Polymerase chain reaction, with sequencing, as a diagnostic tool in culture-negative bacterial meningitis

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Objective: To evaluate the feasibility of using 16S rDNA universal primer PCR (followed by sequencing) and 65-kDa heat shock *Mycobacterium tuberculosis* protein gene PCR as a method to determine a bacterial etiology in culture-negative cerebrospinal fluid (CSF) samples.

Methods: One hundred and forty-nine CSF samples from 128 patients were processed. DNA was extracted from the CSF samples and amplified with the eubacterial 16S rDNA primers P11E and P13B, and with the 65-kDa heat shock protein gene mycobacterial primers. The amplicons were identified by sequencing and specific oligoprobe hybridization.

Results: Overall, a microbiological diagnosis was made in 11 of 125 ultimately culture-negative cases. The use of 65-kDa heat shock protein gene PCR was needed to improve the diagnosis of tuberculous meningitis; in four patients, prospectively studied, the outcome of antituberculous therapy could also be followed.

Conclusions: In culture-negative bacterial meningitis it is possible to improve the microbiological diagnosis by use of 16S rDNA amplification and sequencing, together with amplification of a more specific gene in mycobacteria.

Key words: Meningitis, PCR, sequencing, hybridization, 16S rDNA, 65-kDa protein gene

INTRODUCTION

Acute bacterial meningitis is a rapidly progressive potentially lethal condition, the causative agents of which, when it is community-acquired, are strikingly age related and usually well predictable, while those of nosocomial origin vary much more. Focused therapy requires rapid detection of the bacteria in the cerebrospinal fluid (CSF): standard techniques may miss the microbiological diagnosis in at least 13% of cases [1,2]. Subacute or chronic meningitis is less frequent: the most common cause is *Mycobacterium tuberculosis*, for which a definitive diagnosis is made by culture, positive in up to 80% of cases, but in 2–8 weeks, while direct microscopy is positive in only 10–40% of cases [1,2]. In Italy it seems that a microbiological diagnosis in suspected bacterial meningitis is not made in 40–64% of cases [3].

In the present study, PCR with the broad-range 16S rDNA primers P11E and P13B [4] (16S PCR), followed by sequencing of positive amplicons, was used retrospectively in stored samples and in a small prospective series of CSF samples. In addition, a pair of primers of the 65-kDa heat shock protein mycobacterial gene [5] was used to verify the sensitivity of the 16S PCR in detection of *Mycobacterium tuberculosis*.

MATERIALS AND METHODS

In total, 149 CSF samples from 128 cases of suspected meningitis were examined. Of these, 70 CSF samples

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had been collected, between 1986 and 1990, from 61 patients with suspected community-acquired meningitis in a secondary referral unit, and most had received initial chemotherapy. Another 61 CSF samples had been obtained, during 1991–95, in a general hospital, from 57 patients, including cases of nosocomial infection as well as fresh admissions. In both these groups, standard microscopy and culture had not yielded a microbial etiology (although other parameters were compatible with infective meningitis); the exceptions were a late yield of *M. tuberculosis* in culture in each of the two groups, and one positive microscopy result for acid-fast bacilli, with negative culture, in the first group (see Table 1). The remaining 18 CSF samples came from 10 patients with chronic meningitis of unknown etiology (in some cases with a clinical suspicion of tuberculous meningitis) studied at the time of their illness in 1995–96. One of these patients later yielded a positive culture for *M. tuberculosis*.

As positive controls we used CSF samples from 12 patients which had yielded positive cultures (three *Streptococcus pneumoniae*, three *Listeria monocytogenes*, three *Neisseria meningitidis*, two *Escherichia coli*, and one *Acinetobacter baumannii*). CSF samples from 15 neurologic patients with non-bacterial disease were used as negative controls (six aseptic meningitis syndrome, three cerebral hemorrhages, four multiple sclerosis, two herpes simplex encephalitis).

All CSF samples were stored at −20°C until PCR was performed (1995–96).

