THE DIAGNOSIS OF TUBERCULOUS MENINGITIS USING THE POLYMERASE CHAIN REACTION

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ABSTRACT

Aim: DNA amplification by the polymerase chain reaction (PCR) was evaluated as a means for rapid diagnosis of tuberculous meningitis (TBM).

Methods: A 240 bp region (nts 460-700) from the MPB 64 protein coding gene specific for Mycobacterium tuberculosis (TB) was selected for amplification. Nineteen clinical samples were studied. Six were obtained from patients with TBM diagnosed by culture (4/6) or by response to therapy (2/6). The remaining 13 samples were obtained from patients with febrile seizu es (8/13), aseptic meningitis (3/13) and septic meningitis (2/13), and these served as negative controls.

Results: We detected TB DNA in all the 6 CSF specimens obtained from patients with TBM. PCR alone was sufficient to detect TB DNA in 5 of these 6 samples. However, one sample was positive only when PCR was followed by oligonucleotide hybridisation. In the 2 patients whose CSF were obtained only after commencement of TB therapy, TB cultures were negative but positive on PCR nd oligoprobe labelling. The diagnosis of TBM was confirmed based on their remarkable response to therapy. Twelve of the thirteen negative controls were TB DNA negative. There was one false positive sample, which was thought to be due to TB DNA contamination.

Conclusion: Taken together, our results indicate that DNA amplification using PCR, followed by oligonucleotide hybridisation offers a rapid (5 working days) means of diagnosis of TBM, provided care is taken to ensure that cross contamination of DNA samples is avoided.

Keywords: tuberculous meningitis, polymerase chain reaction, oligonucleotide hybridisation.

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INTRODUCTION

Tuberculous meningitis (TBM) caused by *Mycobacterium tuberculosis* (TB) can be diagnosed by clinical manifestations, smear examination and culture growth of the bacteria. Although detection by direct acid-fast staining for bacilli offers the most rapid means of detection of TB organisms, the relatively few organisms in the CSF make detection difficult as a smear examination requires a minimum of 10,000 organisms/ml[1]. The absolute diagnosis of TBM therefore continues to depend on culture techniques which require 6-8 weeks for culture identification. As a result, its diagnosis is often presumptive, and TB treatment may either be delayed or started unnecessarily.

Immunological methods used to detect *M. tuberculosis* antigens have been developed as an alternate and rapid means of diagnosis of tuberculosis[2,3]. Unfortunately, the specificity of these tests have been affected by the cross reactivity of *M. tuberculosis* antigens with other mycobacterial species[4], and attempts at improving specificity by limiting antigen detection to only those specific for *M. tuberculosis* have resulted in a reduction of test sensitivity[5].

Knowledge of the mycobacterium genomic sequences coupled with the sensitive DNA amplification technique by the polymerase chain reaction (PCR)[6,7] have recently allowed rapid detection of *M. tuberculosis* DNA. Mycobacterial DNA in clinical samples have been detected using PCR by amplification of the MPB 64 protein[8,9], the 65 kDa heat shock protein[10,11] and the IS6110 insertion DNA sequence[12,13]. In this study, we evaluated the usefulness of DNA amplification using PCR for the detection of the MPB 64 protein specific for *M. tuberculosis* in CSF specimens in the diagnosis of TBM.

MATERIALS AND METHOD

Bacterial cultures

M. tuberculosis cultures were kindly provided by the TB laboratory, Tan Tock Seng Hospital (TTSH). Non-mycobacterial cultures of *Pseudomonas* species, *Klebsiella* species *and ß-streptococcus A and B and G* were obtained from the Microbiology Department, National University Hospital (NUH).

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Clinical specimens

A total of 19 CSF specimens from patients hospitalised at the NUH and TTSH were evaluated. CSF samples were obtained from 6 patients with TBM, diagnosed either by a positive TB culture (4/6) or by the patients' remarkable response to treatment. The latter 2 samples were likely to be TB culture negative as they were obtained after commencement of therapy. The non-tuberculous specimens were obtained from patients with febrile fit (8/13), aseptic (3/13) and septic meningitis (2/13), and these acted as negative controls.

