

Diagnostic Significance of Polymerase Chain Reaction as Compare to Culture and Direct Microscopy in Cases of Pulmonary Tuberculosis

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Abstract

Background: Tuberculosis (TB) is presently an important health problem worldwide. Situation has changed in recent years due to AIDS pandemic. Hence, it urges the need to introduce specific methods for rapid diagnosis to avoid unnecessary or improper treatment. At present, sputum microscopy is the sole rapid diagnostic method available. Sputum culture may require more than 2 weeks to confirm diagnosis. Hence, detection of *Mycobacterium tuberculosis* by enzymatic amplification (polymerase chain reaction [PCR]) has been found useful in diagnosis of pulmonary TB.

Materials and Methods: The present study includes 45 sputum samples of patients presenting to TB chest OPD with productive cough for more than 2 weeks. Diagnostic tests are applied on sputum samples - direct microscopy by Ziehl–Neelsen staining, culture by modified Petroff's method, and PCR by commercially available kit (Bio basic INC).

Results: Of 45 sputum sample, TB was confirmed by culture in 19 patients, acid-fast bacilli detected in 18 of 19 patients, and all of these patients were positive for PCR.

Conclusion: PCR is extremely helpful in detecting *Mycobacterium TB* in sputum sample as compare to culture and direct microscopy.

Key words: Culture, Polymerase chain reaction, Pulmonary tuberculosis, Ziehl–Neelsen staining

INTRODUCTION

Tuberculosis is presently an important health problem throughout the world.^[1] Despite its progressive decrease, situation has changed in recent years due to AIDS pandemic.^[2] Classically, there is correlation between the presence acid-fast bacilli (AFB) in clinical samples and the isolation and culture of *Mycobacterium TB*. Moreover, the increase the incidence of atypical mycobacterium in patients with AIDS urges the need to introduce specific method for rapid diagnosis to avoid unnecessary or improper treatment.

At present, microscopic examination is the sole rapid diagnostic method available. The technique is simple and may be performed in any laboratory. However, the preparation and reading of the smear is time consuming and detects only 40–80%^[3] of pulmonary TB (PTB) cases and only in more advanced cases.^[4] Diagnosis of patients at an earlier stage, while still smear negative, would be advantages because they are less contagious^[5,6] and have lower morbidity and mortality.^[7] Culture through new radiometric systems,^[8] biphasic culture^[9] may require more than 2 weeks to confirm diagnosis. Hence, the detection of *Mycobacterium TB* by enzymatic amplification (polymerase chain reaction [PCR]) has been found useful in diagnosis of PTB.^[10-12] Several PCR procedures have been described to detect *Mycobacterium tuberculosis* genome, the target sequence being the main difference,^[13-22] due to influence on specificity and sensitivity. Although the insertion sequence IS6110, specific for *M. tuberculosis* complex and repeated several times in the chromosome is the most frequently used target for diagnosis and epidemiologic purposes, the investigation of groEL gene

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may also prove important for initial study in areas where atypical mycobacterium is prevalent in immunocompromised patients. However, insertion sequence IS6110 specific for *M. tuberculosis* is most frequently used for diagnosis^[23-25] and has high sensitivity and specificity.

Aims and Objectives

1. To evaluate all TB patients for direct microscopy, culture, and PCR.
2. To compare statistically each of these above tests.

MATERIALS AND METHODS

The study includes the patients of TB presenting to the OPD of Department of Medicine, TB and Chest, NSCB Medical College, Jabalpur (M.P.).

The present study includes 45 sputum samples of patients presenting to OPD with productive cough for more than 2 weeks. Diagnosis of TB is based on the isolation and identification of *M. tuberculosis* in samples. Samples are subjected for testing at RNTCP laboratory, NSCB Medical College, Jabalpur, for direct microscopy by Ziehl–Neelsen (ZN) staining, culture by modified Petroff’s method, and PCR extraction ZN were done by commercially available kits (Bio basic INC).

Smear Preparation

The clinical specimens of fluids collected in sterilized bottles and sent to laboratory. Clean sterilized slides were taken, and appropriate portion of sample was transferred to the center of slide using inoculating wire loop. Thin smear was made by spreading the sample and then air dried for 15 min. Slide was then heat fixed on an electric slide warmer at 80°C for 20 min. Inoculating loop was sterilized by flaming for further use.

Acid-fast Staining

Mycobacteria retain the primary stain even after exposure to decolorizing with acid- alcohol, hence, termed “acid fast.” A counterstain is employed to highlight the stained organisms for easier detection. There are several methods of determining the acid-fast nature of mycobacteria. In the carbol fuchsin (ZN) procedure, acid-fast organism appears red against blue background.

