Application of nested polymerase chain reaction (nPCR) using MPB 64 gene primers to detect *Mycobacterium tuberculosis* DNA in clinical specimens from extrapulmonary tuberculosis patients

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**Background & objectives:** The conventional culture technique for diagnosis of extrapulmonary tuberculosis is time consuming. In order to find a sensitive and rapid technique nested polymerase chain reaction (nPCR) targeting the conserved MPB 64 gene of *Mycobacterium tuberculosis* was evaluated for detection of *M. tuberculosis* DNA directly from clinical specimens of extrapulmonary origin.

**Methods:** A total of 400 clinical specimens from clinically suspected cases of extrapulmonary tuberculosis and 30 control specimens of nontuberculous aetiology were processed by smear and culture and by nPCR technique for detection of *M. tuberculosis*. The specimens were divided into 3 groups, (group I-280 specimens [104 peritoneal fluid (PF), 120 cerebrospinal fluid (CSF), 44 lymph node biopsies 3 pericardial fluid and 9 other biopsy specimens], group II - 120 aqueous humour (AH) from idiopathic granulomatous uveitis cases, and group III - 30 control specimens (10 CSF and 20AH).

**Results:** The conventional culture was positive only in 16 of 400 specimens. The overall positivity of nPCR was 35.2 per cent (141/400). Among the 280 specimens from extrapulmonary lesions (group I), 15 were bacteriologically positive, while 115 of 265 bacteriologically negative specimens (43.4%) were positive by nPCR. All the 30 control specimens were negative by nPCR.

**Interpretation & conclusion:** The nPCR using MPB64 gene primers might be a rapid and reliable diagnostic technique for detection of *M. tuberculosis* genome in clinically suspected extrapulmonary tuberculosis specimens, as compared to the conventional techniques.

**Key words** Body fluids - DNA detection - extrapulmonary tuberculosis - *Mycobacterium tuberculosis* - nested polymerase chain reaction

An essential element in the management of extrapulmonary tuberculosis is the availability of rapid, sensitive and specific identification of the causative agent. The laboratory diagnosis is largely based on direct microscopy and culture for mycobacterium. Direct microscopic examination to detect acid-fast bacilli (AFB) has very low sensitivity and often less specific. The gold standard of isolation
of mycobacterium is not only time-consuming but also has low sensitivity in case of extrapulmonary tuberculosis. As an alternative to these classical methods, new nucleic acid-based technologies showed promises as more rapid, sensitive and specific means of detection and identification of mycobacteria. Several groups have reported the usefulness of PCR using IS6110 primers to detect M. tuberculosis complex DNA in archival specimens and specimens from patients with pulmonary and extrapulmonary tuberculosis. We found PCR with this most widely used IS6110 primers though specific, but with low sensitivity. In studies from Tuberculosis Research Centre, Chennai in south India, 42.7 per cent isolates of M. tuberculosis were shown to either lack or have only a few copies of IS 6110. Other workers from India and other geographical regions of the world have also reported similar results. We undertook the present study with the objective to improve the diagnostic ability using more sensitive nested PCR technique on extrapulmonary clinical specimens for detection of M. tuberculosis genome with primers targeting the MPB64 gene for the immunogenic protein found only in culture filtrates of M. tuberculosis and occasional isolates of M. bovis BCG. We earlier applied this method on ocular specimens for detection of mycobacterial DNA. This nPCR was absolutely specific and sensitive enough to detect even a single bacillus in the specimen. In this study the diagnostic value of this nPCR technique was evaluated for detection of M. tuberculosis DNA in different types of clinical specimens of paucibacillary conditions from clinically confirmed tuberculous lesions of extrapulmonary origin.

**Material & Methods**

The study was carried out from January 1999 to December 2002.

**Clinical specimens:** Three groups of extrapulmonary clinical specimen (430) were included in the study. Group I, consisting of 280 clinical samples of extrapulmonary origin [104 peritoneal fluids (PF), 120 cerebrospinal fluids (CSF) 44 cervical lymph node biopsies as fine needle aspiration biopsy (FNAB), 9 tissue biopsy specimens (liver - 4, endometrium - 1, synovium - 2, cyst wall of ovary - 1 and paravertebral tissue - 1), and 3 pericardial fluid] was processed for detection of acid-fast bacilli (AFB) by smear, culture and for M. tuberculosis genome by nPCR. All the specimens of this group were from lesions clinically and/or radiologically diagnosed as tuberculosis. The extrapulmonary specimens collected by the clinicians in sterile containers were received in our laboratory.