DNA extraction

DNA was extracted as described by Pao et al [5] with some modifications: 0.5 mL of CSF was mixed with 0.5 mL of ×2 extraction buffer with lysozyme (×2 extraction buffer: 40 mM EDTA; 800 mM NaCl; 1.5 M sucrose; 100 mM Tris-HCl, pH 9; lysozyme 1 mg/mL).The mixture was incubated at 37°C for 1 h, and sodium dodecylsulfate at 1% and proteinase K (Sigma Chemical Co., St Louis, MO, USA), to a final concentration of 100 mg/mL were then added. After 1 h of incubation at 55°C, the DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and with chloroform.

DNA was precipitated with 0.1 volume of 2 M sodium acetate and two volumes of absolute ethanol overnight at −20°C. After centrifugation at 10 000*g* for 20 min, the pellet was dried in a vacuum desiccator and dissolved in 100 µL of Tris-EDTA buffer (10 mM Tris, pH 8; 1 mM EDTA).

PCR

For 16S rDNA amplification, P11E and P13B primers, which amplify a 220-bp region of bacterial 16S rDNA, selected by Relman et al [4], were used at 25 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min as final extension.

For 65-kDa amplification, the primers described by Pao et al [5], which amplify a 165-bp region of 65-kDa heat shock mycobacterial protein, were used at 40 cycles: 94°C for 1 min, 63°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min as final extension.

The PCR was performed with a heat-stable native-DNA polymerase (*Taq* polymerase; Perkin Elmer, Norwalk, Conn., USA). The final PCR mixture composition was: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 0.5 mM (each) primers, 1.0 mM $MgCl_2$ and 2.5 U of *Taq* polymerase per 50 µL reaction volume. Separate laboratory rooms were used for preparing samples and manipulating the amplification reaction mixture; post-PCR analysis (electrophoresis, dot and Southern blotting) was performed on another floor; dedicated micropipettes and tips were used for all procedures; positive displacement pipettes were used in sample preparation and amplicon manipulation. Each PCR assay was performed in duplicate and the result was considered positive only if both assays gave a clear gel signal. In every PCR amplification, *E. coli* DNA or *M. tuberculosis* DNA was used as positive control, and sterile double-distilled water as negative control.

The sensitivity of amplification with the universal primers was examined at 25 cycles. Serial 10–fold dilutions of *E. coli* DNA were added to the reaction mixture. The 65-kDa amplification sensitivity was tested at 40 cycles with serial 10–fold *M. tuberculosis* DNA dilutions.

Sequencing

The 16S amplicons from negative-culture CSF samples were directly sequenced. Amplified DNA was purified with Agarase according to the manufacturer's instruction (Amersham, Little Chalfont, UK). Nucleotide sequences were determined by the dideoxy chain termination method [6] using a modified Sequenase protocol [7]. Sequence determination was carried out in duplicate on both strands with primers P11E and P13B.

Data analysis

The sequences were compared with the bacterial 16S rDNA in Genbank and EMBL databases with the DNAsis program (Hitachi software CD data genetic database, Pharmacia, Uppsala, Sweden).

Hybridization

Southern blot and dot-blot hybridization were per-

formed on 65-kDa amplicons with a digoxygenin-labeled *M. tuberculosis*-specific oligoprobe [5] according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

RESULTS

The universal 16S rDNA primers, at 25 cycles, gave reproducible amplicons with 100 genomes of *E. coli*; the same sensitivity was obtained in the mycobacterium-specific 65-kDa heat shock protein gene amplification at 40 cycles with *M. tuberculosis* DNA (Figure 1). The positive control CSF samples were all 16S rDNA PCR positive. Sequencing gave the same microbiological identification as culture. The negative control CSF samples were all 16S and 65-kDa PCR negative.

Overall, there were 11 patients in whom a microbiological diagnosis was obtained only with the proposed methods (Table 1). False-positive amplifications were found in seven cases, identified as *Staphylococcus* spp. or *Corynebacterium* spp. (four and three respectively) by sequencing. They were from the oldest stored samples, characterized as possible community-acquired meningitis, but not nosocomial, complicated or secondary meningitis (see Materials and Methods).