ZN staining

- Slides were placed on a staining rack.
- Slide was first flooded with carbol-fuchsin and heated slowly using intermittent heat for 5 min.
- Slides were rinsed by running water till the stain was washed away.
- Then, the slides were flooded with decolorizing solution for 2–3 min, again rinsed with water and excess of water was drained from slides.

- Slides were then counterstained with methylene blue for 30 s, rinsed thoroughly with water and excess water was drained off the slides.
- Finally, slides were air dried and observed under oil emersion.

Culture of Samples

Modified Petroff’s method

The majority of clinical specimens submitted to laboratory are contaminated, to varying degree, by more rapidly growing normal flora organisms. These would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow. The specimens must, therefore, be subjected to a proper digestion and decontamination procedure that liquefies the organic debris and eliminates the unwanted normal flora.

Processing

- Sample was taken and 4% NaOH (double the amount of samples) was added to it.
- Cap of the bottle was tightened and kept on shaker for 15 min.
- After shaking, it was centrifuged at 3000 rpm for 15 min.
- After centrifugation, supernatant was discarded in a 5% phenol jar (discard jar).
- 20 ml sterilized distilled water was added to the pellet.
- Sample was then recentrifuged at 3000 rpm for 15 min supernatant was again discarded in phenol jar and pellet was used for inoculation of slopes of LJ Medium.

Two LJ medium slopes were taken per sample and inoculated with centrifuged sediment (pellet obtained by modified Petroff’s method over the entire surface of the medium using a sterilized inoculating wire loop). Loop was sterilized after inoculating each sample. Cap of bottles was tightened to minimize evaporation and drying up of medium. All the inoculated cultures were then kept in incubator at 35–37°C for 8 weeks. Examination and reading of cultures were done at regular weekly intervals. Typical colonies of *M. tuberculosis* appeared as, rough, crumbly, waxy, non-pigmented (buff colored), and slowly growing, i.e., only appeared 2–3 weeks after inoculation. Cultures were reported as positive or negative on the basis of growth of *M. tuberculosis* on the medium.

PCR

DNA extraction

The extraction of DNA was done by commercially available kit (Bio basic INC) Manufacturer’s instruction was followed. Briefly, the procedure was

1. 200 µl TE buffer was added in 100 µl sample.
2. 400 µl of digestion solution was added to sample from step and mixed well.

3. 3 µl of proteinase K solutions was added and incubated at 55°C for 15 min.
4. 260 µl of 100% ethanol was added and mixed well. The mixture was applied to column that is in a 2.0 ml collection tube. Spin at 10,000 rpm for 2 min.
5. Flow thoroughly was discarded in the collection tube, then 50 µl of waste solution was added and spin at 10,000 rpm for 2 min.
6. The wasting step was repeated (5).
7. The flow thoroughly was discarded and was spin at 10,000 rpm for an additional min. to remove residual amount 0 waste solution.
8. The EZ - 10 columns were placed into a clean 15 ml microcentrifuge tube and 30–50 µl elution buffer was added into the center part of membrane in the column. The tube was incubated at 37°C or 50°C for 2 min. Incubation at 37°C or 50°C could increase recovery yield.
9. For elution of DNA, the column was spin at 10,000 rpm for 2 min.

Aliquot of purified genomic DNA was kept at 20°C for long time storage.

PCR mixture

Required volume calculated as under:

Initial conc. × Vol. required = Final conc. × Vol. of reaction mix. PCR mixture can be prepared as follows:

Component	Volume
Water	13 µl
10×buffer	2.5 µl
MgCl ₂	1.5 µl
dNTPs mix	3.0 µl
Primers (F)	0.5 µl
(R)	0.5 µl
Taq polymerase	2.0 µl
Sample	2.0 µl
Total volume	25 µl

Primers used for IS6110:

- 5'-CCTGCGAGCGTAGGCGTCGG and
- 5'-CTCGTCCACGCCGCTTCGG

PCR Program

Step I: Preheating at 94°C 5 min.

Step II: Denaturation at 95°C for 1 min.

Step III: Annealing at 60°C 30 s.

Step IV: Extension at 72°C for 1 min

(Step II, III, and IV 40 cycles)

Step V: Final Extension at 72°C for 7 min.

Step VI: At 4°C forever.

Electrophoresis

- PCR product with 50 bp DNA ladder is loaded on 1.5% agarose gel (w/v) with ethidium bromide 0.5 mg/ml.

- Gel is electrophoresed at 100 V for 30 min.
- Gel is observed on gel documentation system.
- Gel photograph is taken and results are noted.
- Product size - 123b.

Results were noted for all samples.

Inclusion Criteria

Sputum samples of patients who are having productive cough for more than 2 weeks with clinical suspicion of TB.