Group II consisting of 120 aqueous humour (AH) aspirates from idiopathic granulomatous uveitis was also tested to exclude tuberculosis. Group III consisted of 30 clinical samples collected from lesions that had definitive evidences of non tuberculous disease conditions, was included as negative control. They were from clinically diagnosed cases of viral meningitis - 9 (CSF), cysticercosis of meninges - 1 (CSF), and 20 AH from persons undergoing cataract surgery.

**Bacteriological procedures:** All specimens were processed by standard bacteriological procedures for detection of AFB. In brief, the aspirated fluid specimens like ascitic fluid, cerebrospinal fluid were concentrated by centrifugation and if visible sediments were not present in the specimens, cytospun smears were made to concentrate the cellular material and infective agents present in the specimen. A part of the deposit thus obtained was stored at -20°C in a biofreezer until processed for nPCR within 24 h and rest was processed for smear stained by Ziehl-Nielsen method and culture on Lowenstein Jensen medium (LJ) in duplicates for AFB. Cytospinned smears and cultures of ocular specimens were directly made on microscopic slides and inoculated onto LJ medium in duplicates. Tissue biopsy specimens were cut into tiny bits using sharp curve scissors and Bard-Parker blades (Glasvan, Hindustan Syringes and Medical Devices Ltd, Faridabad, India) in sterile petriplates and homogenized in a glass tissue grinder and direct smears were made from the homogenized suspension and inoculated onto LJ medium in duplicates.

**Nested polymerase chain reaction (nPCR):** nPCR using primers targeting for MPB 64 protein of M. tuberculosis was performed as described earlier by us. Nested primers (custom synthesized by Bangalore Genei, Bangalore, India) included outer sense primers for first round of amplification consisting of one outer
set of primers and one inner set of primers. upstream outer primer: 5’ TCCGCTGCCAGTCGTCTTCC 3’ and downstream outer primer: 5’ GTCCTCGCGAGTCTAGGCCA 3’, and inner primer set for the second round consisting of upstream primer 5’ ATTTGTGAAGGTGAACTGAG 3’ and downstream primer: 5’ AGCATCGAGTCTCGATCGCGGA 3’. Outer primer set codes for 240 bp region (nucleotides 460-700) from MPB64 gene, while the inner primer set codes for a region (200 bp) within the 240 bp (481-661 nucleotides). The procedures followed for extraction of DNA from the standard strains of mycobacteria and from the clinical specimens were the same as carried out in our earlier studies. The nPCR was found to be specific and sensitive enough to detect 0.25 femto gram of H37Rv genomic DNA corresponding to a single bacillus of \textit{M. tuberculosis}. nPCR amplified \textit{M. tuberculosis} DNA to give 240 bp product after the first round and 200 bp product after the second round. Analysis of the results on agarose gel electrophoresis and visualization of the amplified products over the UV transilluminator (Pharmacia, Sweden) was done for both the 1st and 2nd round amplification products.

Precautions to prevent amplicon contamination: Adequate and rigorous precautions were taken to prevent amplicon contamination. Separate rooms were used for preparation of DNA, its amplification and analysis of the amplified product. PCR preparation was performed in a laminar flow workbench with single use aliquots of reagents, and dedicated pipettes. The microfuge tubes and mineral oil aliquots were double sterilized.

Statistical analysis: The results were analysed by Non-parametric binomial test.

