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Evaluation of nested PCR method for diagnosis of meningitis due to *Neisseria meningitidis* and *Haemophilus influenzae*

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Abstract: Meningitis due to *Neisseria meningitidis* and *Haemophilus influenzae* is a common infectious disease among infants and children that can be fatal in the absence of rapid and proper diagnosis and treatment. Therefore, a rapid and sensitive method for diagnosis is a high priority. The aim of this study was to compare the performance of culture, conventional polymerase chain reaction (PCR), and nested PCR methods for diagnosis of meningitis caused by *N. meningitidis* and *H. influenzae*. A total of 106 cerebrospinal fluid samples were collected from infants and children suspected of having meningitis. A portion of these samples were used for culture and DNA extraction. The extracted DNA was placed in a PCR reaction that contained specific primers for *N. meningitidis* and *H. influenzae*. PCR products from negative samples were reamplified with internal primers by nested PCR reaction. The results indicated that among the 106 samples evaluated for *N. meningitides*, 1 (0.9%) sample was positive by culture, whereas 3 (2.8%) samples were positive by PCR and 7 (6.6%) were positive by nested PCR. For *H. influenzae*, 2 (1.9%) samples were positive by culture, whereas 3 (2.8%) were positive by PCR and 9 (8.5%) were positive by nested PCR methods. In conclusion, among the culture, conventional PCR, and nested PCR methods evaluated, nested PCR showed higher sensitivity than culture or PCR methods.

Key words: Bacterial meningitis, *Neisseria meningitidis*, *Haemophilus influenzae*, PCR, nested PCR

Introduction

Bacterial meningitis is a serious and fatal infection affecting the central nervous system. About 60% of all meningitis cases occur among children, and *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumonia* are the pathogens responsible for 80%–90% of cases (1). Mortalities due to meningitis are mainly related to delay in diagnosis and treatment, and there is a critical demand to improve methods for rapid diagnosis of meningitis. The clinical signs of bacterial meningitis vary depending on age. In children 6–10 years old, the main symptoms include meningeal irritation and neck stiffness. In younger children signs of meningitis include fever, irritability, lethargy, and sleepiness, particularly if associated with signs of meningeal irritation or high intracranial pressure. Among the routine laboratory tests used for the diagnosis of bacterial meningitis, culture is considered the gold standard; however, it is a time-consuming

technique that requires viable microorganisms, and its sensitivity is affected by antibiotic treatments initiated before spinal puncture. Other rapid tests, such as latex agglutination, have low sensitivity.

Currently, molecular techniques such as polymerase chain reaction (PCR) are being widely used for the etiologic diagnosis of different infections. Kristiansen et al. showed the presence of *N. meningitidis* DNA in the culture-negative cerebrospinal fluid (CSF) sample from a patient with meningococcal disease after antibiotic treatment (2). Ni et al. studied the use of PCR in diagnosing meningococcal meningitis (3). Their results showed specificity and sensitivity of 91%; moreover, neither sensitivity nor specificity was affected by previous antibiotic treatment. Radstrom et al. described a nested PCR strategy for the simultaneous detection of *N. meningitidis*, *H. influenzae*, and *Streptococcus* spp. in CSF (4). Their study demonstrated the possibility of simultaneous amplification of 3 major pathogens that cause bacterial meningitis; however, their method had certain shortcomings such as false-positive results due to contamination and false-negative results due to the presence of PCR inhibitors in CSF, which compromised the diagnostic reliability of the technique. Additional studies linking clinical and laboratory findings to PCR results are essential to confirm the diagnostic value of PCR. Nested PCR is the reamplification of PCR product with primers that are internal to the first PCR primers. This technique is of higher sensitivity and specificity than single-step PCR (5). In the present work, we compare the reliability of nested PCR and routine culture for detection of *N. meningitidis* and *H. influenzae* in patients suspected of having meningitis.

Materials and methods

After an informed consent form was signed by parents or guardians, 106 children with suspected bacterial meningitis referred to the Children's Hospital of Tabriz in northwestern Iran were enrolled in the present study. CSF samples were divided into 2 portions for culture and PCR methods.

Culture of the samples

All CSF samples were quickly transferred to the laboratory and inoculated onto blood agar (Merck, Darmstadt, Germany) and chocolate agar, incubated at 37 °C, and monitored daily for 2 weeks for bacterial growth. Positive cultures were tested by selected biochemical tests, including the Kovacs oxidase test and carbohydrate utilization, for identification of *N. meningitides* and X and V factor requirements for *H. influenzae*.

