

DETECTION OF *HAEMOPHILUS INFLUENZAE* TYPE B IN CEREBROSPINAL FLUID OF SUSPECTED CHILDREN WITH MENINGITIS BY PCR

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ABSTRACT

Background: A polymerase chain reaction (PCR) assay with primers from “hpd” gene was compared with culture for the detection of *Haemophilus influenzae* type b (Hib) in CSF samples from children suspected of meningitis.

Methods: 300 cerebrospinal fluid (CSF) samples from suspected meningitis cases were studied by culture (Leventhal & Chocolate Agar) and PCR.

Results: The latter method could detect Hib in all 5 culture positive and in 2 of 295 culture negative cases, showing sensitivity, specificity, and an accuracy index of 100%, 99% and 99%, respectively. The PCR result was available within a day.

Conclusion: This study has shown that PCR is a rapid, sensitive, and specific diagnostic test for Hib from CSF samples. Furthermore, to maximize management of meningitis cases to reduce the morbidity, mortality, and complications of invasive Hib infection a combination of culture and PCR is necessary for the detection of Hib infection. The incidence of Hib meningitis among children in Children's Medical Center in Tehran based on culture and PCR results were 2.4 % and 1.7% respectively. Based on culture results, Hib causes 15.6% of all cases of bacterial meningitis in children.

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Keywords: *Haemophilus influenzae* type b; Meningitis; PCR; culture; CSF; Iran; Incidence.

INTRODUCTION

Bacterial meningitis is a serious and sometimes fatal infection affecting the central nervous system.¹ *Haemophilus influenzae* type b (Hib) is an important

cause of meningitis, resulting in significant morbidity and mortality in children aged less than five years in countries where children are not vaccinated against Hib.² Encapsulated strains are responsible for a variety of invasive diseases, the most frequent being meningitis, but epiglottitis, arthritis, pneumonia, and cellulitis also occur.³ A recent study in Children's Medical Center in Tehran during 2001-2002 showed that Hib is the cause

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of 15.6 % of cases of bacterial meningitis. The exact burden of invasive Hib disease in Tehran is unknown, since optimum and rapid laboratory facilities are not routinely available for detecting the etiologic agent of meningitis, the most important disease caused by Hib.² In many industrialized countries, the incidence of invasive Hib disease has fallen sharply with the introduction of Hib conjugate vaccines into routine immunization programs.² Since the widespread use of *Haemophilus influenzae* capsular type b (Hib) conjugated vaccines in the United States began in 1987, the incidence of invasive Hib disease in children less than 5 years old has declined drastically.⁴ Despite the success of the Hib vaccination program, the re-emergence of invasive Hib disease has been noted in a well vaccinated population, implying the necessity for continuous surveillance in the post-vaccination period.³ Diagnosis of acute bacterial meningitis by direct microscopy is rapid but non-specific and has a low sensitivity. On many occasions, it fails to provide definitive evidence of infection due to a particular species.² Traditional laboratory diagnostic methods (Culture-CSF) take up to 36 hours or more and may show negative results in approximately 30% of cases due to antibiotic use prior to sampling.⁴ Furthermore, it has been observed that following an increase in the practice of starting antimicrobial therapy prior to clinical samples collection, the ability to confirm the pathogenic microorganisms of bacterial meningitis has decreased by approximately 30%. The need for rapid, sensitive and specific methods for the diagnosis of meningitis due to Hib is becoming more urgent, since strains of Hib resistant to ampicillin, chloramphenicol, and co-trimoxazole, emerged in different parts of the world, questioning the appropriateness of empirical antimicrobial therapy. Moreover, estimation of the burden of Hib disease and the efficacy of Hib vaccination is imprecise without an opti-

imum diagnostic test.⁵ The development of a PCR assay for target gene amplification has enabled the detection of low numbers of pathogens in clinical samples.⁵ We carried out this study to detect Hib directly from the CSF of patients suspected of meningitis by culture and PCR assay.

MATERIAL AND METHODS

Study population

The study population included children less than five years old admitted to Children’s Medical Center of Tehran. CSF samples of 100 µL were gathered from suspected cases. A child with signs and symptoms of meningitis, such as high fever, lethargy, convulsions, bulged fontanelles, neck stiffness, etc., was considered a case of meningitis, and a lumbar puncture (LP) on that child was obtained.⁵

Clinical samples

300 Cerebrospinal fluid (CSF) samples were collected for detection of Hib with PCR and culture (Leventhal and Chocolate Agar medium).