Exclusion Criteria

- Old diagnosed case of PTB.
- Old treated case of PTB.
- History of ATT or the second line ATT intake.

RESULTS

The study was done to compare the tests - direct microscopy, culture, and PCR statistically for detection *M. tuberculosis* in samples. 45 patients with cough more than 2 weeks attending the TB and chest OPD were selected and sputum samples were collected. Mean age of presentation was 34 years (13–66) with 28 male patients and 17 females patients, out of these patients, TB was confirmed by culture in 19 patients, all of these patients were positive for PCR and AFB were detected in 18 of 19 patients on ZN staining [Table 1].

The sensitivity of PCR was 100% with specificity of 76.92% and positive predictive value 76%. The ZN staining was 94.74% sensitive and 82.46% specific.

Comparing the tests according to the clinical diagnosis of TB based on the radiological, clinical, and epidemiological data, 25 patients fulfilled the criteria for diagnosis of TB [Table 2].

PCR test was able to detect DNA of mycobacterium in all the samples (100%) of patients with clinical diagnosis, growth was seen in 19 (76%) of 25 patients, and AFB were found in 22 (88%) of 25 patients. The specificity of each of these tests was 100% [Figure 1].

DISCUSSION

In our study, it was observed that the sensitivity of PCR in all samples was higher than culture and direct microscopy.

Table 1: Comparison of results obtained by PCR and ZN staining in patients confirmed by culture

	PCR positive	PCR negative	AFB positive	AFB negative
Culture positive	19.00	0.00	18.00	1.00
Culture negative	6.00	20.00	4.00	22.00

PCR: Polymerase chain reaction, AFB: Acid-fast bacilli

Table 2: Comparison of culture PCR and ZN stain in patients diagnosed by clinical criteria

Clinical diagnosis	PCR positive	PCR negative	Culture positive	Culture negative	AFB positive	AFB negative
TB	25.00	0.00	19.00	6.00	22.00	3.00
Non-TB	0.00	20.00	0.00	20.00	0.00	20.00

PCR: Polymerase chain reaction, AFB: Acid-fast bacilli, TB: Tuberculosis, ZN: Ziehl-Neelsen

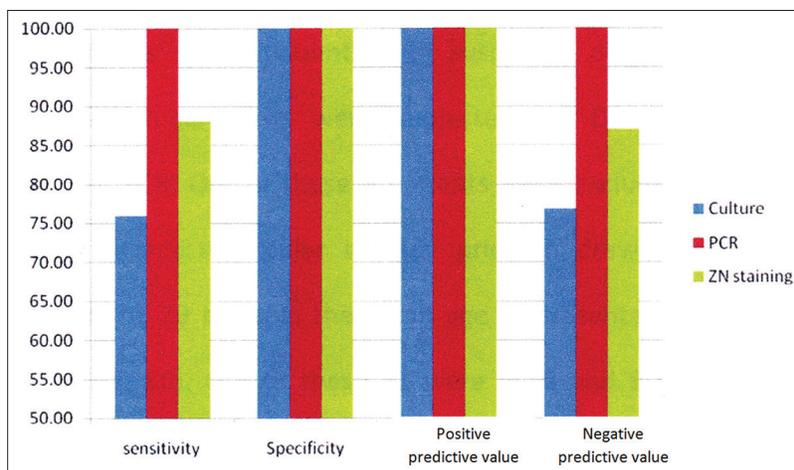


Figure 1: Comparison of tests statically for pulmonary samples

The sensitivity of PCR for detection of IS6110 sequence in pulmonary samples was 100%. PCR was found to be specific test considering clinical diagnosis. The results obtained for PCR in sputum samples in this study were comparable with the results obtained by other authors - Eisenach *et al.*^[26] and Brisson-Noël *et al.*^[14]

There were 19 patients of PTB confirmed by culture. All were positive for PCR, making PCR 100% sensitive test, but the specificity of PCR was found to be 76.92%. Considering the clinical diagnosis, the PCR test was 100% specific and 100% sensitive.

Eisenach *et al.*^[13] studied 162 sputum samples and found a correlation between culture and PCR are 100% of non-treated patients.

Brisson-Noël *et al.*^[14] using amplification of groEL gene and the insertion sequence IS6110 also found a correlation between PCR and clinical or bacteriologic data in 97.4% of cases. Other studies have been made using genomic sequences different to groEL gene and insertion sequence IS6110 with similar sensitivity results.

CONCLUSION

Considering the results obtained and available literature, it is concluded that PCR is extremely helpful in detecting *M. tuberculosis* in sputum samples as compare to culture and direct microscopy. It can be conducted in short time allowing us to timely intervene and prevent complications.

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