EFFICIENCY OF POLYMERASE CHAIN REACTION FOR THE DIAGNOSIS OF TUBERCULOUS MENINGITIS

Abdolnasser Rafi1 and Behroor Naghily2

¹Research Center for TB, Tabriz University of Medical Sciences, Tabriz; ²Department of Infectious Diseases, Tabriz University of Medical Sciences, Tabriz, Iran

Abstract. The early diagnosis of tuberculous meningitis (TBM) is very important. In this study, 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%), whereas only one of 29 was acid-fast microscopy (AFM) positive (3.4%), and 5 out of 29 were culture-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 the application of PCR should be extremely useful in the diagnosis of TBM.

INTRODUCTION

The most dangerous form of extrapulmonary tuberculosis is tuberculous meningitis (TBM), which occurs in 7-12% of tuberculous patients in developing countries (Tandon, 1978). 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 (Braude, 1981). In spite of the availability of effective chemotherapy, the motality and morbidity of TBM remain high (Molavi and LeFrock, 1985).

Conventional bacteriology, such as direct microscopy and culture, are not sufficient for the diagnosis of TBM because there are too few bacilli in the cerebrospinal fluid (CFS) to be demonstrated by direct microscopy and on the other hand, successful culture identification of tubercle bacilli takes about 7 weeks. Fortunately, molecular techniques have been developed as sensitive and reliable diagnostic tools for the identification

E-mail: rafi_dr@yahoo.com

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 CFS specimens from highly probable TBM patients.

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

MATERIALS AND METHODS

Clinical specimens

A total of 29 CFS specimens from highly probable TBM patients received at the Research Center for TB and Pulmonary Diseases of Tabriz for confirmation by PCR within 3 years, were tested. Direct microscopy and culture examination were performed on the same specimens for comparison with PCR. CFS specimens from the 6 non-tuberculous patients (the control group) were examined by the above-mentioned methods.

Correspondence: Dr A Rafi, Research Center for TB of Tabriz, University of Medical Sciences, Farabi (Chaikenar) Street, Tapali Bagh Station, Tabriz 51547, Iran.

Acid-fast microscopy (AFM)

All fixed CFS 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 light microscope using an oil immersion(x 100) lens.

Culture

All CFS 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 (Boom *et al*, 1990)

Nine hundred microliters of lysis buffer (containing GuSCN 120 g; 0.1 M Tris-HCl at pH 6.4, 100 ml; 0.2 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 seconds, then allowed to stand at room temperature for 10 minutes. Next, it was shaken again, then spun at 12,000g for 15 seconds. 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 minutes. One hundred milliliters 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 minutes at 56°C. It was again mixed and spun at 12,000g for 2 minutes, and 5 µl of the supernatant was used for PCR.

Preparation of chromosomal DNA

Mycobacterium tuberculosis DNA was purified from a fresh culture of *M. tuberculosis* by boiling method (McFadden, 1990), as follows: a colony of *M. tuberculosis* was added directly to 500 μ l of distilled water, boiled for 10 minutes, spun at 12,000g for 2 minutes, and 5 μ l 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* (1990) from sequences which are repeated several times in the chromosome of *M. tuberculosis*. The sequences of the primers (synthesized by Oswel DNA Service, Edinburgh, UK), which amplify a 123-bp fragment of the repetitive sequence, were: 5'-CCTGCGAGCG TAGGCGTCGG-3' and 5'-CTCGTCCAGCGC CGCTTCGG-3'.

PCR procedure

Briefly, 5 μ l of each prepared CSF specimen was incubated in a 45 μ l reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂ 0.1% gelatin, 1 μ M of each of the 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 μ l of sterile mineral oil (Eisenach *et al*, 1990).

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 μ l of the chromosomal DNA in the amplification mixture (De Wit *et al*, 1991).

The reaction was performed using an automated thermal cycler. The samples were denatured at 94°C for 5 minutes, and then 30 amplification cycle were performed. Each cycle consisted of denaturation at 94°C for 2 minutes, annealing of primers at 68°C for 2 minutes, and primer extension at 72°C for 2 minutes. After the 30th cycle, the extension reaction was continued for another 5 minute at 72°C (Eisenach *et al*, 1990). The presence of the 123-bp amplification product was sought by electrophoresis of 5 μ l of the amplified mixture at 60 V for 40 minutes on agarose gel (1%). The DNA was stained with ethidium bromide $(0.5 \ \mu g/ml)$ 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 CFS 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 1. Of the 29 CFS 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 2 shows that 20 cases were positive only by 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 AFM only or culture only. Significant differences were obtained amongst the positive results of the three diagnostic methods (p<0.0001, Q=41.3333).

In total, the PCR positivity rate 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 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, provide a powerful tool for the specific and rapid diagnosis of paucibacillary situations.

In this study, the 86.2% positivity rate by PCR in a small proportion of patients suspected of TBM is remarkable. Since there are normally few bacilli in CSF specimens from TBM patients to be demonstrated by direct microscopy, and

					Table 1					
Evaluation of	PCR	in CSF	specimens	from	patients	suspected	of	tuberculous	meningitis	(TBM).

Specimen	No. tested	Positive by:			
			Culture	PCR ^b	
CSF from highly probable TBM patients CSF from non-tuberculous patients (control group)	29 6	1 (3.4%) 0	5 (17.2%) 0	25 (86.2%) 0	

^aAFM, acid-fast microscopy; ^bPCR, polymerase chain reaction.

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

Table 2 The results of patients suspected of TBM, in detail.

culture examination of CSF specimens from TBM patients takes several weeks, PCR will prove useful as an efficient technique for the rapid diagnosis of TBM, even though conventional diagnostic methods are less expensive. PCR can specifically identify *M. tuberculosis* in a clinical specimen within 7-8 hours. The use of PCR thus saves valuable time in the early identification of mycobacteria and implementing effective treatment (Telenti *et al*, 1993; Sechi *et al*, 1999).

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 PCR only (Table 2). However, statistical analysis of positive results of the AFM, culture and PCR using Cochran's Q test showed significant differences amongst the three diagnostic methods (p<0.0001) indicating that each method alone has retained its own value.

In conclusion, the result of our study suggest that, given 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.

REFERENCES

Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; 28: 495-503.

- Braude Al. Medical microbiology and infectious diseases. Vol 2. Philadelphia: Saunders, 1981: 1239-42.
- De Wit MY, Faber WR, Krieg SR, *et al.* Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. *J Clin Microbiol* 1991; 29: 906-10.
- Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis. J Infect Dis* 1990; 161: 977-81.
- McFadden J, ed. Molecular biology of mycobacteria. London: Surrey University Press, 1990: 139-72.
- Molavi A, LeFrock JL. Tuberculous meningitis. *Med Clin North Am* 1985; 69: 315-31.
- Sechi LA, Dupre I, Sanguinetti M, Fadda G, Zanetti S. Simple and rapid identification of Mycobacteria by PCR. *Mol Cell Probes* 1999; 13: 141-6.
- Tandon PN. Tuberculosis meningitis. In: Vinken PJ, Bruyn GW, eds. Handbook of clinical neurology, Vol. 33. Amsterdam: North Holland Publishing, 1978: 195-262.
- Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993; 31: 175-8.