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ORIGINAL COMMUNICATION

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Rapid diagnosis of tuberculous meningitis by polymerase chain reaction assay of cerebrospinal fluid

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Abstract A polymerase chain reaction (PCR) method for the rapid diagnosis of tuberculous meningitis (TBM) was used to study prospectively 47 cerebrospinal fluid (CSF) samples from 45 patients. Twenty CSF samples were from patients with clinically suspected TBM and another 27 samples came from patients without clinically suspected TBM. Mycobacterial DNA was detected in 15 CSF samples (14 from patients with clinically suspected TBM and 1 from a patient not suspected of having TBM). Of the PCR-positive samples, 4 were also positive for mycobacterial culture. However, 32 PCR-negative samples were all culture-negative. All samples were negative for the acid-fast bacillus by direct smear. The single PCR-positive patient in the clinically unsuspected TBM group was initially diagnosed as suffering from aseptic meningitis on the basis of his clinical features. The mycobacterial culture of his CSF specimen was also positive and a revised diagnosis of an aseptic type of TBM was made. The estimations of specificity and sensitivity in this study were 100% and 70% respectively. The results showed that using a PCR to detect mycobacterial DNA in CSF for the early diagnosis of TBM is not only a rapid but also an accurate method.

Key words Deoxyribonucleic acid
Mycobacterium tuberculosis
Polymerase chain reaction
Tuberculous meningitis

Introduction

The mortality and morbidity of tuberculous meningitis (TBM) remain high, despite the availability of effective chemotherapy. Delay in diagnosis is directly related to poor prognosis [2, 17]. Therefore, early accurate diagnosis is very important in the treatment of TBM. The conventional methods of bacteriology, which require 6–8 weeks to exclude an infection, are inadequate for the early diagnosis of TBM. In the past few years, methods for the rapid diagnosis of TBM have been developed. Immunoassay in the diagnosis of TBM, including the detection of tuberculous antigen, antibody, and/or immune complexes in cerebrospinal fluid (CSF), have been emphasized, but the specificity and sensitivity in most studies has not been assessed [1, 6, 7]. The molecular biology approach using a DNA probe for the rapid diagnosis of tuberculosis has been used but has not proved to be very satisfactory [8, 24].

Recently, a polymerase chain reaction (PCR) method based on deoxyribonucleic acid (DNA) amplification has been used for rapid detection of tuberculous infection [3, 10, 13, 20, 23, 27]. It has also proved to be the most sensitive, specific and economical method for the early diagnosis of tuberculous infection. However, clinical specimens used to identify mycobacterial DNA were often sputum, pleural effusion, blood, lymph node biopsy, and gastric aspiration [3, 4, 13, 15, 23, 27]. Clinical exploration of the PCR in CSF for the early diagnosis of TBM is not common, and all such studies have been retrospective to date [16, 22, 28, 30]. We present our prospective study and results using a PCR assay to identify mycobacterial

DNA in CSF for the rapid diagnosis of TBM in the early stages.

Materials and methods

We used PCR to study prospectively 47 CSF samples from 45 patients to detect mycobacterial DNA for the early diagnosis of TBM. Twenty CSF samples were taken from 18 patients with clinically suspected TBM and 27 samples obtained from 27 patients without clinically suspected TBM served as the control group. We further classified the 18 clinically suspected TBM patients into three subgroups based on clinical features: 8 cases of highly probable TBM, 6 of probable TBM, and 4 of possible TBM. In one patient who was included in the highly probable TBM subgroup, repeated sampling of CSF specimens (3 times) for PCR was performed. The clinical criteria for suspecting TBM and the classifications are shown in Table 1 and were summarized from those of Davis et al. [9], Haas et al. [12], Kennedy and Fallon [17], and Shankar et al. [28]. In the 27 unsuspected TBM patients, CSF samples were obtained from 3 with pyogenic meningitis, 12 with aseptic meningitis, 3 with brain tumours, 3 with epilepsy, 2 with Guillain-Barré syndrome, 2 with transverse myelitis, and 2 with psychogenic weakness of the limbs.

We carefully separated each CSF sample and stored them at –20° C in different refrigerators, processing DNA extraction and amplification in different batches to avoid contamination. However, the PCR assay was performed at least twice for each CSF sample in order to test the consistency of the results.

