Application of enzyme amplified mycobacterial DNA detection in the diagnosis of pulmonary & extra-pulmonary tuberculosis

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Background & objectives: As isolation of Mycobacterium tuberculosis in culture requires a long time, there is a need for simple rapid method for direct detection of M. tuberculosis from clinical specimens. Amplified nucleic acid hybridization assays such as polymerase chain reaction (PCR) have shown promising results. In the present study, the sensitivity of PCR assay was assessed over smear microscopy for rapid diagnosis of tuberculosis in pulmonary and extra-pulmonary samples from patients suspected to have tuberculosis.

Methods: A total of 37 clinical samples from patients with pulmonary tuberculosis and 133 from patients with extra-pulmonary tuberculosis were subjected to Ziehl-Neelsen staining for smear preparation and PCR for detection of mycobacterial DNA.

Results: It was observed that 100 per cent of acid fast bacilli (AFB) positive and 35.7 per cent of AFB negative pulmonary samples and 82.76 per cent of AFB positive and 56.73 per cent of AFB negative extra-pulmonary samples were positive for mycobacterial DNA detection. Total positivity rates of DNA amplification method in pulmonary and extra-pulmonary samples were 75.67 per cent and 61.7 per cent respectively which were significantly higher in comparison with AFB positivity, which was 62.16 per cent in pulmonary and 21.8 per cent in extra-pulmonary samples (P<0.05 and P<0.001 respectively).

Interpretation & conclusion: Routine application of DNA amplification method in diagnosis of AFB negative patients with pulmonary or extra-pulmonary tuberculosis may be a useful tool for detection of M. tuberculosis.

Key words Fine needle aspiration (FNA) - Mycobacterium tuberculosis - polymerase chain reaction (PCR)

Early diagnosis of tuberculosis is an important aspect in the control of tuberculosis. Definitive diagnosis is dependent on the demonstration of acid fast bacilli (AFB) by microscopy and isolation and identification of Mycobacterium tuberculosis by culture methods. As most of the species of mycobacteria, including M. tuberculosis, are slow growing, culture on solid media is time consuming and lacks sensitivity and specificity. The fast track culture methods are expensive and use of a liquid medium with radiometric growth detection system (such as BACTEC radiometric system) and other rapid liquid culture media replaced the traditional methods of isolation1. Though these new methods have decreased the time required for isolation, they are costly and require a special set up for working. To overcome these limitations, procedures based on in vitro amplification of mycobacterial DNA, using polymerase chain reaction (PCR) is in practice in developed countries2-4. Use of PCR in clinical practice in developing countries is not very well evaluated for its validity and reproducibility in detection of M. tuberculosis in clinical specimens. The present study was undertaken...
with the aim to assess the sensitivity of PCR assay over AFB smear examination for rapid detection of *M. tuberculosis*, using different clinical samples in Indian hospital setting.

**Material & Methods**

The study was conducted in the Department of Microbiology, Chhatrapati Sahuji Maharaj Medical University (Upgraded K.G.M.C.) Lucknow during September 2000 to March 2002.

**Inclusion criteria**: The study group comprised prospectively enrolled freshly diagnosed patients suspected to have pulmonary tuberculosis (37) and extra-pulmonary tuberculosis (133) of either sex and all ages, with positive response to anti-tubercular treatment (ATT) after four weeks of follow up.

**Exclusion criteria**: AFB negative patients with pulmonary and extra-pulmonary tuberculosis not responding to ATT after four week of treatment were excluded from the study.

**Sample size**: Globally tuberculosis represents about one third of the world's population. Overall it is estimated that about 10 per cent of the infected persons will eventually develop active tuberculosis. Therefore at 95 per cent confidence level if the prevalence of the disease is 10 per cent the recommended study population is approximately 138 (n=z²₁₋ᵦ²P(1-P)/d²). We have enrolled a total of 170 (pulmonary samples 37 and extra-pulmonary samples 133) samples.  

