One-Step Heminested PCR for Amplification of *Neisseria meningitidis* DNA in Cerebrospinal Fluid

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A one-step polymerase chain reaction (Heminested-PCR) was designed to target the 16S rRNA fragment simultaneously using a set of primers for the universal bacterial group and a *Neisseria meningitidis* species-specific sequence for diagnostic purposes. The diagnostic features of the Heminested-PCR were evaluated in the study of 168 cerebrospinal fluid (CSF) specimens from 84 patients with a *N. meningitidis* infection, meningitis caused by unrelated bacteria and other etiologies (57 patients), or suspicious cases (27 patients) with clinical symptoms of bacterial meningitis but with negative results from bacteriological procedures. About 90% of patients with bacterial meningitis, including those suspicious cases, had prior antibiotic therapy. The sensitivity, specificity, positive, and negative

predictive values found in relation to culture and/or microscopy were 91.7, 100, 100, and 90.5%, respectively. In patients suspected of having bacterial meningitis, the Heminested-PCR revealed 51.9% (14 patients) positive for *N. meningitidis* infection and 40.7% (11 patients) positive for unrelated bacterial infections. The agreement of the Heminested-PCR with culture and/or microscopy was high and ranked as *almost perfect* (kappa indices > 0.856), in contrast to its agreement with other techniques. These findings speak in favor of the molecular diagnosis of meningococcal meningitis in patients who are culture- and/or microscopy-negative, due to their prior antibiotic treatment. J. Clin. Lab. Anal. 14:193–199, 2000. © 2000 Wiley-Liss, Inc.

Key words: *Neisseria meningitidis*; bacterial meningitis; molecular diagnosis

INTRODUCTION

The meningitis caused by *N. meningitidis* has a large geographic distribution and remains a public health problem in many countries (1). In Brazil this infection is hyperendemic; for example, in 1998, 1,036 cases were reported in the municipality of São Paulo, representing an incidence of 5.04 cases per 100,000 inhabitants with 16.5% of these considered lethal from bacterial serogroups B and C (2). In the Emílio Ribas Infectology Institute (São Paulo), about meningitis 1,000 cases were recorded; 51% of these were caused by *N. meningitidis*, 6.8% by *H. influenzae*, 4.1% by *S. pneumoniae*, and about 10% by unknown etiology (3).

The diagnosis of meningococcal infection relies mostly on cultures and direct microscopic examinations, and their shortcomings are related to specimen deterioration from inadequate conditions for its transportation or storage, and from previous antibiotic therapy (4). Because meningococcal meningitis is a life-threatening infection and can often cause neurological sequels, an early diagnosis of

meningitis is of high priority for the implementation of appropriate therapy.

Thus, the molecular diagnosis of *N. meningitidis* infection by using a fast PCR that can amplify fragments of microbial DNA in cerebrospinal fluid (CSF) or blood specimens has been proposed. These diagnostic techniques are considered to be more sensitive and specific than conventional bacteriological and immunological techniques (5).

Target genes that have been most often investigated for the identification of *N. meningitidis* in cultured microorganisms or in CSFs of infected patients are IS1106 (6,7), *porA* (5,8), *siaD* (9), *dhps* (10,11), and 16S rRNA (12–14). There are few

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studies of the latter gene's diagnostic purposes. For instance, Radstrom et al. (13) have used a semi-nested PCR to detect *N. meningitidis* in CSF, whereas Kotilainen et al. (14) used PCR followed by sequencing to detect the same organism in blood specimens, based on 16S rRNA and/or 23S rRNA genes.

We designed a practical one-step PCR technique (Heminested-PCR) for the diagnosis of the *N. meningitidis* infection with a combination of primers which enable the detection of eubacteria in CSF specimens by targeting the universal 16S rRNA gene, concomitantly with *N. meningitidis*, by flanking the species-specific nucleotide sequence. Since this strategy has not been approached before, the evaluation of this molecular diagnostic technique was performed in comparison with conventional diagnosis criteria (with bases on clinical, bacteriological, and immunological antigen-detection data) in the study of patients with and without meningococcal meningitis, including those patients suspected to have bacterial meningitis.

