Evaluation of an in-house-developed PCR for the diagnosis of tuberculous meningitis in Indian children

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Early and rapid detection of the causative organism is necessary in tuberculosis, particularly tuberculous meningitis, as the disease affects mainly children and if untreated or improperly treated can cause severe central nervous system disorders and can often be fatal. An in-house-developed PCR technique was developed for the detection of Mycobacterium tuberculosis DNA, in which the target for amplification was a 340 bp nucleotide sequence located within the 38 kDa protein gene. The test can detect as small an amount of DNA as 10 fg, which is equivalent to two to three organisms, and is highly specific. Amplified product was detected by ethidium bromide staining after electrophoresis and Southern hybridization. Evaluation of test sensitivity and specificity was carried out using acid-fast bacilli-positive sputum samples from patients with pulmonary tuberculosis and an equal number of non-tuberculosis patient samples as negative controls. In a double-masked study 30 cerebrospinal fluid samples from tuberculous meningitis patients and 30 samples from non-tuberculous meningitis patients were investigated. Out of the 30 samples 22 were positive by ethidium bromide-stained gel electrophoresis and 27 gave positive results by Southern hybridization. All of the 30 control samples showed negative results. The sensitivity of this PCR was 90 % and specificity, 100 %.

INTRODUCTION

Tuberculous meningitis (TBM), which occurs in 7–12 % of tuberculosis patients in developing countries, involves the central nervous system (CNS) and is one of the most severe forms of extra-pulmonary tuberculosis (Tandon, 1978). It is common among children and is often a post-primary manifestation that develops 2–12 months after primary infection. Rapid detection of the causative organism is of paramount importance in TBM as the disease can be fatal and clinical outcome depends heavily on the stage at which treatment is initiated (Leonard & Des Prez, 1990).

Diagnosis of TBM is presumptive and is based on clinical symptoms, neurological signs, cerebrospinal fluid (CSF) findings, CT scans and the response to anti-tuberculosis drugs (Kennedy & Fallon, 1979). Conventional methods like microscopy and culture, although considered as gold standards, are quite inadequate. Acid-fast staining requires a large number of organisms (>10⁴ cells ml⁻¹) and it has been reported that the positivity of acid-fast smears in children with tuberculosis is low (Delacourt et al., 1995), and in TBM, in particular, it is only 8–10 % (Davis et al., 1993). Culture requires 6–8 weeks due to the slow growth of Mycobacterium tuberculosis and is often negative.

Various techniques have been reported for the diagnosis of TBM, including adenosine deaminase assay (Lopez-Cortes et al., 1995), radioimmunoassay (Ashtekar et al., 1987; Kadival et al., 1987a) and ELISA (Kadival et al., 1986) for the detection of mycobacterial antigens and antibodies to the mycobacterium (Mathai et al., 1991; Kadival et al., 1994). These techniques show promise but the sensitivity and often the specificity reported is insufficient and needs improvement.

Rapid techniques based on nucleic acid amplification such as PCR are more sensitive and specific as they attempt to detect specific DNA sequences of the organism. We have already described a PCR assay using a 340 bp sequence of the 38 kDa protein gene as the target sequence for amplification (Kadival et al., 1995, 1996). The 38 kDa protein is an important secretory protein of M. tuberculosis (Kadival et al., 1987b; Young et al., 1996); it is involved in phosphate transport and

Abbreviations: AFB, acid-fast bacilli; CNS, central nervous system; CSF, cerebrospinal fluid; ECL, enhanced chemiluminescence; TBM, tuberculous meningitis.
is highly specific for *M. tuberculosis* (Anderson et al., 1990). The objective of the current study was to evaluate the role of this PCR technique in the diagnosis of TBM.

**METHODS**

**Patients and clinical specimen.** CSF samples from 60 patients (28 males, 32 females; age range 5 months to 12 years) were received from the paediatric department of the KEM Hospital, Mumbai. All the samples were received in ice and were stored at $-20^\circ$C until further analysis. Samples were collected on the basis of signs and symptoms that indicated involvement of the CNS. The study was double-masked, i.e. the laboratory was not aware of the clinical data and the clinician was not aware of the laboratory data until all analysis was complete. All the samples, and clinical and other data were kept by one of the authors (M.A.J.) and after coding, the samples were transferred to the laboratory for PCR. The personnel performing the PCR were not aware of the identities of the samples until the test had been performed on all the samples. Acid-fast microscopy was performed by the Ziehl–Neelsen method and culture was performed on Lowenstein–Jensen slants for all samples. Two hundred microlitres of CSF was centrifuged and the precipitate was used for culture.