In the 118 retrospectively studied patients there were six 16S PCR-positive results: sequencing of the

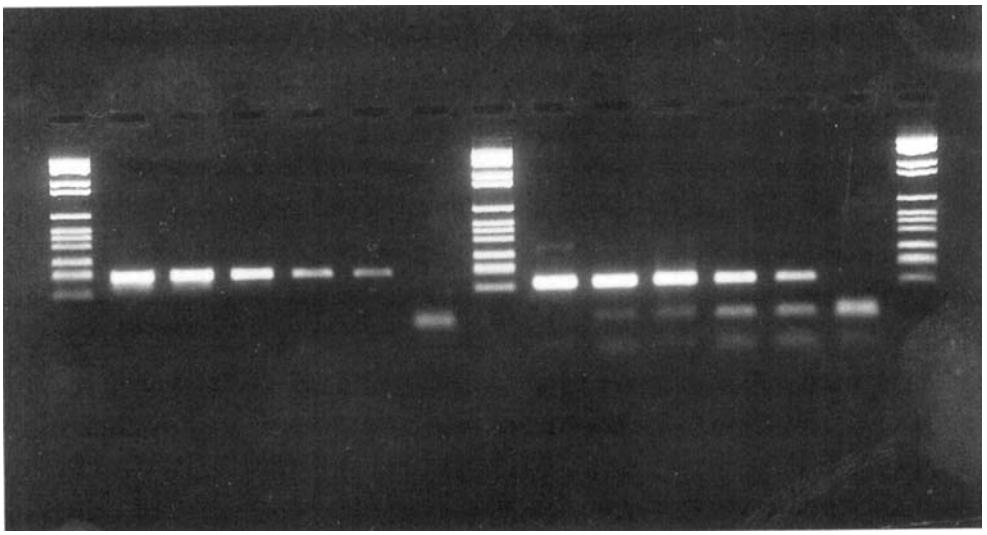

Figure 1 Sensitivity of 16S rDNA PCR. Lane 1: DNA molecular weight marker VI (MW VI Boehringer Mannheim). Lanes 2–7: 10^6, 10^5, 10^4, 10^3, 10^2 and 10 *E. coli* genomes, respectively. Lane 8: MW VI Boehringer Mannheim. Lanes 9–14: 10^6, 10^5, 10^4, 10^3, 10^2 and 10 *M. tuberculosis* genomes, respectively. Lane 15: MW VI Boehringer Mannheim.

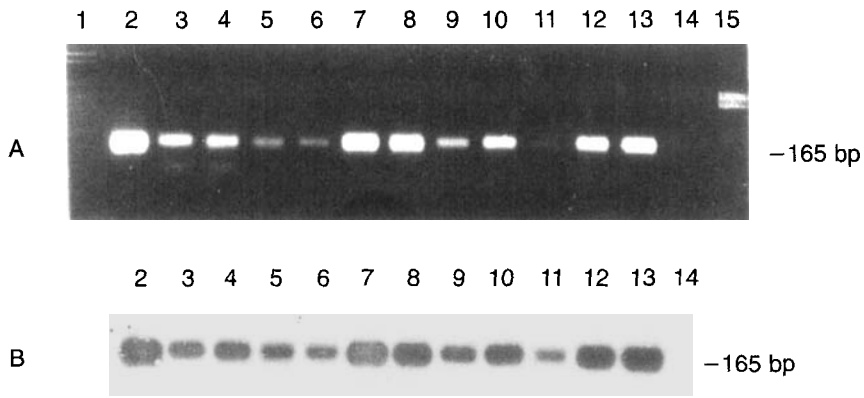

Figure 2 Results in specimens positive in 65-kDa PCR. (A) Lane 1: DNA molecular weight marker VI (MW VI Boehringer Mannheim). Lanes 2–12: 65-kDa PCR-positive samples. Lane 13: positive control. Lane 14: negative control. Lane 15: DNA molecular weight marker V (MW V Boehringer Mannheim). (B) Southern blotting and hybridization of 65-kDa amplicons.