For extraction of DNA, 300 μl CSF was mixed with 300 μl extraction buffer, which contained 0.5% sodium dodecyl sulphate (SDS), 10 mmol/l TRIS hydrochloride (pH 8.0), and 0.1 mol/l ethylenediaminetetraacetic acid (EDTA), for 30 min at 95° C. All CSF samples were then extracted with phenol/chloroform, after which the DNA was precipitated with ethanol and redissolved in 30 μl of 10 mmol/l TRIS hydrochloride containing 0.1 mmol/l EDTA (pH 7.6). The procedure for amplification was performed using a pair of primers with the upstream sequence of 5´-TCCGCTGCCAGTCGTCTTCC-3´ and the downstream sequence 5´-GTCCTCGCGAGTCTAGGCCA-3´ [27]. These primers amplify a 240-base-pair (bp) DNA fragment within the gene encoded for specific MBP 64 protein of the *Mycobacterium tuberculosis* complex [14, 29]. Briefly, 100 μl of reaction mixtures containing 10 mmol/l TRIS hydrochloride (pH 8.3), 50 mmol potassium chloride, 1.5 mmol/l magnesium chloride, 10 mg/l gelatin, 200 μmol/l each of deoxyribonucleotides triphosphate, 50 pmol of each primer, 2.5 units of *Taq* polymerase (Promega), and about 10 μl DNA extracted from CSF were amplified by 35 cycles at 94° C (1 min), 50° C (1 min), and 72° C (2 min) in a thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Controls with purified mycobacterial plasmid DNA and without DNA were included in each experiment.

Thereafter, electrophoresis was carried out on 20 μl of amplified products in a 2% agarose gel containing 0.5 μg/ml of ethidium bromide and visually inspected under ultraviolet light for bands of DNA of appropriate sizes. If the initial result was negative, a portion of the first amplified reaction mixture was re-amplified for another 35 cycles under the same conditions with freshly supplemented deoxyribonucleotide triphosphate, primers, and *Taq* DNA polymerase [23]. Alternatively, DNA from the initial round of amplification was then Southern blotted on to nylon membranes (Amersham, Buckinghamshire, UK) by the standard method [26]. Hybridization was carried out at 42° C for 8 h with 2×10^5 cpm/ml (^{32}P)-5´-labelled (specific activity 1–3 mCi/pmol) oligonucleotide probe from the midportion of the sequence used for amplification (5´-CTTCAACCCGGGGGAGT-3´) [27]. The membranes were washed in 2 × SSC in 0.5% SDS twice at room temperature for 5 and 15 min respectively, followed by another washing with 0.1 × SSC in 0.5% SDS twice at 62° C for 30 min. The membranes were then exposed to X-ray film (Kodak X-OMATE) overnight at –70° C with an amplifying screen.

Results

The results of detecting mycobacterial DNA, culture for mycobacteria, and acid-fast bacillus by direct smear in the CSF samples are shown in Table 2. None of the CSF samples was positive by direct smear. Only 3 CSF samples (15%) showed positive cultures for *M. tuberculosis* in the

Table 1 Clinical criteria and classification for suspected tuberculous meningitis *(TBM)*; *ESR* erythrocyte sedimentation rate, *PPD* purified protein derivative

Clinical evidence
- fever, headache, and stiff neck for more than 2 weeks

Laboratory evidence
1. CSF study (WBC > 20 cells/mm^3, protein > 100 mg/dl, or glucose < 45 mg/dl or CSF/blood glucose below 40%)
2. CT findings (basal exudate, hydrocephalus, or focal brain abnormalities such as infarction or intracranial tuberculoma)
3. Tuberculosis outside the CNS or positive PPD skin test
4. Hyponatraemia or elevated ESR

Definition of clinically suspected TBM
- clinical evidence and 1 or more than 1/4 laboratory evidence(s)

Classification of clinically suspected TBM
- highly probable TBM: clinical evidence and 3 or 4/4 laboratory evidences
- probable TBM: clinical evidence and 2/4 laboratory evidence
- possible TBM: clinical evidence and 1/4 laboratory evidence

Table 2 Results of mycobacterial culture, acid-fast bacillus *(AFB)* by direct smear, and polymerase chain reaction *(PCR)* of CSF specimens

Group	No (%) CSF specimens with positive results			
	n	AFB	Culture	PCR
Clinically suspected TBM group	20	0	3 (15%)	14 (70%)
highly probable TBM	10[a]	0	2	8
probable TBM	6	0	1	4
possible TBM	4	0	0	2
Clinically unsuspected TBM group	27	0	1 (4%)[b]	1 (4%)
Total	47	0	4	15