**Specimens**: Following clinical samples were collected from the patients.

- **Pulmonary tuberculosis**– Sputum (36), broncho alveolar lavage (BAL,1).
- **Extra-pulmonary tuberculosis**– Samples collected from 133 patients of extra-pulmonary tuberculosis (i) Samples other than tissue and fine needle aspirates (FNA): ascitic fluid(7), blood(2), bone marrow(2), cerebro spinal fluid (CSF, 5) gastric aspirate(2), pleural fluid(16), pus(44), stool(1), synovial fluid(1) and urine(7) and (ii) FNA and tissue biopsies: tissue biopsy(4) and fine needle aspirates from cervical lymph nodes(42).

**Negative controls**: Sputum (5) from patients with asthma and CSF (5) from proven cases of viral encephalitis were taken.

**Gold standard**: Response to anti-tubercular treatment (feeling of well being/weight gain/improved appetite/disappearance of fever) was taken as gold standard in the study.

**Sample processing**: The samples were processed for examination by Ziehl-Neelsen staining to look for AFB and PCR for detection of *M. tuberculosis* DNA. Three sputum samples from each patient with pulmonary tuberculosis were examined. If any of the samples contained>3 bacilli it was labelled as AFB positive.

Except for CSF and FNA, Petroff’s method was used for decontamination of the samples. Sputum and pus samples were treated with equal volume of 4 per cent NaOH for 30 min. After washing with saline twice, pellet was used for smear preparation. The remaining pellet was used for DNA extraction. Various body fluids were concentrated by centrifugation before decontamination and were treated with NaOH for 10 min. CSF deposits and FNA were directly used for smear preparation and DNA extraction. Tissue biopsy material was homogenized in TE buffer (Tris 10 mM, EDTA 1 mM pH 8.0) and suspension was treated with proteinase K, (Bangalore Geni, Bangalore, India) (10mg/ml) at 56°C overnight. Finally the pellet was decontaminated and used for smear preparation and PCR assay.

**Extraction of mycobacterial DNA from samples**: DNA isolation from clinical specimens was performed as previously described. Briefly, sample pellet was suspended in 200µl of TE buffer and boiled for 10 min. Equal volume of lysis buffer (Tris 10 mM, EDTA 2 mM, NaCl 0.4 M and triton x-100, 0.5%) pH 8.0 was added and vortexed thoroughly. Tubes were incubated at 65°C for two hour after adding 10µl of proteinase K (10 mg/ml). Samples were again boiled for 10 min to inactivate the proteinase K, followed by DNA purification with phenol: chloroform (24:1 v/v) and then by chloroform alone. Aqueous phase was finally transferred in 2.5 volumes of chilled ethanol and sodium acetate (0.3 M final concentration) was added. Tubes were kept at –20°C overnight for precipitation of DNA. Samples were
centrifuged at 12,000 g for 15 min at 4°C and DNA pellets were washed with 70 per cent ethanol by centrifugation at 12,000 g for 10 min and allowed to air dry. DNA pellet of each sample was suspended in 20µl of sterile triple distilled water and 2 µl of it was used for PCR assay.

Amplification of mycobacterial DNA: A pair of oligonucleotide primers with insertion sequence IS6110 amplifying a 123 bp fragment specific of *M. tuberculosis* complex, was used in this study; Primer 1:5’CCTGCGAGCGTAGGCGTCGG3’ (forward primer) and Primer 2:5’ CTCGTCCAGCGCCGCTTCGG3’ (reverse primer) (Bangalore Geni, Bangalore, India). Amplification was carried out in a final volume of 20 µl containing 10mM TrisHCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 250 µM of each dNTPs, 50 pico moles of each primer and one unit of taq DNA polymerase (reagents from Bangalore Geni, Bangalore). 2 µl of sample DNA was added to 20 µl of reaction mixture. A positive control having DNA sample from a standard strain of *M. tuberculosis* and a negative control with distilled water substituted for DNA were also included in every batch of experiment. Amplification cycles were performed on automated thermal cycler (Techne, UK).

Amplification protocol: The cycling profile used was as described earlier, i.e., initial denaturation at 94°C for 10 min followed by 35 cycles, each consisting of three steps: (i) denaturation at 94°C for 60 sec, (ii) annealing at 60°C for 60 sec, and (iii) primer extension at 72°C for 60 sec. Final extension was done at 72°C for 10 min. All steps of sample processing, DNA extraction, PCR reagent preparation and analysis of PCR product were carried out in separate rooms to avoid the carry over contamination.

