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EVALUATION OF DIRECT MICROSCOPY, CULTURE, AND POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF TUBERCULOUS MENINGITIS

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ABSTRACT

In view of the importance of early diagnosis of tuberculous meningitis (TBM), the efficiency of the polymerase chain reaction (PCR), one of the most reliable and sensitive DNA-based assays, was compared with conventional methods (acid-fast microscopy and culture) for the detection of *M. tuberculosis* in cerebrospinal fluid (CSF) specimens from patients suspected of TBM.

Of the 29 CSF specimens from highly probable TBM patients (based on clinical features), 25 were positive by PCR (86.2%), was acid-fast microscopy (AFM) positive (3.4%), positive (17.2%).

No positive results were found by AFM, culture or PCR in the non-tuberculous control group. The results of this study indicate that application of PCR should be extremely useful for the diagnosis of TBM.
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INTRODUCTION

The most dangerous form of extrapulmonary tuberculosis is tuberculous meningitis (TBM) which occurs in 7-12% of tuberculous patients in developing countries.[1] TBM can occur at any age except in the newborn. Patients with TBM always have a focus of infection elsewhere, but one in four have no clinical or historical evidence of such an infection. Occasionally, the onset is much more rapid and may be mistaken for a subarachnoid hemorrhage.[2] In spite of the availability of effective chemotherapy, the mortality and morbidity of TBM remain high.[3]

Conventional bacteriology such as direct microscopy and culture are not sufficient for the early diagnosis of TBM because there are too few bacilli in the cerebrospinal fluid (CSF) to be demonstrated by direct microscopy and on the other hand, successful cultural identification of tubercle bacilli will take about 7 weeks. Fortunately, new molecular techniques have been developed as sensitive and reliable diagnostic tools for the identification of tubercle bacilli. The most important advance in the usefulness of molecular methods, especially in diagnostic application, is the polymerase chain reaction (PCR), which has in many cases increased not only the speed of DNA-based assays, but also greatly enhanced their sensitivity.

In the study described here, PCR was performed to detect specific *M. tuberculosis* DNA in CSF specimens from highly probable TBM patients.

The aim of this study was to investigate whether PCR detects tubercle bacilli in CSF specimens that are missed by direct microscopy and culture, and if so, whether PCR has significant diagnostic value compared to conventional methods.

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MATERIALS AND METHODS

Clinical specimens

A total of 29 CSF specimens from highly probable TBM patients received at the Bacteriology Division of UCL Medical School for confirmation by PCR (from 1992 until mid-1994) were tested. Direct microscopy and culture examination were performed on the same specimens for comparison with PCR. CSF specimens from the 6 non-tuberculous patients as the control group were examined by the above-mentioned methods.

Acid-fast microscopy (AFM)

All fixed CSF smears were stained by Ziehl-Neelsen (ZN) method for acid-fast bacilli (AFB) using strong carbolfuchsin, acid-alcohol as a decolorant, and methylene blue as the counterstain. After staining, more than 20 fields of each smear were examined carefully by the light microscope using the oil immersion (× 100) lens.

Culture

All CSF specimens were inoculated onto Lowenstein-Jensen (LJ) media without delay, and incubated at 37°C for 6-8 weeks and the slopes were examined weekly. The positive cultures were tested by selected biochemical tests for the definitive identification of *Mycobacterium tuberculosis*.

DNA extraction from clinical specimens[4]

900 µl of lysis buffer (containing GuSCN 120 g; 0.1 M Tris-HCl at pH 6.4, 100 ml; 0.2 M EDTA at pH 8.0, 22 ml; and Triton X-100, 2.6 g) was mixed with 40 µl of diatom suspension in a 1.5 ml Eppendorf microcentrifuge tube and shaken briefly. The CSF specimen (50 µl) was added to the above mixture, shaken for 5 sec, then allowed to stand at room temperature for 10 min. Next, it was shaken again, then spun at 12000 g for 15 sec. The supernatant was discarded, and the nucleic acid (NA)-pellet was washed twice with washing buffer (containing GuSCN 120 g; and 0.1 M Tris-HCl at pH 6.4, 100 ml), twice with 70% ethanol and once with acetone. Then acetone was removed and the NA-pellet was dried at 56°C for 10 min. 100 ml of TE buffer (Tris-HCl at pH 8.0, 10 mM; and EDTA at pH 8.0, 1mM) was then added to the NA-pellet, mixed and incubated for 10 min at 56°C. It was again mixed and spun at 12000 g for 2 min, and 5 µl of the supernatant was used for PCR.

Preparation of chromosomal DNA

Mycobacterium tuberculosis DNA was purified from fresh culture of *M. tuberculosis* by boiling method,[5] as follows:

Table I. Evaluation of PCR in CSF specimens from patients suspected of tuberculous meningitis (TBM).

Specimen	No. tested	Positive by:		
		AFM *	Culture	PCR **
CSF from highly probable TBM patients	29	1 (3.4%)	5 (17.2%)	25 (86.2%)
CSF from nontuberculous patients (control group)	6	0	0	0

* AFM, acid-fast microscopy
** PCR, polymerase chain reaction

Table II. The results of patients suspected of TBM in detail.

Total No. of patients suspected of TBM	No. (%) of TBM patients with positive results by:				
	only AFM	only culture	only PCR	both culture & PCR	all three AFM, culture & PCR
29	0 (0%)	0 (0%)	20 (69%)	4 (13.8%)	1 (3.4%)

A colony of *M. tuberculosis* was added directly to 500 µl of distilled water, boiled for 10 min, spun at 12000 g for 2 min, and 5 µl of the supernatant used as a positive control in every PCR examination.