Laboratory diagnosis of Hib

Patients with suspected bacterial meningitis were subjected to lumbar puncture for collecting CSF. All CSF samples were cultured on Chocolate Agar and Leventhal medium. The suspected bacterial colonies were identified by typical Gram stain morphology, catalase, oxidase and requirement test of factors X and V.⁴ The *Haemophilus influenzae* isolates were serotyped by Hib-specific antiserum.⁸

Detection of Hib by PCR

One hundred µL of CSF was taken in a microcentrifuge tube and was heated in a boiling water bath for 15 min-

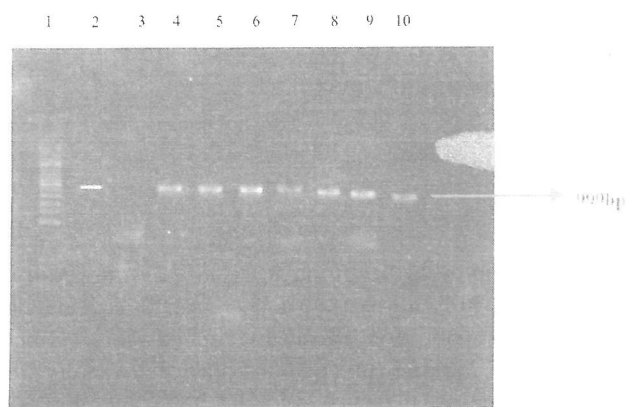


Fig. 1. Gel electrophoresis of positive Hib samples. (1) DNA size marker, (2) positive control, (3) *E. coli* DNA as negative control, (4-10) positive samples of *Haemophilus influenzae* type b.

Table I. Comparison of results of PCR with that of culture for detection of *H. influenzae* type b from CSF of suspected meningitis cases.

PCR	Culture		Total (n= 300)
	Positive (n= 5)	Negative (n= 295)	
Positive	5	2	7
Negative	0	293	293

The sensitivity of PCR from CSF was 5 (100%) of 5 for culture positive *H. influenzae* type b (Hib). The specificity was 293 (99%) of 295 for culture negative Hib. The test had a positive predictive value for 5 (71%) of 7 and a negative predictive value of 293 (100%) out of 293.

utes and centrifuged at 10000 g for 1 minute. Ten μL of supernatant was used as template DNA.⁸

PCR primers

The selection of primers was based on the published work of Sasaki and Munson. The primers were constructed from the "hpD" gene encoding outer membrane protein, which acts as a glycerophosphodiester phosphodiesterase. The primers for detecting Hib were Haem Right (primer 1) [5'-CAGTAAATACACCTGTTGCCCTG-3'], and Haem Left (primer 2) [5'-GCCATTCATCAAATA-3'], and were similar and complementary to the nucleic acid sequence of conserved regions of the "hpD" gene specific for b serotype of *H. influenzae*. Both primers were comprised of 24 bases.⁸

PCR assay

PCR was performed in a reaction mixture of 50 μL containing 1.5 μL (1.5 mM) MgCl_2 , 1 μL (0.2 mM) dNTP, 5 μL (1X) 10X PCR buffer (KCl, Tris-HCl, Magnesium), 0.5 μL (2.5U) of Taq DNA Polymerase (Cinnagen, Lot.810017), 10 μL DNA Template (*Haemophilus influenzae*, ATTC 35056 Difco as control positive), 40 μL D.W, and primers (Primers 1,2: 2 μL or 40 pm) per reaction tube. The reaction mixture was processed in a programmable DNA thermal cycler (Ependorff, Germany) consisting of 35 cycles with the following parameters: (a) denaturing for two minutes at 94°C, (b) annealing for two minutes at 56°C, and (c) extension of primers for two minutes at 72°C. The primary denaturaton took 10 minutes at 94°C and final extension 8 minutes at 72°C. At the end of 35 cycles, the samples were kept at 4°C until analyzed. PCR products for Hib were detected by 1.5 % agarose gel electrophoresis.

RESULTS

Over a period of one year (Dec. 2001- Dec. 2002), 300 children suspected of meningitis aged less than five years were included in the study. Hib was isolated from CSF cultures. Five of the 300 CSF samples were positive for Hib, both by culture and CSF-PCR. The latter also detected Hib in 2 of 295 culture-negative CSF samples (Fig 1). Compared to the CSF culture results, CSF-PCR yielded 2 false positive and no false negative results (Table I). The sensitivity and specificity of CSF-PCR was 100% and 99%, respectively. Based on PCR and culture results the incidence of Hib among children referring to the Children's Medical Center of Tehran were 2.4% and 1.7% respectively. Based on culture results, 15.6% of the cases of bacterial meningitis in children were due to

Haemophilus influenzae type b. Age at diagnosis ranged from 10-42 months (median, 22 months). Incidence among males and females based on culture and PCR results were 20%, 14% and 80%, 86% respectively.