DNA extraction

Bacterial strains

DNA was extracted from bacterial cultures using sodium dodecyl sulphate (SDS) and proteinase K. A loopful of bacteria was resuspended in Tris-EDTA. SDS and proteinase K were added to final concentrations of 0.5% and 100 µg/ml. The mixture was incubated for 1 hour at 37°C. Cell wall debris and remaining proteins were removed by precipitation with cetyltrimethylammonium bromide (CTAB, Aldrich, Milwaukee USA) for 10 minutes at 65°C. DNA was purified using phenol-chloroform-isoamyl alcohol extractions.

Cerebrospinal fluid

CSF specimens were aliquoted into one ml volumes and stored at -70°C. SDS and proteinase K were added to final concentrations of 0.2% and 120 µg/ml. The mixtures were incubated for 1 hour at 65°C, and then for a further 5 hours at 37°C. DNA was purified using phenol-chloroform-isoamyl alcohol extractions.

DNA extracted from both bacteria and CSF were precipitated in 3 mol/L sodium acetate and ethanol. Aqueous DNA was washed in alcohol, dried and then solubilised in double distilled water.

DNA amplification by the polymerase chain reaction

DNA amplification of the 240 bp region from the MPB 64 protein coding gene specific for *M. tuberculosis*[8] was carried out using two oligonucleotide primers, 5'-TCCGCTGCCAGTCGTCTTCC-3' and 5'-GTCCTCGCGAGTCTAGGCCA-3. The PCR protocol involved 40 cycles of amplification performed at 94°C (denaturation, 1 min), 60°C (annealing, 2 min) and 71°C (extension, 2 min). Amplified DNA was electrophoresed in 1% agarose gels at 100V for 1 hour and then transferred to HyBond-N membranes (Amersham International, England).

Oligonucleotide hybridisation

The oligonucleotide probe 5'-CTTCAACCCGGGGGAGT-3' from the midportion of the amplified sequence[14] was labelled at the 5' end with T4 polynucleotide kinase (Amersham International, England) using α-32 P-dCTP[15]. Membranes were prehybridised for 1 hour at 42°C in buffer containing 0.54 M NaCl, 30 mM NaH_2PO_4, 3 mM EDTA, 5X Denhardt's solution, 0.5% SDS and 1 mg/ml salmon sperm DNA. Hybridisation was carried out in 6X SSC (1X SSC: 0.5 M NaCl, 15 mM sodium citrate) containing 0.05% sodium pyrophosphate at 42°C. Hybridised membranes were washed once at room temperature for 10 minutes and for 20 minutes at 51°C. The membranes were then exposed to X-ray films (Kodak X-omat AR, Kodak) at -70°C overnight.

RESULTS

Our overall results are summarised in Table I. The PCR technique detected TB DNA in 5/6 TBM specimens. PCR and oligonucleotide hybridisation enabled the detection of TB DNA in all the 6 specimens obtained from the TBM patients. When compared with standard TB culture, PCR followed by oligonucleotide hybridisation was also more sensitive, especially if CSF specimens were obtained after commencement of TB therapy. In 2 such cases, TB DNA was present on PCR and oligonucleotide hybridisation, but negative on TB culture. The diagnosis of TBM in these patients was confirmed by their remarkable response to TB therapy.

Table I - Overall summary for the diagnosis of TBM

	TB culture	Amplified 240bp DNA (PCR)	RC and oligonucleotide hybridisation
TBM (n=6)	4/6*	5/6	6/6
Negative controls (n=13)	ND **	1/13	1/13

* In the 2 negative specimens, CSF were obtained from patients after initiation of TB therapy.

** ND=not done. All the negative controls did not receive TB therapy.

Fig 1 – Gel electrophoresis of PCR products. DNA was extracted from CSF specimens, and amplified by PCR using primers for the MPB 64 protein. The 123bp ladder, (lane 1), DNA from *M tuberculosis* (lane 2), TBM positive specimens (lanes 3-7), negative controls (lanes 8-11) are shown.