MATERIALS AND METHODS

Cultured Bacterial Strains

The following bacterial strains were studied in the Heminested-PCR standardization process: *Neisseria meningitidis* serogroup A (CDC 1894), serogroup B (CDC 2991), serogroup C (CDC 1628), and serogroup W135 (Marseille) from the Adolfo Lutz Institute (São Paulo, Brazil). The bacterial controls included *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC 11111), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 13813), *Streptococcus pneumoniae* (ATCC 33400), *Haemophilus influenzae* (ATCC 10211), and *Mycobacterium tuberculosis* H37Ra (ATCC 25177).

Patients and Specimens

The cerebrospinal fluids (CSF) were obtained from 168 patients at the Emílio Ribas Infectology Institute (IIER, National Reference Hospital for Infectious Diseases) as well as at the Clinical Hospital of São Paulo of the Medical School, São Paulo University. Ethical committees from both institutions authorized the study. The clinical histories of the patients and epidemiological data were recorded, and the CSF was submitted to the conventional examinations, consisting of evaluation of its physical aspects, leukocyte count, protein and lactate concentrations, culture and microscopy, and bacterial antigen detection by latex agglutination (LA) and counterimmunoelectrophoresis (CIE) techniques. Five groups were formed based on the clinical and laboratory data according to Ni et al. (6). Group I (n = 19) included patients with diagnoses of meningococcal meningitis (*N. meningitidis* serogroup A, B, C or W135) defined by clinical, culture and/or microscopy, and antigen detection (15) criteria. Group II

(n = 65) included patients with a high probability of *N. meningitidis* infection, showing gram-negative diplococci and/or the presence of antigen in CSF by LA or CIE, but negative by culture. Nevertheless, the clinical symptoms and other laboratory CSF examinations such as leukocyte count and both protein and lactate concentration were consistent with meningococcal meningitis. Group III (n = 20) had patients with unrelated bacterial meningitis caused by *Mycobacterium sp.*, *S. pneumoniae*, *H. influenzae b*, and *Klebsiella sp.* Group IV (n = 37) consisted of patients with nonbacterial infections caused by *C. neoformans* and other etiologies presenting lymphocytic pleocytosis meningitis. Group V (n = 27) gathered patients with consistent clinical symptoms of bacterial meningitis with leukocyte count and both protein and lactate concentration. However, they were culture- and microscopy-negative for different microorganisms. About 90% of patients, excluding those from group IV, had previously received antibiotic therapy.

Cell Lysis

DNA from cultured mycobacteria, gram-positive, and gram-negative bacteria were extracted as previously described (16–18). However, in CSF specimens, DNA was detected directly in cell lysates, as reported by Kertesz et al. (19). Briefly, the specimen was centrifuged and the cell pellet was suspended in 100 µL lysing buffer (5% Nonidet P-40, 5% Tween 80, and 4 ng proteinase K per µL, 1 mg gelatin per mL in PCR buffer, pH 8.3, containing 500 mM KCl, 100 mM Tris-HCl), and it was incubated for 20 min at 56°C and then for 10 min at 95°C.

Primers

N. meningitidis was distinguished from other bacteria with the combination of 3 primers targeting small (17 to 23 oligonucleotides) bacterial species-specific and universal subunits of 16S rDNA. The reaction amplified a universal bacterial fragment, represented by a 370-bp amplicon, with primers RW01 and DG74, and the specific *N. meningitidis* sequence corresponded to a 279-bp amplicon, with primers COR28 and DG74 (7,8), as illustrated in Figure 1.