**Diagnosis.** For all of the patients a detailed medical history was obtained that included: (i) presentation of clinical signs and symptoms, such as fever, headache, neck stiffness, vomiting and alterations of sensorium; (ii) BCG vaccination status, past history of tuberculosis or presence of a contact; (iii) general and systemic examination including a detailed CNS examination; (iv) routine laboratory tests that included complete blood count, CSF cytology, proteins and sugar, erythrocyte sedimentation rate, Mantoux test and chest X-ray; (v) CT scans of the brain, both plain and contrast. Criteria generally used to classify patients as TBM are given in Table 1 (Doerr et al., 1995; Lang et al., 1998). The patient population classified as TBM fulfilled major criteria A and B and any two of the minor criteria (1, 2, 3, 4 and 5).

**TBm.** CT scans of the brain revealed basal exudates and hydrocephalus.

**Table 1.** Criteria used for classification of the likelihood of TBM

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<td>(A) Abnormal neurological signs and symptoms, e.g. fever, neck stiffness, altered sensorium, convulsions</td>
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<tr>
<td>(B) Abnormal CSF cytology and biochemistry; pleocytosis (&gt;10 leucocytes ml$^{-1}$), elevated protein concentration (&gt;40 mg dl$^{-1}$), amount of glucose in CSF less than 60% of serum glucose</td>
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<td>(C) Positive Ziehl–Neelsen carbol fuschin smear and culture-positive for <em>M. tuberculosis</em> but negative for any other bacteria</td>
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<th>Minor criteria</th>
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<td>(1) Close contact with a known case of active tuberculosis</td>
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<td>(2) Positive Montoux skin test</td>
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<td>(3) Microbiological or radiographic evidence of active tuberculosis at extra-neural site</td>
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<td>(4) Abnormal findings in CT</td>
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<td>(5) Clinical response to anti-tuberculous drugs</td>
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typical of TBM, in six and 25 of the 30 TBM patients, respectively. Chest X-ray revealed the presence of progressive primary complex in two patients while one had extra pulmonary tuberculosis (tuberculous cervical lymphadenopathy). Four patients had tuberculosis contacts in the family and two were positive by the Mantoux test (>10 mm). CSF cytology showed pleocytosis with lymphocyte predominance and increased proteins (>40 mg dl$^{-1}$). Most importantly all patients showed clinical improvement with anti-tuberculosis treatment, leading to improved signs and symptoms. All samples from TBM cases were, however, negative for acid-fast bacilli (AFB) and culture.

**Controls.** Thirty patients were classified as non-TBM controls. None of the patients were positive for AFB, while three grew other bacterial cultures (*Staphylococcus aureus* in one and *Pseudomonas aeruginosa* in two others). The Mantoux test was negative in all of them and chest X-rays did not show any abnormality. There was no evidence of tuberculosis in any of the patients. CSF cytology revealed pleocytosis with polymorphonuclear cells predominating. Twenty-four of the controls were classified as cases of pyogenic meningitis, four were viral encephalitis, one was aseptic meningitis and one was neurosarcoidosis.

**Preparation of CSF for PCR.** Two hundred microlitres of neat CSF sample was treated with proteinase K ($10$ mg ml$^{-1}$, in $200$ mM Tris/HCl, pH 8:3) at $65^\circ$C overnight and boiled for $10$ min. Ten microlitres of the sample was directly used for PCR.

**Oligonucleotide primers and probes.** Primers for amplification of the $340$ bp region of the $38$ kDa protein gene of *M. tuberculosis* were procured from Isgen. The primers were synthesized by an automated DNA synthesizer on the basis of phosphoramidite chemistry. The two primers were designated KD1 (5’ CCA AGC AGG ATC TGC GCC GAG GCT 3’) and KD2 (TTG ATG ATC CCC TAG CCG TTC CTC 3’) and in addition a biotinylated internal probe KD3 (5’ TGC GCC GAG GAC ACA CCG GGC TGC GTG GCC TAT 3’) was also synthesized.