Table 1 Results of direct microscopy, culture, broad-range bacterial PCR with sequencing and *M. tuberculosis*-specific PCR with hybridization in CSF samples

Patient number[a]	Direct microscopy	Culture	65-kDa PCR and hybridization	16S PCR and sequencing
1a	–	–	–	*Streptococcus pneumoniae*
1b	–	–	–	*Streptococcus pneumoniae*
2	–	–	–	*Neisseria meningitidis*
3	–	–	–	*Neisseria meningitidis*
4	–	–	–	*Acinetobacter* spp.
5	–	–	–	*Streptococcus pneumoniae*
6	–	–	–	*Streptococcus pneumoniae*
7a	–	*M. tuberculosis*	+	–
7b	–	–	+	–
7c	–	–	+	–
7d	–	–	–	–
8a	–	–	–	–
8b	+	–	+	–
8c	–	–	+	–
9	–	–	+	–
10a	–	–	+	–
10b	–	*M. tuberculosis*	+	–
10c	–	–	+	–
11a	–	–	+	–
11b	–	–	–	–
11c	–	–	+	–
11d	–	–	–	–
12a	–	–	–	–
12b	–	–	+	–
12c	–	–	+	–
12d	–	–	–	–
13a	–	–	–	–
13b	–	*M. tuberculosis*	+	*M. tuberculosis*
13c	–	–	+	*M. tuberculosis*
14a	–	–	+	–
14b	–	–	+	–

[a]Letters indicate the temporal sequence of specimens from numbered patients.

220-bp amplicons gave four *Streptococcus pneumoniae*, two *N. meningitidis* and one *Acinetobacter* species. The *Acinetobacter* species was from a neurosurgical patient from whom a later CSF sample gave a positive culture for *A. baumannii*. Positive 65-kDa PCR and hybridization results for *M. tuberculosis* (Figure 2) were obtained in the CSF samples of four additional patients (of whom two were also culture positive—see Materials and Methods). In the retrospectively studied samples there were eight of 118 microbiological diagnoses which were overlooked by standard techniques and achieved by gene amplification using two sets of primers (Table 1, patients 1–10).

In the small prospective series of 10 patients with chronic meningitis, the two amplification tests allowed three microbiological diagnoses of tuberculous meningitis missed by standard methods. From the 10 patients, 18 CSF samples were processed; there were eight positive results from four patients with the 65-kDa PCR and hybridization. Two of these CSF samples gave a positive result also with the 16S PCR, while *M. tuberculosis* was cultured in only one CSF sample (Table 1, patients 11–14). In these patients an effect of antituberculous therapy on PCR results was evident. The sequencing of the 16S amplicons identified the causative organisms as *M. tuberculosis*.

DISCUSSION

Amplification using 16S rDNA primers has already been used in culture-negative bacterial diseases [8–12]; it has been suggested as a strategy for the diagnosis of culture-negative bacterial meningitis and applied in the diagnosis of meningitis caused by *N. meningitidis*, *Haemophilus influenzae* and *Streptococcus* spp. [11,13–15]. Hall et al [14] used a semi-nested design in an experimental demonstration of the feasibility of amplifying the 16S–23S spacer region as a method of diagnosis of community-acquired bacterial meningitis. Druel et al [16] have already used bacterial 16S rDNA

primers and sequencing of the PCR products in the diagnosis of a bacterial etiology of 'aseptic meningitis' in nosocomial cases.

With the universal primers P11E and P13B, a relatively narrow region of the 16S rDNA gene of bacteria may be amplified and directly sequenced. Our use of this PCR suggests that about 5% of culture-negative non-tuberculous bacterial meningitis, of variable etiology, can be diagnosed by a 16S rDNA amplification and sequencing technique, but only with the use of 65-kDa protein gene-specific mycobacterial primers is it possible to diagnose most tuberculous meningitis cases. This allowed the detection of two cases of tuberculous meningitis (clinically suspected but missed by standard techniques) among the 118 patients in the retrospectively studied series.The sensitivity of 16S rDNA primers is lower in tuberculous meningitis, probably because *M. tuberculosis* has only one 16S rDNA gene copy in the chromosome, but two 65-kDa heat shock protein genes [17,18]. The lower sensitivity of the 16S rDNA primers as opposed to 65-kDa protein gene primers is also explained by the number of amplification cycles used: in the former it was 25, as suggested by Relman et al [3] in order to exclude false-positive results, while in the latter it was 40, because the specificity of the gene allowed more amplification cycles. In the two tuberculous meningitis cases in the prospective series which were 16S rDNA PCR positive, the sequencing of the amplification products identified the causative organism as *M. tuberculosis*.