[a] In one patient three CSF samples were taken of which the first two were negative for DNA amplification and the third was positive
[b] The single PCR-positive patient in the unsuspected TBM group who was initially diagnosed as aseptic meningitis was also positive for mycobacterial culture. A revised diagnosis of aseptic type of TBM was made

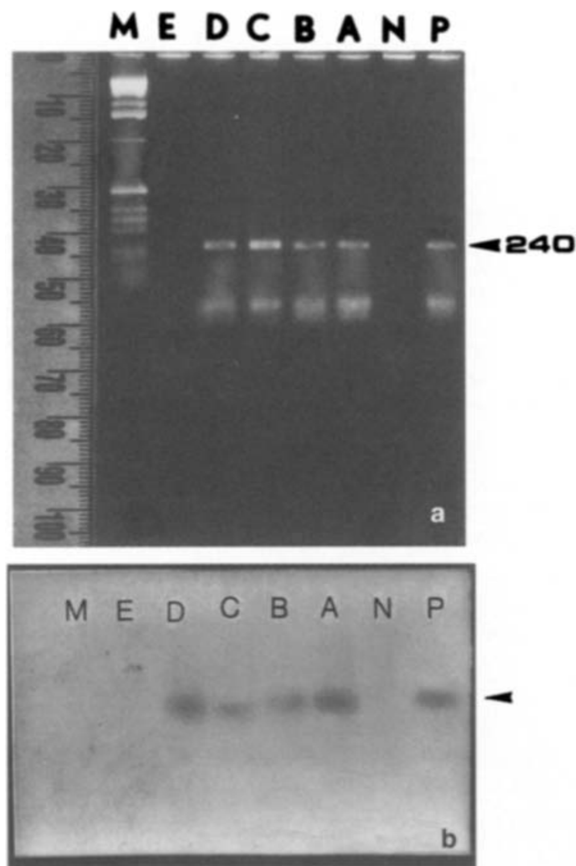

Fig. 1 **a** Determination of mycobacterial DNA in cerebrospinal fluid (CSF) by the polymerase chain reaction (PCR) method. A 240 bp band indicates the presence of the DNA of *Mycobacterium tuberculosis (arrow). Lanes A, B and C* were prepared from the CSF of highly probable, probable and possible tuberculous meningitis patients, respectively. *Lane D* was prepared from the CSF of a patient with an initial clinical diagnosis of aseptic meningitis. *Lane E* shows the CSF from a patient with pyogenic meningitis. *Lane P* is mycobacterial plasmid DNA (Genemed Biotech, San Francisco) that was used as a positive control. *Lane N* is a negative control and *lane M* a DNA marker of the 1 kb ladder (BRL, Gaithersburg). **b** Southern blot hybridization of amplified products with the internal oligonucleotide probe. The lanes are similar to those in the upper portion of the figure. The *arrow* indicates the 240 bp bands

clinically suspected TBM group, whereas there were 15 positives in the CSF samples by the PCR method, including 14 (70%) in the clinically suspected TBM group and 1 in the clinically unsuspected TBM group (Fig. 1, upper). In the PCR-positive CSF samples, detection of the mycobacterial DNA of the former showed that 8 samples were from highly probable TBM patients, 4 from probable TBM patients, and 2 from possible TBM patients. The latter was initially diagnosed as having an aseptic meningitis on the basis of clinical features, but the mycobacterial culture was finally positive after 8 weeks. In all PCR-positive CSF samples, a 240 bp amplification product was seen on ethidium bromide stained agarose gel, which was then confirmed by Southern hybridization (Fig. 1, lower). All CSF samples were tested at least twice in different batches using the same procedure, and all revealed the same results.

Of the 15 PCR-positive CSF samples, 4 concomitantly had positive cultures for *M. tuberculosis*; 2 were in the highly probable TBM subgroup, 1 in the probable TBM subgroup, and 1 had an initial diagnosis of aseptic meningitis. However, none of the PCR-negative CSF samples were positive for mycobacterial culture.

One highly probable TBM patient in our study had three CSF samples taken. The first two samples were negative for DNA amplification and the third yielded a positive result. Three CSF samples were negative for mycobacterial culture. Our study also revealed that 6 CSF samples from patients with clinically suspected TBM were PCR-negatives. These patients were finally diagnosed as having TBM on the basis of clinical manifestations, laboratory findings and response to antituberculous therapy (Table 3).