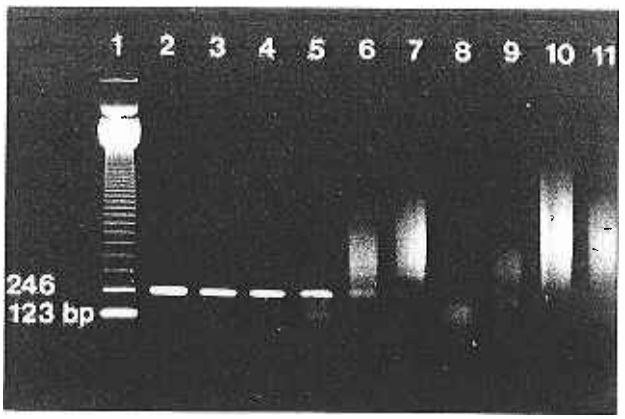


Fig 2 – Autoradiograph of oligonucleotide hybridisation with the MPB 64 protein probe. DNA from the electrophoresed gel shown in Fig 1 was transferred to nylon membrane by Southern blotting. The membranes was hybridised with radiolabelled (^{32}P) oligonucleotide probe. Positive bands are seen in lanes 2-7, which correspond to *M tuberculosis* DNA, and 5 positive TBM specimens respectively. The negative lanes (8-11) correspond to negative controls.

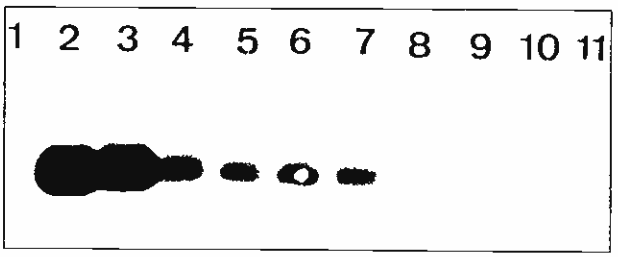


Fig 3 – Gel electrophoresis of PCR products. DNA was extracted from non mycobacteria and amplified by PCR using primers for the MPB 64 protein. The 123 bp ladder (lane 1), DNA from *Escherichia coli*, *ß Streptococcus A, B, G, Klebsiella sp*, and *Pseudomonas sp* (lanes 3-8), and *M tuberculosis* (lanes 2 and 9) are shown.

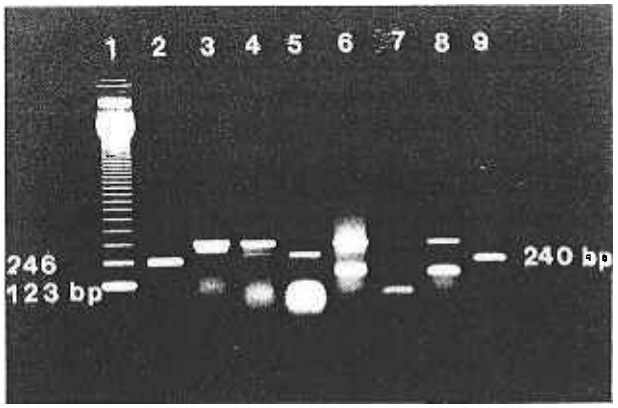
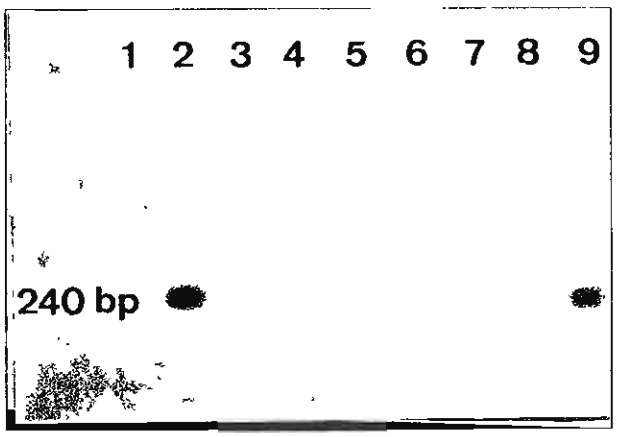


Fig 4 – Autoradiograph of oligonucleotide hybridisation with the MPB 64 protein probe. DNA from the electrophoresed gel shown in Fig 3 was transferred to nylon membrane by Southern blotting. The membranes was hybridised with radiolabelled ^{32}P) oligonucleotide probe. Positive bands are seen in lanes 2 and 9 correspond to *M tuberculosis* DNA, the negative lanes (3-8) correspond to *Escherichia coli, ß Streptococcus A, B, G, Klebsiella sp*, and *Pseudomonas sp*, respectively.