One-Step PCR Amplification (Heminested-PCR)

Cell lysate was added to a PCR mixture containing 2.0 mM deoxynucleotide triphosphate, primers RW01, DG74, and COR28, 2.0 U Taq polymerase, and 3.0 mM MgCl₂. The amplification was carried out for 5 min at 95°C, using a hot-start procedure, with 30 cycles that each consisted of a 1-min denaturation at 94°C, 1-min annealing at 56°C, and 1-min extension at 72°C. The amplified products were submitted to a 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by UV fluorescence.

Fig. 1. *Neisseria meningitidis* identification for primer locations. 16S rRNA, minor subunit of ribosome; DG74, "primer 1" to universal bacterial fragment identification; RW01, "primer 2" to universal bacterial fragment

identification; COR28, "primer 3" to *N. meningitidis* fragment identification; dark bands, conserved regions; light bands, variable region.

Controls

The *N. meningitidis* reference strain was grown on blood agar supplemented with 5% hemoglobin and a suspension was prepared based on 0.5 MacFarland-scale turbidity, corresponding to 10^8 UFC/mL. To check the sensitivity of the Heminested-PCR, decreasing concentrations of bacteria ranging from 10^7 up to 10^0 UFC/mL were added to CSF samples. DNA was extracted from an aliquot of 10 μ L of each bacterial concentration and amplified by Heminested-PCR technique. To study the specificity of this technique, DNA from aforementioned bacterial reference strains unrelated to *N. meningitidis*, but also from causative agents of bacterial meningitis, were assayed. In each amplification process, negative, blank, and positive controls were included. The negative control was a CSF specimen from a patient without bacterial meningitis, the blank control consisted of sterile water (Mili Q), and the positive control was a CSF specimen from a patient with a defined diagnosis of meningococcal meningitis. The possible interference of inhibition factors present in CSF was also investigated by adding 10 ng of *N. meningitidis* DNA in negative CSF specimens.

Diagnostic Performance of Heminested-PCR

The diagnostic performance of this technique was evaluated in terms of sensitivity, specificity, and both positive and negative predictive values (20). The agreement of Heminested-PCR with other conventional techniques was expressed in kappa index (21,22) and rank (23). Also, 95% confidence (24) was determined for the proportions of positive or negative results.

RESULTS

In the first part of the study, optimal conditions that amplify a 16S ribosome subunit, common to all bacteria group concomitantly with a species-specific sequence of *N. meningitidis*, were standardized for the one-step Heminested-PCR technique.

The purified DNA from cultured *N. meningitidis* was used as a template, and it was found that the amplification technique using primers RW01, DG74, and COR28 was sensitive enough to detect bacterial concentrations as low as 10^1 CFU per ml in CSF. But reproducible results were obtained from the concentration of 10^2 CFU per ml.

The specificity of the same primers was checked assaying heterologous DNA extracted from the nine aforementioned bacteria strains other than *N. meningitidis*, which showed no false positive results. Figure 2 presents DNA fluorescence patterns after the gel electrophoresis stained with ethidium bromide. The DNA fragment amplified, using universal primers RWO1 and DG74, is a band of 370 bp, whereas the species-specific amplification of *N. meningitidis* DNA carried out by primers COR28 and DG74 resulted in a 279-bp amplicon. These patterns were also reproducible using CSF patients with bacterial meningitis and meningococcal meningitis, respectively.

Detectable inhibitor factors in the CSF specimens, which might affect the efficiency of Heminested-PCR, was not observed in this study.

In the second part of study, the diagnostic features of the Heminested-PCR was evaluated using CSF specimens from patients with well-defined diagnoses of meningococcal meningitis, other bacterial meningitis, and other unrelated meningitis, as well as from cases with a clinical suspicion of bacterial meningitis but with negative bacteriological data.