Discussion

Because there are few organisms in the CSF of patients with TBM, positive cultures for mycobacteria are infrequent, ranging from 5%–22.9% [5]. Conventionally, the rapid diagnosis of *M. tuberculosis* with AFB by direct smear is often negative in patients with TBM. In contrast, PCR can amplify very small quantities of selected sequences of DNA [25], and has proved to be a highly specific and sensitive method for the early diagnosis of tuberculous infection [3, 4, 10, 13, 15, 20, 23, 27]. As in our study, all CSF samples in those studies were negative for AFB by direct smear, and there were only 15% positive cultures for *M. tuberculosis* in the suspected TBM patients. The PCR, however, detected 70% of the clinically suspected TBM CSF specimens in our study, which meant that it was much more sensitive than the conventional methods for the early diagnosis of TBM.

Although the PCR can amplify the specific DNA sequence by thousands of times within a few hours, the exquisite sensitivity of the PCR is also its main potential drawback. False-positive results caused by the cross-contamination and contamination with amplified DNA product in the laboratory are well-recognized problems [15, 25, 28]. Standard procedures including preparation of samples in a separate room or biosafety hood, autoclaving of solutions, dividing reagents into aliquots to minimize the number of repeated samplings, avoiding splashes, using positive displacement pipettes, and multiple assay blanks with the inclusion of positive and negative controls in each run have been suggested to avoid false-positives with PCR [18, 19].

In this study, a PCR-positive finding was noted in the CSF of an initially unsuspected TBM patient who was di-

Table 3 Clinical picture and outcome of PCR-negative patients with clinically suspected TBM (*P/L* polymorphonuclear cells/lymphocytes, *WBC* white blood cells/ mm^3, *AFB* acid-fast bacillus, *AMI* acute myocardial infarction, *ESR* erythrocyte sedimentation rate, *N* normal, *PPD* purified protein derivative, *TB* tuberculous bacillus, *VP* ventriculoperitoneal

No.	Age/Sex (years)	Clinical classification	CSF study				Brain CT findings
			WBC	P/L (%)	protein (mg/dl)	glucose (mg/dl)	
1.	23/M[a]	Highly probable TBM	20	2/98	150	35	Leptomeningeal enhancement, hydrocephalus
2.	23/M[a]	Highly probable TBM	88	10/90	186	24	Leptomeningeal enhancement, hydrocephalus
3.	27/M	Probable TBM	112	58/42	85	26	Leptomeningeal enhancement, hydrocephalus
4.	25/M	Probable TBM	75	36/64	130	47	Hydrocephalus, basal exudate
5.	81/M	Possible TBM	24	30/70	83	50%[b]	Insignificant finding
6.	15/F	Possible TBM	2	–	48	55	Hydrocephalus, intracranial tuberculoma

[a] In this patient three CSF samples were taken; the first two were PCR-negative
[b] Value of CSF/blood glucose

No.	TB outside the CNS	ESR	Hyponatraemia	PPD skin test	ABF	TB culture	Responses to antituberculosis therapy and outcome
1.	–	N	–	+	–	–	1. Complicated by communicating hydrocephalus received VP shunt 2. Complete recovery 3 months later
2.	–	N	–	+	–	–	Same as case 1
3.	–	N	–	–	–	–	Mild cortical dysfunction remained 1 year later
4.	–	N	–	–	–	–	1. Complicated by communicating hydrocephalus received VP shunt 2. Complete recovery 4 months later
5.	+[a]	N	–	–	–	–	Gradually improved, but finally died of AMI 4 months later
6.	–	N	–	–	–	–	Complete recovery 2 months later