**DNA amplification by PCR.** Fifty microlitres of PCR mixture, containing $10$ mM Tris/HCl, pH 8:3, $50$ mM NaCl, $0:01$% gelatin, 0:2 mM of each dNTP, 0:5 mM of each primer KD1 and KD2, 1 U of Taq DNA polymerase (AmpliTaQ, Perkin Elmer, Cetus) and 50 ml of mineral oil, was added to each tube. Ten microlitres of the treated CSF sample was added last. The test was carried out in duplicate where the second sample was spiked with $100$ fg of *M. tuberculosis* H37 Rv DNA. The mixtures were then subjected to $40$ cycles of PCR in a programmable thermal cycler (MJ Research). Each cycle comprised denaturation at $94^\circ$C for $1$ min, annealing at $64^\circ$C for $1$ min and primer extension at $72^\circ$C for $1$ min. After the $40$ cycles were completed, additional extension for $10$ min at $72^\circ$C was carried out.

**Detection of amplified product.** An aliquot (15 ml) from the PCR was analysed by gel electrophoresis in 2% agarose gel in Tris/borate EDTA (TBE) buffer for $2$ h at $70$ V then stained with ethidium bromide and visualized under a transilluminator. For Southern hybridization the gel was soaked in $0:25$ M HCl for $10$ min and rinsed with distilled water. The gel was denatured in $0:4$ M NaOH for $30$ min and the DNA transferred overnight to a nylon membrane using $10$ X SSC by a capillary method. The membranes were exposed to UV light for $3$ min for immobilization of DNA. Pre-hybridization was carried out at $60^\circ$C for $4$ h in a solution containing $6$ X SSC, $0:5$% SDS, $5$ X Denhardt’s reagent and $100$ mg ml$^{-1}$ of salmon sperm DNA as a blocking reagent. Hybridization was carried out at $60^\circ$C overnight in a solution containing biotinylated internal probe KD3. Subsequently the membranes were washed twice with $0:1$ X SSC and $0:1$% SDS at room temperature for $5$ min and twice at $60^\circ$C for $10$ min. The membranes were then incubated in $1:4000$ diluted Strepavidin-POD conjugate at $42^\circ$C for $1$ h and washed with $2$ X SSC and $0:5$% SDS twice at $42^\circ$C and once at room temperature for $5$ min. Hybrids were visualized using an
enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech) after the membranes were exposed to X-ray film for autoradiography results.

RESULTS AND DISCUSSION

PCR was performed in duplicate for each sample and one tube was spiked with standard M. tuberculosis DNA to identify inhibition of Taq polymerase. A test was considered as inhibited if both sample and spike gave negative PCR, as positive if both gave positive PCR, and as negative if the test gave negative and the spike gave positive PCR. Twenty-seven of the 30 TBM samples were positive (sensitivity 90 %) while all the controls were negative (specificity 100 %). Out of the 27 positive results, 22 samples showed a strong band of amplification product at 340 bp in ethidium bromide-stained agarose gel and five samples showed uncertain weak bands which were confirmed as positive by Southern hybridization with the internal oligonucleotide probe by the ECL technique. Thus our test gave 73.3 % sensitivity when amplicon detection was done by ethidium bromide staining alone (22/30) and increased to 90 % sensitivity (27/30) when Southern hybridization was also used. Fig. 1 presents the Southern hybridization results of 14 samples from the TBM group. The samples in lanes 2, 5, 8, 12 and 13 showed poor or no band on ethidium bromide staining (Fig. 1a) but showed a clear band on Southern hybridization (Fig. 1b). None of the samples showed inhibition. The positive predictive value thus was 100 % and the negative predictive value was 90 %.

TBM is one of the common clinical manifestations of extra-pulmonary tuberculosis. The incidence of TBM in developing countries like India has shown an upward trend during the past two decades. Though TBM can occur at any age, it is common in infants and children. The population in this study was a paediatric population between 6 months and 12 years. In an earlier study 20 % of paediatric patients who died in the past two decades. Though TBM can occur at any age, it is common in infants and children. The population in this study was a paediatric population between 6 months and 12 years. In an earlier study 20 % of paediatric patients who died in 30 TBM samples were positive (sensitivity 90 %) while the 30 patients diagnosed with TBM 27 showed positive PCR results, i.e. a sensitivity of 90 % was achieved. One of the reasons for the high sensitivity of our study could be that the patients were classified as advanced cases of TBM.

Definitive diagnosis of TBM is possible by AFB and culture. However, in TBM in children many reports indicate positive results in only 8–10 % and 29–48 % for AFB and culture, respectively (Stamos & Rowley, 1995). In the Indian sub-continent, smear and culture positivity was observed in only 15–20 % of patients with TBM (Tandon, 1978).