Results

\textbf{PCR and conventional bacteriological investigations:} The over all positivity of nPCR in groups I and II was 35.2 per cent (141 of 400) whereas the culture was positive only in 16 samples (4%) (Table). The specimen wise analysis of sensitivity of nPCR showed a higher percentage of clinical sensitivity in PF (60.5%), in CSF (30.8%) and in AH (10.83%) and in 50 per cent of other biopsy specimens than by culture PF (2.9%) CSF (0.8%) except in lymph node specimen where the culture was positive in 20 per cent of the cases. All the 30 control specimens were negative by nPCR. Among 280 specimens from group I (from extrapulmonary lesions clinically of tuberculous origin), 15 were bacteriologically positive (1 by smear and 14 others by culture). In this group, 115 (43.4%) of 265 bacteriologically negative specimens were positive by nPCR. One peritoneal fluid was negative by nPCR, though it grew \textit{M. tuberculosis}. nPCR was performed on this specimen after spiking it with \textit{M. tuberculosis} DNA and PCR inhibitors were not present in it. Among the 9 tissue biopsy specimens, 1 paravertebral tissue was negative by smear, positive by culture and nPCR, and 4 by nPCR (one each of liver, cyst wall of the ovary, synovium and paravertebral tissue). In cases of clinically suspected meningitis cases, of the 119 microbiologically negative specimens by conventional techniques nPCR detected \textit{M. tuberculosis} genome in 32.4 per cent (34 CSF) increasing the sensitivity by 31 per cent (Non-parametric binomial test showed significant difference, \textit{P}<0.0001). In our study, in group I cases where proper clinical assessment was made for a clinical diagnosis of tuberculosis of the 104 peritoneal fluid specimens, only nPCR approach was able to detect the genomic DNA of \textit{M. tuberculosis} in 100 specimens (62.9%), though there was one case negative by PCR but was positive by culture. Thus, only in 17 of 400 (4.2%) (positive by smear in 1 and by culture in 16) specimens conventional microbiological diagnosis was possible whereas nPCR detected presence of mycobacterial DNA in 126 (35.5%) additional cases significantly increasing the diagnostic sensitivity by 31.1 per cent. One of the 3 pericardial fluid specimens was nPCR positive. Of the 44 lymph node FNAB specimens, 10 (22.7%) were bacteriologically positive, nPCR was negative in an AFB smear positive specimen and the bacterium could be a non-tuberculosis mycobacterium (NTM). Ten (29.4 %) of 34 smear and culture negative lymph node FNAB samples were nPCR positive (Non-parametric binomial test showed significant difference, \textit{P}=0.05 in 34). Except one lymph node FNAB specimen (smear and culture positive for \textit{M. tuberculosis}) which was positive by the end of first round of nPCR, all other nPCR positive specimens were positive only after the 2nd round of the amplification procedure, indicating the paucibacillary condition of the specimens.
Among the 120 AH specimens from patients of idiopathic granulomatous uveitis (group II), 2 were smear, culture and nPCR positive, and 11 (9.3%) of 118 bacteriologically negative AH specimens were nPCR positive for *M. tuberculosis* (Non-parametric binomial test showed significant difference, \(P<0.0001\)). All the 30 control specimens from non tuberculous lesions (CSF-10, AH-20) were negative by both bacteriological methods and nPCR. The results of agarose gel electrophoresis of the first and second set of nPCR of some of the clinical specimens are shown in the Fig.

### Discussion

Our results clearly demonstrated that nPCR had a significant advantage over the conventional techniques to detect the presence *M. tuberculosis* in samples of clinically suspected extrapulmonary tuberculosis patients as a rapid and sensitive diagnostic test. The single step PCR using primers targeting MPB 64 gene applied by Manjunath *et al*\(^\text{15}\) on pulmonary TB specimens was reported to be a highly specific and sensitive technique. Seth *et al*\(^\text{16}\) conducted a double-blind study on extrapulmonary specimens and evaluated the PCR with one round primers of MPB 64 gene considering clinical assessment as the gold standard in cases of tuberculous meningitis, and reported that PCR was positive in 85 per cent samples.