DNA extraction from CSF samples

DNA extraction was performed by the SDS-proteinase K phenol-chloroform method (6). Briefly, samples were centrifuged at 10,000 rpm for 10 min, and the pellet was dissolved in 300 µL of TE buffer (Tris-HCl, 10 mM; EDTA, 1 mM) containing sodium dodecyl sulfate (SDS) and proteinase K. The reaction was incubated at 55 °C for 60 min followed by extraction with phenol-chloroform-isoamyl alcohol (24:24:1). Finally, the pellet was washed with 70% ethanol, dried at 37 °C for 10 min, and dissolved in 30 µL of TE buffer. The concentration of DNA was measured at 260 nm, and 1–2 µL (50–100 ng) from each sample was used for PCR.

PCR procedure

The sequence of primers used for specific amplification of *N. meningitidis* and *H. influenzae* is shown in Table 1. PCR

Table 1. The sequence of oligonucleotide primers used in this study.

Organisms	Gene target	Primer sequences	Size of product (bp)
N. meningitidis	*ctrA*-PCR	**F**:5'-TGGCCTTTCTTCGATGGG-3' **R**:5'-CGTTGAACCACCTACCGCAG-3'	313
	ctrA-nested PCR	**F**:5'-GAGCAGTTGGTCACGGCACG-3' **R**:5'-ACACACGCTCACCGGCTGC-3'	232
H. influenzae	*bexA*-PCR	**F**:5'-GCGTAAATAATGTATGTAAGAAGTATCAC-3' **R**: 5'-CGTCTCGTTGTAGTATTGATACGC-3'	638
	bexA-nested PCR	**F**: 5'-GGCGAAATGGTGCTGGTAA-3' **R**: 5'-CCCTGATGAGTACTTCTTCACTGG-3'	256

was carried out as described by Okay and Özcengiz (7). Briefly, 1–2 μL of each DNA sample was entered into a 25-μL reaction mixture containing 2.5 10X PCR buffer [10 mM Tris-HCl (pH 8), 50 mM KCl], 1.5 mM MgCl2, 10 pM of each primer, 0.2 mM of each dNTP, and 1 unit of Taq DNA polymerase. A control tube containing no target DNA (as the negative control) and a tube containing DNA of *N. meningitidis* (ATCC 13077) and *H. influenzae* (ATCC 49766) (as positive controls) were included in all PCR sets. PCR amplification was carried out at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 60 s, and extension at 72 °C for 50 s with a final extension cycle at 72 °C for 5 min. The reaction was performed using an automated thermal cycler (Eppendorf, Germany). For nested PCR reactions, 1 μL of PCR product was used as a template along with internal primers. PCR products were electrophoresed on 1% agarose gel and stained by ethidium bromide. DNA bands were visualized under an ultraviolet light (UV transilluminator) and documented.

Results

All 106 CSF samples (suspected meningitis patients) were tested by culture, PCR, and nested PCR methods. The summary of results is shown in Table 2. Among the 106 CSF specimens tested, 16 (15%) samples were positive by nested PCR; 7 specimens (6.6%) contained *N. meningitidis* DNA and 9 specimens (8.5%) contained *H. influenzae* DNA. Of the 16 nested positive cases, 3 cases (18.7%) were positive by culture; 1 (6.2%) was positive for *N. meningitidis* and 2 (12.5%) were positive for *H. influenzae*. Conventional PCR identified the correct pathogen in 6 (37.5%) of the 16 cases (Figures 1 and 2). Comparison of the results from the 3 methods showed that 3 cases (18.75%) were positive by all 3 methods, whereas 6 cases (37.5%) were positive by both conventional PCR and nested PCR, and 10 specimens (62.5%) were positive only by nested PCR. The positivity rate of conventional PCR was 2 times higher and the nested PCR positivity rate was 5 times higher than the positivity rate of the culture method (Figures 1 and 2).

Discussion

Rapid diagnosis of bacterial meningitis is of critical importance for controlling the mortality rate of the disease. The definitive diagnosis of meningitis is based on isolation of the bacteria causing meningitis from CSF or blood. However, the culture method takes at least 24 h, and in cases where patients have already received antimicrobial therapy, the sensitivity of the culture is noticeably reduced (8). In recent years, molecular methods, especially PCR, have revolutionized the diagnosis of infectious diseases due to their sensitivity and rapidity. In this study we compared the diagnostic efficacy of the culture and PCR methods for the diagnosis of bacterial meningitis due to *N. meningitides* and *H. influenzae*. The results of this study showed that the efficiency of PCR is significantly higher than culture for early diagnosis of bacterial meningitis (Table 2).

Our results showed that the culture method detected infection in 2.8% of cases; *N. meningitidis* and *H. influenzae* were responsible for 0.9% and 1.8% of these cases, respectively. In comparison, the PCR method was positive for *H. influenzae* in 3.8% of cases and for *N. meningitidis* in 1.9% of cases. The highest sensitivity was obtained by nested PCR; with this method, 8.5% of cases were positive for *H. influenzae* and 6.6% for *N. meningitidis*.