DISCUSSION

PCR is increasingly being used in clinical microbiology laboratories for the detection of agents of infectious disease, since the conventional diagnostic methods, such as microscopy, culture, and serology, fail to identify the responsible pathogen in many occasions.¹⁰ In the present study, we found PCR to be a useful technique for the detection of Hib "hpD" DNA in the CSF samples from suspected meningitis patients. The "hpD" gene has been used as target DNA for amplification by PCR. The primers were sensitive and specific for the detection of Hib in CSF samples. The selected primer showed excellent specificity with non-Hib strains. PCR was also negative with other common agents of meningitis such as *Pneumococcus* and *S. agalactiae*. It provided a result within a day from the clinical samples, whereas the culture methods took up to 36 hours or more. In a recent study, the DNA extraction method of Shoma et al. was used for CSF samples.¹² The limitation of the study is that we used agarose gel electrophoresis for screening PCR products, which appears to be a less-sensitive method for detecting PCR products than hybridization.^{1,4,12} DNA extraction by a commercial kit may also increase the yield of DNA sufficiently for PCR amplification. It has been observed in our clinical microbiology laboratory, as in many other laboratories,¹² that there is a growing discrepancy between the numbers of clinically suspected and culture-confirmed cases of bacterial meningitis. To address this problem, non-culture methods, such as CSF-PCR, have been employed and shown to detect additional cases of Hib meningitis in our study. CSF-PCR showed a sensitivity of 100% compared to the conventional culture method. Thus, the sensitivity of CSF-PCR was superior to that of bacterial culture. It is a good diagnostic test for the detection of Hib in CSF samples. It is relatively simple and requires a short period of time compared to culture. However, determining the antibiotic susceptibility of Hib isolates is a problem in the PCR technique. Further development of a multiplex PCR for detecting both Hib and the antibiotic resistance gene would be extremely useful.¹² Both culture and PCR tests detected Hib in CSF samples. We did not detect other serotypes of *H. influenzae* by culture from CSF,

suggesting that Hib is the primary cause of invasive infection such as meningitis among children in Tehran. Following diagnosis of meningitis, administration of early therapy with a proper antimicrobial agent is the key factor to reduce mortality and morbidity and sequels of invasive Hib disease, particularly meningitis.

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REFERENCES

1. Corless CE, et al: Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol* 39: 1553-1558, 2001.
2. Salisbury DM: Summary statement: the First International Conference on *Haemophilus influenzae* type b infection in Asia. *Pediatr Infect Dis J* 17(Supple): S93-5, 1998.
3. Dabernat H, et al: *Haemophilus influenzae* carriage in children attending French day care centers: Molecular Epidemiological Study. *J Clin Microbiol* 41: 1664-1672, 2003.
4. Leslye L, et al: Identification of *Haemophilus influenzae* serotypes by standard slide agglutination serotyping and PCR-based capsule typing. *J Clin Microbiol* 41: 393-396, 2003.
5. Dalton HP, Allison MG: Modification of laboratory results by partial treatment of bacterial meningitis. *Am J Clin Pathol* 49: 410-413, 1986.
6. Saha SK, Rikitomi N, Ruhulamin M, Wtanabe K, Ahmad K, et al: The increasing burden of disease in Bangladesh children due to *Haemophilus influenzae* type b meningitis. *Ann Trop Paediatr* 17: 5-8, 1997.
7. Hassan-King M, Baldeh I, Adegbola R, et al: Detection of *Haemophilus influenzae* and *Streptococcus pneumoniae* DNA in blood culture by a single PCR assay. *J Clin Microbiol* 34: 2030-2, 1996.
8. Van Ketel RJ, de Wever B, van Alphen L. Detection of *Haemophilus influenzae* in cerebrospinal fluid by polymerase chain reaction DNA amplification. *J Med Microbiol* 33: 271-6, 1990.
9. Hassan-King M, Adegbola R, et al: A polymerase chain reaction for diagnosis of *Haemophilus influenzae* type b disease in children and its evaluation during a vaccine trial. *Pediatr Infect Dis J* 17: 309-12, 1998.
10. Galil K, Singleton R, Levine OS, et al: Re-emergence of invasive *Haemophilus influenzae* type b disease in a well vaccinated population in remote Alaska. *J Infect Dis* 179: 101-6, 1999.
11. Barenkamp SJ, et al: Comparison of outer-membrane protein subtypes and biotypes of isolates of *Haemophilus influenzae* type b. *J Infect Dis* 144: 480, 1981.
12. Shreen SH, Mahbubur R: Rapid detection of *Haemophilus influenzae* type b in Bangladesh children with pneumonia and meningitis by PCR and analysis of antimicrobial resistance. *J Health Popul Nutr* 19(4): 268-274, 2001.