Results obtained from five groups of patients by Heminested-PCR in CSF were compared with those of culture, microscopy, LA and CIE (Table 1). In the PCR procedures amplification technique, the band of 370 bp corresponding to a universal bacterial fragment was seen in all CSF specimens from group I patients who were culture-positive for *N. meningitidis* and in those in group II who were strongly suspected of having meningococcal meningitis with gram-negative diplococci (detected by microscopy). The 370-bp band was also seen in 89.0% of group V patients. On the other hand the amplicon

of 370 bp, together with 279 bp, representing specificity for *N. meningitidis*, was detected in 100% of CSF samples from group I patients, in 89.2% of group II patients, and in 51.8% of group V patients. In general, LA and CIE gave low positivity for meningococcal infection, except in group I patients whose positivity (84.2%) found by LA was similar to microscopy findings.

In seven CSFs from group II patients that were Heminested-PCR-negative, the presence of inhibitor factor was checked by adding a detectable limit of purified DNA from cultured *N. meningitidis* to the specimens, and retesting these specimens in the same amplification technique. Positive results were obtained showing the absence of inhibition factor in those specimens.

The diagnostic features of Heminested-PCR varied from 9.4 to 100% according to different reference techniques (Table 2). When the culture, LA, and CIE were used as reference techniques, Heminested-PCR gave 100% sensitivity and low specificity ranging from 52.5 to 59.8%. Taking microscopy as the reference technique, the DNA amplification technique showed 91.4% sensitivity and 95% specificity. The best diagnostic performance of the Heminested-PCR was observed when both culture and microscopy were taken as reference techniques, giving 91.7, 100, 100, and 90.5% for sensitivity, specificity, and both positive and negative predictive values, respectively.

The agreement of the Heminested-PCR with conventional techniques for the diagnosis of meningococcal meningitis was observed to vary from 57.4 to 95.0% (Table 3). Kappa (κ) indices were calculated and null hypothesis was tested. It was found that most of the z values obtained for κ indices were higher than the critical z (1.96) at 95% confidence, except those for Heminested-PCR and culture, and for Heminested-PCR and CIE that gave no significant z values. The obtained κ index value also enabled the ranking of the agreement of Heminested-PCR with a combination of culture and microscopy, or microscopy alone, as *almost perfect*. Nevertheless, the agreement of the PCR with LA was ranked as *moderate*, and those with culture or with CIE as *slight*.

DISCUSSION

The Heminested-PCR technique was standardized to be performed in a one-step reaction for the detection of any bacteria, simultaneously discriminating *N. meningitidis* in CSF specimens.

The selection of primers was seen to be of critical importance for obtaining efficient DNA amplifications. In a preliminary attempt, NM1 and NM2 primers, homologous to the *N. meningitidis* gene-encoding dihydropteroate syntetase, were assayed but unspecific results were found. Data reported by Kristiansen et al. (11) also show the low specificity given by these primers, although specific results had been observed in their earlier work (10).

Fig. 2. Primer specificities (RW01, DG74, and COR28). EGA 1.5% post-PCR samples. Column 1, molecular weight (100 bp); columns 2, 3, 5, 6, and 7: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycobacterium tuberculosis*; columns 4 and 8, *N. meningitidis* B and W135, respectively.

TABLE 1. Positivity found by Nm-PCR, bacteriological, and antigen detection techniques in the study of 168 cerebrospinal fluids from patients with or without meningococcal meningitis

Group of patients	Technique					
	Nm-PCR ^a (%)	Pact-PCR ^b (%)	Cult. ^c (%)	Microsp. ^d (%)	LA ^e (%)	CIE ^f (%)
I. Meningococcal meningitis (N = 19)	100	100	100	84.2	84.2	42.1
II. High probability of <i>N. meningitidis</i> infection (N = 65)	89.5	100	0	100	27.7	13.8
III. Unrelated bacterial meningitis (N = 20)	0	100	0	0	0	0
IV. Non-bacterial meningitis (N = 37)	0	0	0	0	0	0
Suspicious of bacterial meningitis (N = 27)	51.8	89.0	0	0	0	0

^aPCR for the diagnosis of *N. meningitidis* infection.^bPCR for the diagnosis of unspecified bacterial meningitis.^cCulture.^dMicroscopy.^eLatex agglutination technique.^fCounterimmunoelectrophoresis technique.