Table 2. Results of culture, PCR, and nested PCR methods in CSF samples collected from children suspected of having meningitis.

Bacterial agent	Total	Positive by:					
		culture; no. (%)	PCR; no. (%)	nested PCR; no. (%)	culture + PCR; no. (%)	culture + nested PCR; no. (%)	culture + PCR + nested PCR; no. (%)
H. influenzae	106	2 (1.9)	1 (0.9)	6 (5.7)	3 (2.8)	8 (7.5)	9 (8.5)
N. meningitidis	106	1 (0.9)	2 (1.9)	4 (3.8)	3 (2.8)	5 (4.7)	7 (6.6)
Total	106	3 (2.8)	3 (2.8)	10 (9.5)	6 (5.6)	13 (12.2)	16 (15.1)

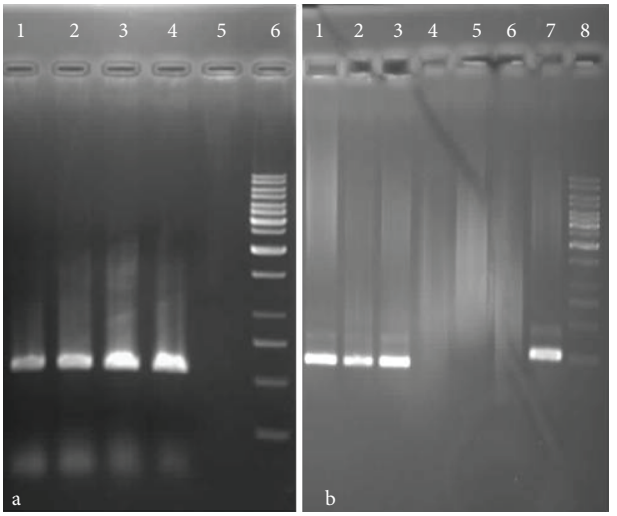

Figure 1. PCR (a) and nested PCR (b) amplification of *N. meningitides* from CSF samples.

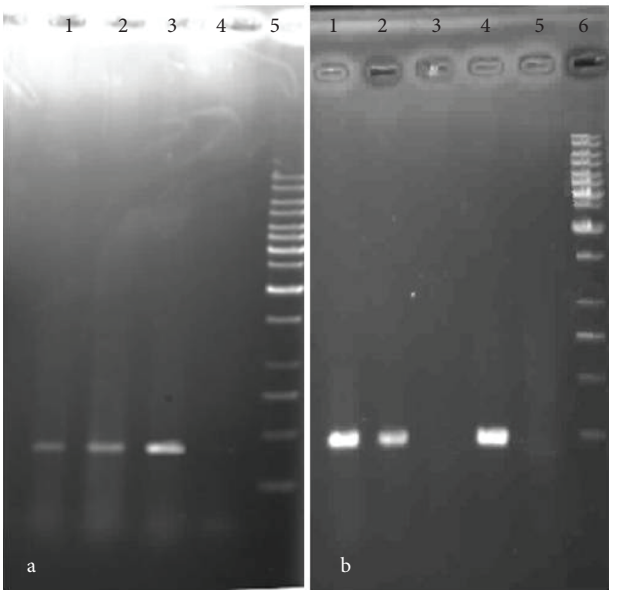

Figure 2. PCR (a) and nested PCR (b) amplification of *H. influenzae* from CSF samples.

These findings are consistent with previous reports regarding the sensitivity of molecular methods for the diagnosis of infectious diseases (9,10). Fredricks and Relman demonstrated that PCR is an accurate method for detection of bacteria in sterile body fluids such as blood and CSF (11). Diggle and Clarke showed that nested PCR is a very sensitive method capable of detecting patients with meningococcal meningitis (12). Du Plessis et al. pointed out that there is generally not enough CSF to conduct most laboratory tests (13), whereas a few microliters of CSF are sufficient for nested PCR. Papavasileiou et al. reported that among 56 meningitis cases, the culture method was positive in only 21% of cases, whereas 77% of cases were detected only by PCR (14). Rasslan et al. evaluated the performance of culture and PCR for diagnosis of tuberculous meningitis and showed that PCR was positive in all culture-positive cases. Furthermore, PCR has shown positive results in smear-positive, culture-negative cases (9). Parija and Gireesh stated a preference for nested PCR over culture for early and sensitive diagnosis of tuberculous meningitis (10). Our study also revealed that among culture, conventional PCR, and nested PCR methods, nested PCR has higher sensitivity.

In conclusion, the results of this study show the high sensitivity of nested PCR for diagnosis of bacterial meningitis. Implementation of PCR assays as a diagnostic procedure in public health laboratories is suggested.

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