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

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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...
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 °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, 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).

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

<table>
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<tr>
<th>Major criteria</th>
<th>Minor criteria</th>
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<td>(A) Abnormal neurological signs and symptoms, e.g. fever, neck stiffness, altered sensorium, convulsions</td>
<td>(1) Close contact with a known case of active tuberculosis</td>
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<td>(B) Abnormal CSF cytology and biochemistry; pleocytosis (&gt;10 leucocytes mm⁻³), elevated protein concentration (&gt;40 mg dl⁻¹), amount of glucose in CSF less than 60 % of serum glucose</td>
<td>(2) Positive Montoux skin test</td>
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<tr>
<td>(C) Positive Ziehl–Neelsen carbol fuschin smear and culture-positive for <em>M. tuberculosis</em> but negative for any other bacteria</td>
<td>(3) Microbiological or radiographic evidence of active tuberculosis at extra-neural site</td>
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**TBM.** CT scans of the brain revealed basal exudates and hydrocephalus.

**Preparation of CSF for PCR.** Two hundred microlitres of neat CSF sample was treated with protease K (10 mg ml⁻¹, in 200 mM Tris/HCl, pH 8.3) at 65 °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 Losen. The primers were synthesized by an automated DNA synthesizer on the basis of phosphoramidite chemistry. The two primers were designated KD1 (5′ CCA AGC AAG ATC CCG AGG GCT 3′) and KD2 (TTG ATG ATC CCG TAG CCG GTG TOC 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 µM of each primer KD1 and KD2, 1 U of Taq DNA polymerase (AmpliTaq, Perkin Elmer, Cetus) and 50 µl 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 °C for 1 min, annealing at 64 °C for 1 min and primer extension at 72 °C for 1 min. After the 40 cycles were completed, additional extension for 10 min at 72 °C was carried out.

**Detection of amplified product.** An aliquot (15 µl) 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 10X 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 °C for 4 h in a solution containing 6× SSC, 0.5 % SDS, 5× Denhardt’s reagent and 100 µg ml⁻¹ of salmon sperm DNA as a blocking reagent.

Hybridization was carried out at 60 °C overnight in a solution containing biotinylated internal probe KD3. Subsequently the membranes were washed twice with 0× SSC and 0 × 5 % SDS, 5× Denhardt’s reagent and 100 µg ml⁻¹ of salmon sperm DNA as a blocking reagent. Hybrization was carried out at 60 °C overnight in a solution containing biotinylated internal probe KD3. Subsequently the membranes were washed twice with 0× SSC and 0.1 × 5 % SDS, 5× Denhardt’s reagent and 100 µg ml⁻¹ of salmon sperm DNA as a blocking reagent.

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enlarged 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 1, 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 had involvement (Udani & Dastur, 1970). Classical TBM evolves through three stages: (1) prodromal stage with non-specific symptoms, (2) stage of meningeal irritation with headache and vomiting, (3) stage with diffuse or focal cerebral involvement with unconsciousness, stupor or coma, and raised intracranial pressure. All the patients in the TBM group were either in stage 2 or stage 3, i.e. in advanced stages 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 (Miorner *et al.*, 1995; Caws *et al.*, 2000; Narayan *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%), Miorner *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
procedures 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