Thus, the strategy in this work was to detect the presence of unspecified bacteria with the use of RWO1 and DG74 primers while simultaneously amplifying the 16S rRNA universal fragment resulting in a 370-bp amplicon, along with the use of COR28 and DG74 primers which amplify the species-specific *N. meningitidis* providing a 279-bp amplicon. So, in the identification of *N. meningitidis*, the association of these amplicons (370 and 279 bp) created a pattern requiring investigation.

The above primers showed sensitive and specific results when purified DNA from homologous and heterologous cultured bacteria was assayed. These primers are similar to those reported (25,26) for the PCR carried out in two-step reactions consisting of amplification followed by hybridization in a southern-blot technique, used mostly for the identification of *N. meningitidis* in cultured bacteria.

The designed Heminested-PCR technique proved to be practical by allowing the detection of both *N. meningitidis* and unrelated-and-nonspecified bacteria in a one-step reaction, thereby reducing the reaction time, and also decreasing considerably the chance of contamination. Moreover there is no need to use radioactive probes or restriction enzymes. There are few reports on PCR techniques for the *N. meningitidis*

detection in CSF specimens. These techniques use different primers or probes based on two-step reactions such as the seminested-amplification, amplification and hybridization (25,26), amplification and DNA sequencing (14), amplification and immunoenzymatic assay (9), or yet, in a three-step reaction with consecutive amplifications (5).

In patients with meningococcal meningitis with culture-positive (group I), the Heminested-PCR was 100% positive. In general, DNA amplification techniques give positive results in culture-positive CSF or blood specimens from patients with meningococcal meningitis (7,13). In patients with a high probability of meningococcal infection (group II), gram-negative diplococci were observed in their CSF, but the DNA amplification technique gave 89.5% positivity for *N. meningitidis* infection and the remaining 10.5% were positive for unrelated bacterial infection. Thus, it seems likely that these latter specimens, which showed diplococci by microscopy, were false-positives resulting from the microorganism morphological distortion caused by the previous antibiotic treatment (12,27), or by unsuitable storage or transportation conditions of specimens (4). In all patients with proved bacterial meningitis other than *N. meningitidis* (group III), Heminested-PCR revealed only one fluorescence band of 370

TABLE 2. Diagnostic performance of the Nm-PCR for *N. meningitidis* infection in relation to culture and microscopy techniques, in the study of 141 cerebrospinal fluids from patients with and without meningococcal meningitis

Reference technique	Sensitivity (%)	Specificity (%)	Predictive of positive (%)	Predictive of negative (%)
Culture	100	52.5	24.7	100
Microscopy	91.4	95.0	96.1	89.1
Culture and/or microscopy	91.7	100	100	90.5
Latex agglutination	100	59.8	44.2	100
Counterimmunoelectrophoresis	100	57.4	22.1	100

TABLE 3. Overall agreement of Nm-PCR with different techniques for the diagnosis of meningococcal meningitis

Nm-PCR ^a with other technique	Agreement (N = 168) (%)	Kappa index	Rank
Culture	58.9	0.230	Slight
Microscopy	92.9	0.856*	Almost perfect
Culture plus microscopy	95.0	0.899*	Almost perfect
Latex agglutination	69.5	0.418*	Moderate
Counterimmunoelectrophoresis	57.4	0.205	Slight

^aPCR for the diagnosis of *N. meningitidis* infection.

* $P < 0.05$.

bp, showing no false-positive results. Also, no false-positive results were found in patients with nonbacterial meningitis (group IV).