[a] Infiltration on chest x-film, response to antituberculous therapy

agnosed as an aseptic meningitis on the basis of his clinical features. This result was confusing, in spite of the fact that we followed the advisable standard procedures and took extreme care during the PCR. We carried out multiple retests of the patient's CSF and also repeated the amplification with another set of primers that amplified a 165 bp DNA fragment within the gene encoded a 65 kDa antigen of *M. tuberculosis* [23], but always obtained the same result. Fortunately, the mycobacterial culture of the patient was revealed as positive 8 weeks later. Because the coincidence of accidental contamination in different laboratories was most unlikely, we excluded the possibility of a false-positive PCR in the patient's CSF sample and finally revised the diagnosis to an aseptic pattern of TBM. This picture of tuberculosis as an unusual cause of transient infection of the central nervous system had been mentioned previously in the literature [11, 17, 21], particularly when small numbers of mycobacterial bacilli are carried in the blood-stream to the brain. Emond [11] published the findings of four patients suffering from acute meningitis who spontaneously recovered from their illness within a few days without specific treatment. The bacterial culture of all CSF specimens resulted in growth of *M. tuberculosis*. He concluded that tuberculosis could also be a cause of transient aseptic meningitis. Kennedy and Fallon [17] reported that one of their 52 TBM patients, a child, had transient meningitis with a positive CSF culture for *M. tuberculosis*. Although reports of aseptic patterns of TBM in the literature are rare, we postulate that this pattern may not be uncommon if routine investigations of the CSF of patients with clinically suspected aseptic meningitis using the PCR for amplification of mycobacterial DNA are carried out.

Although the PCR assay was proven to be a sensitive method to detect small numbers of mycobacteria with 10–100 bacilli present in clinical specimens, one highly probable TBM patient in our study had repeated CSF samples taken and was finally PCR-positive on the third CSF sample. This result may be explained by the fact that there were too few mycobacteria, less than 10 bacilli, in the CSF in the early stage of the mycobacterial infection for them to be obtained in the first two lumbar punctures. Then the PCR-negative finding resulted from the absence of mycobacterial DNA which was prepared from the patient's own CSF for amplification. Not until there was further growth of mycobacteria in the untreated patient did the PCR convert to a positive result in the third CSF sample. Thus, it is advisable to repeat the CSF study again in

clinically suspected TBM patients in order to improve PCR sensitivity.

In addition to the patient with repeated CSF sampling who was PCR-negative in the first two specimens, this study also revealed that another four CSF samples in the clinically suspected TBM group were PCR-negative. Because these four patients promptly received antituberculous therapy after CSF sampling, and they gradually recovered following their illness, we did not repeatedly sample their CSF for further re-amplification. However, these clinically suspected TBM patients were all finally confirmed to have TBM, despite the fact that their PCR assays and mycobacterial culture were both negative.

In our study, 14 out of 20 clinically suspected TBM CSF samples were PCR-positive. Therefore, the sensitivity when using PCR for the early diagnosis of TBM in the clinically suspected TBM group was 70%. All CSF samples that were PCR-negative never had a positive culture for mycobacteria in this study, and therefore we believe that the specificity of this assay in the early diagnosis of TBM was 100%. Kaneko et al. [16] first used the PCR to detect mycobacterial genome in the CSF of a series of TBM patients retrospectively, and revealed that 5 of 6 patients with clinically diagnosed TBM had a PCR-positive reaction; of these, only 2 also had positive mycobacterial culture [16]. Consequently, Shankar et al. [28] showed that PCR in CSF was comparable to conventional bacteriology and an enzyme-linked immunosorbent assay (ELISA) for CSF antibodies in the diagnosis of TBM. Their results showed that PCR could detect 65% of clinically suspected TBM, but ELISA and culture could only detect 44% and 12% of cases, respectively. In addition, they also considered PCR to be a useful rapid diagnostic test for TBM and feasible for use in developing countries, since detection can be achieved by simple agarose gel electrophoresis. From our own experience, only 6–8 h are needed to complete the PCR procedure on CSF, but care must be taken despite the fact that the PCR procedure is simple. Zhuang et al. [30] studied 53 clinically diagnosed TBM cases and revealed that the positive rate for PCR, acid-fast bacillus, and culture in CSF was 51.7%, 8.6%, and 1.7%, respectively. Pan's study [22] revealed that the sensitivity and specificity of his PCR system in about 80 TBM cases and 63 non-TBM cases were 85% and 98.4%, respectively. However, the above studies were all retrospective, unlike our present study, which was prospective. To summarise the reports in the literature and our study, the sensitivity of PCR in detecting mycobacterial DNA in CSF in patients with clinically suspected TBM is around 52%–85%. Using precautions to avoid false-positive results, however, we believe that the specificity of PCR can be 100%, as shown in our study.

In conclusion, this study revealed that PCR is a useful and accurate method for use in the early diagnosis of TBM. In addition to enabling a rapid diagnosis of TBM in the early stages, PCR also has a higher sensitivity and specificity than other methods used in the diagnosis of TBM. However, we must advise clinicians to pay heed to the aseptic pattern of TBM.

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