Selection of primers

The primers used for the specific amplification of *M. tuberculosis* DNA, had been originally designed by Eisenach et al.[6] from sequences which are repeated several times in the chromosome of *M. tuberculosis*. The sequences of the primers (synthesized by Oswel DNA Service, Edinburgh, U.K.), which amplify a 123-bp fragment of the repetitive sequence, were:
5'-CCTGCGAGCGTAGGCGTCGG-3' and
5'-CTCGTCCAGCGCCGCTTCGG-3'

PCR procedure

Briefly, 5µl of each prepared CSF specimen was incubated in a 45 µl reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 1 µM each of primers, 0.2 mM each of deoxynucleotides dATP, dGTP, dCTP, and dTTP (Pharmacia) and 1.25 units of Taq polymerase (purchased from Perkin-Elmer Cetus). The reaction mixtures were covered with 40 µl of sterile mineral oil.[6]

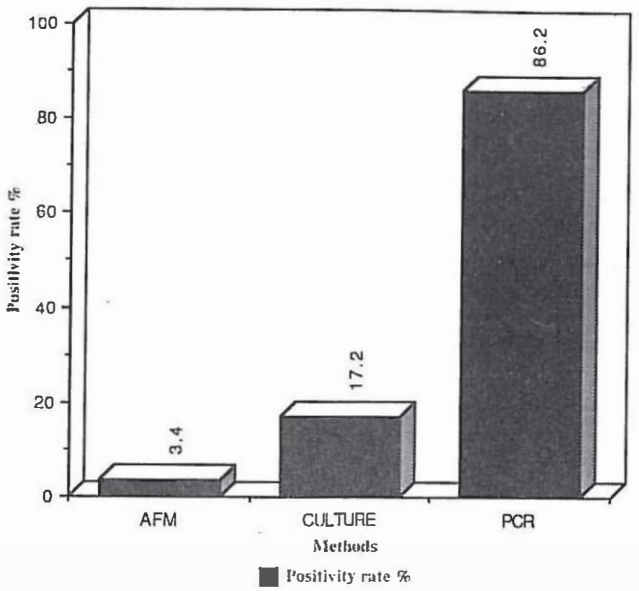

Fig. 1. Efficiency of PCR in comparison with acid-fast microscopy and culture for the diagnosis of TBM.

A control tube containing no target DNA as a negative control, with another tube containing chromosomal DNA of *M. tuberculosis* as a positive control were included with every set of tests. Precautions were taken to avoid contamination with extraneous DNA. In order to control for the presence of PCR inhibitors, PCR-negative specimens were retested by using 2 µl of the chromosomal DNA in the amplification mixture.[7]

The reaction was performed using an automated thermal cycler. The samples were denatured at 94°C for 5 min, and then 30 amplification cycles were performed. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 2 min. After the 30th cycle, the extension reaction was continued for another 5 min at 72°C.[6] The presence of the 123-bp amplification product was sought by electrophoresis of 5 µl of the amplified mixture at 60 V for 40 min on an agarose gel (1%). The DNA was stained with ethidium bromide (0.5 µg/ml) and visualized on a 302-nm UV transilluminator. The molecular size marker used in this experiment was 123-bp DNA Ladder (Sigma).

RESULTS

A total of 29 CSF specimens (from 29 highly probable TBM patients) were tested by direct microscopy for AFB, culture, and PCR. No PCR inhibitors were found in the PCR-negative specimens. The results are summarized in Table I. Of the 29 CSF specimens, only one was positive by AFM (3.4%) and five were culture-positive (17.2%), whilst 25 specimens were found to be PCR-positive (86.2%) and produced the 123-bp fragments.

AFM, culture and PCR were negative in the 6 non-tuberculous patients (control group).

Table II shows that 20 cases were positive by only PCR (69%), whereas 4 cases were positive by both culture and PCR (13.8%) and only one case was positive by all three (AFM, culture, and PCR) (3.4%). No positive results were found by either only AFM or only culture.

In total, the positivity rate of PCR was 5 times higher than the positivity rate of culture, and 25 times higher than the positivity rate of direct microscopy for TBM patients.

DISCUSSION

This study shows that the efficiency of PCR is significantly higher than microscopy and culture for the early diagnosis of TBM (Table I and Fig. 1). The accuracy of this study is demonstrated by the fact that the control group gave negative results.

The repetitive nature of the target sequence amplified by the PCR described here probably contributes to the high sensitivity, and fewer than 10 bacilli can be detected by application of this PCR. The above-mentioned detection limit of this PCR combined with the use of the best method of DNA extraction from clinical specimens,[4] provide a powerful tool for the specific and rapid diagnosis of paucibacillary situations.

In this study the 86.2% positivity rate of PCR in a small proportion of patients suspected of TBM is remarkable. In view of the fact that there are few bacilli in CSF specimens from TBM patients to be demonstrated by direct microscopy, and on the other hand cultural examination of CSF specimens from TBM patients takes several weeks, it will be useful to carry out PCR as an efficient technique for the rapid diagnosis of TBM, even though conventional diagnostic methods are cheaper than PCR. PCR can specifically identify *M. tuberculosis* in a clinical specimen within 7-8 hours. The use of PCR for the identification of mycobacteria has recently been published[8] and this, when developed further, might enable referral TB laboratories to identify all species of mycobacteria in a matter of hours rather than months.

The results of this study also indicate that PCR can be used alone as a reliable test for the diagnosis of TBM, considering that 20 CSF specimens out of 29 (69%) were positive by only PCR (Table II).

In conclusion, the results of our study suggest that in view of the specificity, sensitivity, and rapidity of the PCR described here, it can be applied as a reliable

method for the diagnosis of difficult cases of tuberculosis such as TBM.

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