The diagnostic features of the Heminested-PCR were evaluated in the study of our patient groups I, II, III, and IV. The sensitivity of this technique was high, ranging from 91.4 to 100% in relation to conventional diagnostic techniques. However, the specificity varied from 52.5 to 100%. As expected, the specificity of the Heminested-PCR was low when the culture, LA, and CIE were individually taken as a reference technique because they are not sensitive when patients had previous antibiotic therapy, and thus, the tests yield negative results. The Heminested-PCR showed good diagnostic performance when evaluated in relation to culture and/or microscopy, presenting 91.7% sensitivity and 100% specificity, as well as high predictive positive and negative values of 100 and 89.1%, respectively. These findings were higher than those obtained in relation to each of the individual techniques, i.e., microscopy, LA, culture, and CIE.

In order to compare our findings with those reported by investigators using different types of PCR techniques, we calculated 95% confidence intervals based on their published data. So, the sensitivity of the Heminested-PCR did not differ from those sensitivities found for different PCR techniques, targeting 16S rRNA, 16S rRNA and/or 23S rRNA, IS1106, porA, and siaD, which varied from 81 to 100%, usually taking culture and/or microscopy as reference techniques (5–7,9,13,14). In these reported evaluations, however, the number of studied specimens from patients with meningococcal meningitis was low (5 to 35). Also the specificity of our DNA technique did not differ from those reported PCR techniques which were performed in anywhere from 45 to 289 patients without meningococcal meningitis.

Also, we calculated the agreement between reported PCR techniques with the conventional diagnosis criteria for meningococcal meningitis adopted by authors (5–7,9,13,14). The κ index as well as null hypothesis for these indices was also calculated based on their reported data. The κ index found for Heminested-PCR ranked as *almost perfect* in similar fashion to those reported for semi-nested PCR using different primers targeting 16S rRNA gene (13), PCR amplifying of a IS1106 gene designed for blood specimen (7), and PCR sequencing of a porA gene (5). Nevertheless, the κ index of the

Heminested-PCR was better than those calculated for PCR targeting of a IS1106 gene for CSF specimens (6) and PCR targeting of a siaD (9) as shown in their values 0.74 and 0.79, respectively; both ranked as *substantial*.

The findings show that the Heminested-PCR was not affected by previous antimicrobial therapy since 90% of the studied patients had taken antibiotic before their hospital admission. The CFS specimens were submitted to molecular procedure within five to six hours, depending on whether or not the patient had received previous antimicrobial therapy.

The results obtained in 27 specimens from patients who were suspected, upon clinical evaluation and other nonspecific CSF analysis, of having bacterial meningitis (group V) but whose cultures and microscopies were negative, here emphasize the relevance of the Heminested-PCR for ascertaining the meningococcal meningitis. The Heminested-PCR revealed that 89.0% (24 patients) had bacterial infections by the amplification with universal primers, including 51.8% (14 patients) with *N. meningitidis* infection, which were identified by species-specific primers. In order to compare our finding of meningococcal meningitis with those reported data, 95% confidence intervals calculated based on published results. Thus, the detection of *N. meningitidis* in 51.8% group V patients did not differ significantly from those 53.6% found by PCR using IS1106 as a target gene (7) in a group of patients with negative results by culture and microscopy. Also statistically, the 71.4% detected by PCR of porA gene as a target (8) are similar to our findings. In group V, we found three CSF specimens completely negative by Heminested-PCR. There are several explanations for these results: the degradation of DNA fragments in the CSF, the meningitis was not caused by a bacterial agent, or, the amount of bacteria in the biological fluid was less than that which is a detectable concentration for the DNA amplification.

We concluded that the Heminested-PCR technique is useful for the detection of any bacterial meningitis, specifically *N. meningitidis*. It is helpful, too, in confirming clinically suspected cases of meningococcal or bacterial meningitis. In other words, this strategy for DNA amplification by PCR in one step is particularly aimed at patients with negative results by culture and/or microscopy (frequently observed in those with acute *N. meningitidis* infection) and who have previously had antibiotic therapy.

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