With regard to the 13 negative controls, there was one false positive result. This false positive specimen was obtained from a patient with febrile seizure. He recovered uneventfully from his acute illness and did not receive anti-TB therapy.

Fig 1 and 2 show the results of a typical experiment. The 240 bp DNA fragment of the MPB 64 protein amplified with DNA extracted from *M. tuberculosis* (Fig 1, lanes 2 and 3), and three TBM culture positive CSF specimens (Fig 1, lanes 4-6) were observed as distinct bands after gel electrophoresis and ethidium bromide staining. The specimen obtained from a patient who responded to TB therapy but was TB culture negative, was negative on PCR (Fig 1, lane 7). The remaining 4 lanes (Fig 1, 8-11) which did not show any bands were PCR products from 4 negative controls. After hybridisation of the 240 bp PCR products with the oligonucleotide probe, DNA from *M. tuberculosis*, and the DNA from the 4 TBM patients, including the patient who was negative on PCR alone, gave a distinct band (Fig 2, lanes 2-7).

In several experiments, DNA extracted from six non-mycobacteria (*E. coli, Klebsiella* spp., *Pseudomonas* spp., *ß-Streptococcus* A, B and G) showed only non-specific amplified fragments (Fig 3, lanes 3-8), and did not produce a band after autoradiography (Fig 4, lanes 3-8). In contrast, DNA from *M. tuberculosis* showed distinct bands on PCR and probing (Figs 3 and 4, lanes 2 and 9).

DISCUSSION

The detection of the DNA encoding for the MPB 64 protein using PCR and oligonucleotide hybridisation was evaluated as a means for the diagnosis of TBM. Our results indicate that PCR with oligonucleotide probing has several advantages over the current methods for the detection of TBM. In particular, it was shown to be a more sensitive test than culture of the mycobacteria. We were not only able to detect *M. tuberculosis* DNA in all the TB culture positive specimens (4/4), but detection was also possible in the 2 specimens which were obtained after commencement of therapy and were TB culture negative (Table I, Figs 1 and 2). Furthermore, the rapidity of obtaining results by PCR, which could be made available within 5 days, contrasts with the long wait of 6 to 8 weeks for culture results.

The extreme sensitivity of the PCR technique is dependent on the number of amplification cycles[15]. In this study, we found 40 cycles to be optimum. Despite these optimal conditions, which gave negative results with non-mycobacteria DNA (Figs 3 and 4), one of the 13 negative control CSF specimens gave a false positive result (Table I). In our earlier experiments, when strict precautions on cross contamination were not observed, we came across specimens which were initially negative on PCR but subsequent repeat testing revealed a positive result (data not shown). We therefore feel the false positive result in this study was due to cross contamination from TB positive specimens. In this regard, Shankar and coworkers[16] had also reported similar problems with contamination of specimens, where an initial PCR study produced 6 false-positives and a subsequent repeat of the same patients with fresh CSF gave negative results. Thus, it cannot be overemphasised that extra precaution is made with regards to cross contamination. As a precautionary step, we now carry out DNA extraction from TB positive and negative specimens in different fume chambers, in addition to performing preamplification and post amplification procedures in separate areas.

In conclusion, we have found PCR with oligonucleotide hybridisation to be a useful technique for the rapid diagnosis of TBM. We are currently evaluating its use for the diagnosis of other forms of TB. In addition, the use of other primers for TB DNA with multiple copies within a genome, such as the *IS6110* insertion element[17] is also being evaluated as a method to increase PCR sensitivity so that oligonucleotide probing may be unnecessary.

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