The 16S rDNA amplification technique was more successful in the prospectively studied series of cases of chronic meningitis. Positive results with this technique also allowed us to follow the effects of specific anti-tuberculous therapy. In one controversial case, of pediatric tuberculous meningitis (Table 1, patient 11) the first CSF sample was 65-kDa PCR positive and antituberculous therapy was started; a later CSF sample was PCR negative and therapy was discontinued; the third was again positive and the specific therapy was resumed; and a fourth CSF sample was negative. We were informed afterwards that there was a slow but substantial clinical improvement, as occurred in patient 12 (Table 1), an adult with clinically possible tuberculous meningitis, who showed a late clear clinical effect of antituberculous therapy, started on the basis of PCR results. There were no other CSF samples taken from patients 13 and 14 (Table 1) because there was evident clinical success of specific therapy.

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References

1. Durand ML, Calderwood SB, Weber DJ, et al. Acute bacterial meningitis in adults. A review of 493 episodes. N Engl J Med 1993; 328: 21–8.
2. Tunkel AR, Scheld WM. Acute bacterial meningitis. Lancet 1995; 346: 1675–80.
3. Di Nola F. Le meningiti. Torino: Edizioni Libreria Cortina, 1990.
4. Relman DA, Loutit JS, Schmidt T, Falkow S, Tompkins LS. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med 1990; 323: 1573–80.
5. Pao CC, Yen TSB, You JB, Maa JS, Fiss EH, Chang CH. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. J Clin Microbiol 1990; 28: 1877–80.
6. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463–7.
7. Winship PR. An improved method for directly sequencing PCR amplified material by using dimethylsulphoxide. Nucleic Acids Res 1989; 17: 1266.
8. Bottger EC. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol Lett 1989; 53: 171–6.
9. Kox LFF, van Leeuwen J, Knijper S, Jansen HM, Kolk AHJ. PCR assay based on DNA coding 16S rRNA for detection and identification of mycobacteria in clinical samples. J Clin Microbiol 1995; 33: 3225–33.
10. Kocagoz T, Yilmaz E, Ozkara S, et al. Detection of *Mycobacterium tuberculosis* in sputum samples by polymerase chain reaction using a simplified procedure. J Clin Microbiol 1993; 31: 1435–8.
11. Radstrom P, Backmann A, Qian N, Kragsbjerg P, Pahlson C, Olcen P. Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis, Haemophilus influenzae* and streptococci using a seminested PCR strategy. J Clin Microbiol 1994; 32: 2738–44.
12. Kirschner P, Rosenau J, Spinger B, Teschner K, Felldman K, Bottger EC. Diagnosis of mycobacterial infections by nucleic acid amplification: 18–month prospective study. J Clin Microbiol 1995; 34: 304–12.
13. Greisen K, Loeffelholz M, Purohit A, Leong D. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J Clin Microbiol 1994; 32: 335–51.
14. Hall LM, Duke B, Urwin G. An approach to the identification of the pathogens of bacterial meningitis by the polymerase chain reaction. Eur J Clin Microbiol Infect Dis 1995; 14: 1090–4.
15. Tenover FC, Huang MB, Rasheed JK and Persing DH. Development of PCR assays to detect ampicillin resistance genes in cerebrospinal fluid samples containing *Haemophilus influenzae*. J Clin Microbiol 1994; 32: 2729–37.
16. Druel B, Vandenesch F, Greeland T, et al. Aseptic meningitis after neurosurgery: a demonstration of bacterial involvement. Clin Microbiol Infect 1996; 1: 230–4.
17. Kong TH, Coates RM, Butcher PD, Hicman CJ, Shinnick TM. *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. Proc Natl Acad Sci USA 1993; 90: 2608–12.
18. De Wit TFR, Bekelie S, Osland A, et al. Mycobacteria contain two groEL genes: the second *Mycobacterium leprae* groEL gene is arranged in an operon with groES. Mol Microbiol 1992; 6: 1995–2007.