Detection of amplified products: Amplified products were resolved electrophoretically on 2 per cent agarose (Bangalore Geni, Bangalore, India) containing ethidium bromide (0.5 µg/ml) and visualised on 260 nm wavelength UV transilluminator (Bangalore Geni, Bangalore, India).

Statistical analysis: The positivity rates by AFB smear examination and DNA amplification were compared using Chi-square test.

**Results & Discussion**

**Pulmonary samples:** Of the 37 pulmonary samples, 23 (62.16%, sputum 22 and one BAL) were positive for AFB by smear examination while 28 (27 sputum and one BAL) were positive for mycobacterial DNA (75.67%) by the PCR assay. PCR identified mycobacterial DNA in all 23 AFB positive pulmonary samples. Of the 14 AFB negative sputum samples, 5 (35.7%) were positive and remaining 9 (64.3%) were negative by PCR. All sputum samples from negative controls were negative for mycobacterial DNA (Table).

**Extra-pulmonary samples:** Of the 87 extra-pulmonary samples other than tissue and FNA, only 13 (14.94%) were detected as AFB positive by smear. Of the 13 AFB positive samples, PCR detected mycobacterial DNA in 12 (91.5%) while one pus sample was negative by PCR assay. *M. tuberculosis* DNA was detected in 9 samples of pleural fluids, 29 pus, 2 ascitic fluid, 1 synovial fluid, 1 CSF, 4 urine, 1 blood and 2 of bone marrow samples (Table).

Of the 46 tissue samples (4 tissue biopsy and 42 FNA), AFB was demonstrated in two tissue biopsy samples and 14 FNA samples. PCR detected mycobacterial DNA in all 4 tissue biopsy samples and 29 of 42 FNA from cervical lymph nodes, while 4 AFB positive FNA samples were negative by PCR. None of negative CSF controls were positive for mycobacterial DNA.

An *in vitro* amplification using IS6110 as a target sequence for detection of mycobacterial DNA from clinical samples showed wide variation in specificity and sensitivity. We found PCR positivity in 100 per cent AFB positive pulmonary and 82.76 per cent of extra-pulmonary samples. Extra detection of *M. tuberculosis* DNA was possible in 5 (35.7%) cases of pulmonary tuberculosis by PCR. Of the 104 AFB negative extra-pulmonary samples 59 (56.73%) were positive by PCR. These samples were not likely to represent false positive results, since these were collected from highly suspected cases of tuberculosis, who responded positively to the anti tubercular treatment. The PCR positivity rates for pulmonary (75.67%) and extra-pulmonary samples (62.16%) were significantly (P<0.05, and <0.001) higher in comparison to AFB smear positivity (62.16% in pulmonary and 21.8% in extra-pulmonary samples). We could not detect mycobacterial DNA in one AFB positive
pus sample and four FNA samples, while AFB could be demonstrated in these by smear examination. All these 5 specimens were tested for the presence of substances inhibiting Taq polymerase by spiking duplicate samples with \textit{M. tuberculosis} DNA and found to be negative for presence of inhibitors. In FNA samples direct smears were made from the aspirated material while the needle washings were used for PCR assay. The only possible reason for negative result by PCR may be unequal distribution of AFB in aspirated material in these samples.

Absence or the presence of fewer copies of target sequence in some strains of \textit{M. tuberculosis} has already been reported\textsuperscript{14-17}. Das \textit{et al}\textsuperscript{18} found that a large number of clinical isolates (40\%) of \textit{M. tuberculosis} from south India had either single copy or no copy of IS6110.

The findings of the present study showed that the procedures based on \textit{in vitro} amplification of mycobacterial DNA, using the PCR might be helpful in the early diagnosis of tuberculosis as these were rapid, sensitive and specific than the bacteriological techniques. Application of PCR could be particularly useful in those cases where the disease was clinically suspected but the histological changes and/or other diagnostic test results were equivocal.

<table>
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<th>Samples</th>
<th>AFB+ve</th>
<th>PCR+ve</th>
<th>AFB+ve</th>
<th>AFB+ve</th>
<th>AFB-ve</th>
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AFB, acid fast bacilli; FNA, fine needle aspirate; BAL, broncho alveolar lavage; CSF, cerebro spinal fluid
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References


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