far, not been carried out in India. Knowledge of the fact that *M. tuberculosis* has the potential to survive intestinal transit will generate data in this regard in the Indian content for the first time. Thus, the research study is very important for primary care physicians in better management of pulmonary tuberculosis.

Conclusion and Key Messages

Our study demonstrates that stool PCR has a moderate correlation with smear microscopy and culture for the detection of swallowed *M. tuberculosis* for the diagnosis of pulmonary tuberculosis. Moreover, stool being a noninvasive specimen is an attractive alternative diagnostic option. However, sample size being small and the study being one of its first kind from this part, more elaborate studies would be required to further standardize stool PCR methods for the diagnosis of pulmonary tuberculosis among inadequate sputum producers and the pediatric population.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients

understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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