The main advantage of using the nested amplification is the minimal detection limits in the range of 10-100 bacilli per sample without the use of radioisotopes and in our set up nPCR was standardized to detect even one single bacillus thus increasing the

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**Table.** Results of microbiological investigations and nPCR (using primers coding for MPB 64 gene) for detection of *M. tuberculosis* on 400 extrapulmonary specimens

<table>
<thead>
<tr>
<th>Groups and types of specimens</th>
<th>Total no.</th>
<th>Smear pos.</th>
<th>Smear neg</th>
<th>Smear pos</th>
<th>Smear neg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n= 400</td>
<td>Culture pos</td>
<td>Culture neg</td>
<td>Culture pos</td>
<td>Culture neg</td>
</tr>
<tr>
<td></td>
<td>n = 8(^\star) nPCR</td>
<td>n = 1 nPCR</td>
<td>n = 8(^\star) nPCR</td>
<td>n = 1 nPCR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pos 0</td>
<td>Neg 1</td>
<td>Pos 8</td>
<td>Neg 0</td>
<td>Pos 7</td>
</tr>
<tr>
<td>Group I</td>
<td>Peritoneal fluid</td>
<td>104</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Extra-pulmonary specimens</td>
<td>Cerebrospinal fluid</td>
<td>120</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>(n=280)</td>
<td>Biopsy tissue</td>
<td>9(^##)</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Pericardial fluid</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lymph nodes (FNAB)</td>
<td>44</td>
<td>-</td>
<td>1(^$)</td>
<td>5</td>
</tr>
<tr>
<td>Group II</td>
<td>Aqueous humour (AH)</td>
<td>120</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Idiopathic granulomatous uveitis.</td>
<td>(n=120)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pos, Positive; Neg, negative. Group I: 115 (43.8 %) of 265 bacteriologically negative specimens were positive by nPCR. \(^\star\) The 16 isolates were identified as *M. tuberculosis*. \(^\text{15}\) *M. tuberculosis* was isolated from this specimen which was negative by nPCR and did not show any PCR inhibitors. \(^\text{16}\)Culture & nPCR in a paravertebral tissue & nPCR only in one each of liver, endometrium, synovium and cyst wall of ovary were positive. \(^\text{17}\)One lymph node specimen showing AFB in smear was negative by nPCR & this bacterium might be a non-tuberculous mycobacterium. Group II: 11 (9.3%) of 118 AH bacteriologically negative specimens from idiopathic granulomatous uveitis were positive by nPCR.
sensitivity of detection\textsuperscript{13}. The specificity of the nPCR was confirmed by the negative results in the 30 specimens from non tuberculous lesions in group III patients.

At present, there is no adequate comparison to evaluate a PCR assay for diagnosis of tuberculosis other than culture results or clinical assessment in cases of extrapulmonary tuberculosis. Cultures often have been negative mainly because of the specimens being paucibacillary or presence of non viable mycobacteria in them. Most often, clinical judgement, Mantoux test and radiological assessment are relied upon for the management of extrapulmonary tuberculosis. In this study, the low positivity (4\%) of the conventional culture technique could be either due to the presence of non viable bacteria in the clinical specimens or due to the use of only the solid LJ medium. We restricted to only LJ medium for isolation of \textit{M. tuberculosis} based on our earlier experience (unpublished observation).

Portillo \textit{et al}\textsuperscript{17} evaluated IS6110 based PCR method to detect \textit{M. tuberculosis} and found 90 per cent sensitivity in CSF and pleural fluid; ascitic fluid and other extrapulmonary specimens greatly enhanced the sensitivity of conventional smear and culture methods, and concluded that PCR could be a highly specific and sensitive technique in the detection of \textit{M. tuberculosis} from extrapulmonary specimens. Few reports\textsuperscript{18,19} are available emphasizing the importance of PCR for diagnosis of intraocular tuberculosis, as establishment of a firm diagnosis of ocular tuberculosis by histological and microbiological methods is difficult. In our study successful therapeutic management was possible in patients with idiopathic granulomatous uveitis positive by nPCR only. The test also was helpful in excluding tuberculous uveitis in others.

We agree with Lazraq \textit{et al}\textsuperscript{20} that PCR results should be interpreted in conjunction with the clinical and radiological data when it is discordant with the conventional methods of results. The decision about when and how to use the PCR technique for diagnosis of tuberculosis should be individualized. The test may enhance diagnostic certainty but should be interpreted in a clinical context based on the local laboratory performance.

To conclude, nPCR using primers for MPB 64 gene appears to be a useful technique for detection of \textit{M. tuberculosis} in paucibacillary extrapulmonary specimens.

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**References**