The use of molecular biology techniques in the diagnosis of tuberculosis started with the use of DNA probes (Grange, 1989), which were less sensitive than even the existing conventional tests. They have been increasingly used for this purpose since the introduction of the PCR technique. The majority of the investigators performing PCR-based diagnosis of tuberculous meningitis have used insertion sequence IS6110 as a target (Miornier et al., 1995; Caws et al., 2000; Narayanan et al., 2001). The principal reason for using IS6110 is the presence of multiple copies in the M. tuberculosis genome (Van Soolingen et al., 1991), which was thought to confer higher sensitivity. It has, however, been shown that there are M. tuberculosis strains originating from India which do not contain IS6110 (Van Soolingen et al., 1993). Our laboratory has reported previously the development of a PCR using the 38 kDa gene as the target sequence. This has been shown to give a sensitivity of 10 fg of DNA, the equivalent of two to three organisms, and is highly specific (Kadival et al., 1995, 1996).

The present study was performed as a double-masked study and demonstrates that PCR is a rapid and powerful technique for the accurate diagnosis of tuberculous meningitis. Of the 30 patients diagnosed with TBM 27 showed positive PCR results, i.e. a sensitivity of 90 % was achieved. One of the reasons for the high sensitivity of our study could be that the patients were classified as advanced cases of TBM.

This sensitivity is comparable to previous studies by Liu et al. (1994) (90 %) and Seth et al. (1996) (85 %) while it is much superior to various other studies: Ahuja et al. (1994) (75 %), Lin et al. (1995) (70 %), Donald et al. (1993) (63 %), Bonington et al. (1998) (60 %), Kox et al. (1994) (48 %), Miornier et al. (1995) (54 %) and Nguyen et al. (1996) (32 %). These earlier studies used IS6110 or the MPB 64 or 65 kDa protein genes as their target for amplification. The study conducted by Lee et al. (1994) showed high false-positives with IS6110 (62 %) and the 65 kDa protein gene (33 %). The lower sensitivities and specificities found by earlier investigators could be due to the methods used for extraction of DNA and amplicon detection.

Most of the earlier investigators isolated DNA from a 12 000 g pellet of CSF using either phenol/chloroform or Boon’s procedure. But M. tuberculosis is present in CSF as free DNA in very small amounts, if at all, and therefore such extraction

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Fig. 1. Southern blot hybridization of amplicon detected by ECL. Samples in lanes 1–14 are PCR products of TBM patient samples and lane 15 is positive control 100 fg M. tuberculosis H37Rv DNA. (a) Ethidium bromide staining of the samples observed on a transilluminator (reverse picture), (b) Autoradiography results after Southern blot hybridization with internal biotinylated probe KDS and ECL detection. Bands in lanes 2, 5, 8, 12 and 13 were not clear in ethidium bromide staining but are seen clearly on hybridization.
processes will result in false-negative results, which reduce the sensitivity. In this study we used whole CSF samples as suggested by Kox et al. (1995) for DNA PCR. Kox et al. (1995) stated that the volume of the CSF sample is very important for good results and at least 2 ml should be processed. However, in the paediatric group that we were dealing with (5 months–12 years), it was difficult to get a large amount of CSF after lumbar puncture and our test has proved that smaller volumes (200 µl) when used directly can give good results.

The sensitivity of PCR was improved by using confirmation by Southern hybridization, which indicates the need for such hybridization rather than detection by ethidium bromide staining alone. All 30 patients, who were classified as non-TBM, were negative by our test, which demonstrates 100% specificity. All the samples showing negative results were true negatives as the spiked samples containing the clinical sample and standard DNA were positive for PCR, thus confirming that there was no inhibition of Taq polymerase. These results showing no false-positives indicate no cross- or carryover-contamination in the PCR test. This was achieved by performing various procedures of the PCR in different rooms (physical separation), dedicated pipettes and other equipment for each laboratory, and enforcing good laboratory practice.

Commercially available kits such as MTD Gene-Probe and Roche AMPLICOR, which are PCR-based tests, when used for diagnosis of TBM have been shown to give low sensitivities of 33% and 60%, respectively (Lang et al., 1998; Bonington et al., 1998).

In conclusion our PCR test is very specific and sensitive, and can be used for rapid and accurate diagnosis of TBM especially in India.

REFERENCES


