

Frequency and Antimicrobial Susceptibility of *Haemophilus influenzae* Type b Isolated from Children Suspected to Meningitis

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Abstract

Background: *Haemophilus influenzae* type b (Hib) has emerged as the predominant cause of meningitis in developing countries wherever it has been studied using good microbiologic procedures, but data regarding frequency, antimicrobial susceptibility and preferable method for detection of *Haemophilus influenzae* type b isolated from Iranian patients were scarce.

Methods: One thousand and eight hundred suspicious CSF samples were received from Children Medical Center Hospital in Tehran, periodically. All samples were cultured on conventional and selective media for *Haemophilus influenzae* type b detection. PCR performed for samples were Hib negative in culture. The susceptibility of the isolates to different antimicrobial agents was determined using the disk diffusion method.

Results: Overall, 6.7% (n= 121) of specimens had positive culture. 15.7% (n= 19) of isolates were diagnosed as *Haemophilus influenzae* type b with both culture and Antiserum test. PCR assay detected 10 isolates of *H. influenzae* type b in samples weren't detected in culture. *H. influenzae* type b isolates were resistance to ampicillin (42.1%), chloramphenicol (36.8%), and co-trimoxazole (52.6%), respectively. The range of antimicrobial susceptibility to cephalosporins was from 52.6% for ceftriaxone to 57.8% for ceftriaxone, ceftazidime, cefixime and ceftizoxime. Multidrug resistances were observed in 31.5% of *H. influenzae* type b isolates.

Conclusion: Although we found high frequency of resistance to the first line drugs for *H. influenzae* type b in Iran, but the frequency of this organism among children meningitis was lower than many Asian and European countries. PCR assay was more sensitive in detection of *H. influenzae* than culture method.

Keywords: *Haemophilus influenzae*, Meningitis, Antimicrobial susceptibility, Iran

Introduction

Following the introduction of *H. influenzae* type b (Hib) conjugative vaccines, incidence of Hib infections declined dramatically, with near elimination of invasive Hib infections in many countries where Hib vaccines are routinely given to all infants. However, the benefit of Hib vaccine is still limited primarily to children of the developed world and data regarding Hib diseases in developing countries are scarce, which has led to delay of the introduction of Hib vaccine in these countries.

In Asian countries such as Iran, Hib rarely reported as a causative agent of bacterial meningitis annually (1-5).

Recent reports on antimicrobial susceptibility of *H. influenzae* have indicated high resistance of this organism to various antibiotics, especially β -lactamase-mediated resistance to ampicillin. However, the frequency of *H. influenzae* resistance to antibiotics varies widely in the world (6-7), but there are a few reports from South Asia (8-9), and no reports from Iran.

The goals of this study were to determine the prevalence, antimicrobial susceptibility patterns of *H. influenzae* isolates collected from Children Medical Center hospital (CMC) and to search the diagnostic value of polymerase chain reaction (PCR) for the detection of bacterial DNA in CSF specimens in comparison to conventional culture method.

Materials and Methods

Bacterial isolation and identification

Cerebrospinal fluids (CSF) from 1800 cases with clinical signs of meningitis (10) received from CMC hospital during Sep 2001 to Jan 2007. The CSF samples prepared according to the standard methodology. Briefly, the sediment of CSF samples cultured on the chocolate agar, blood agar, and levinthal medium plates and incubated for 24-48 h at 37° C in a CO₂ incubator or candle jar. Colonies suspected to *H. influenzae* on chocolate agar and levinthal medium were further confirmed on the basis of their growth requirement for hemin and Nicotinamide dinucleotide (NAD), using the “X” and “V” factor disks (Oxoid, UK) on trypticase soy agar plate for 24 h.

Serotype identification for a, b, c, d, e, and f serotypes were performed by slide agglutination test (Murex ZM20–25; Wellcome, Dartford, UK). CSF gram staining is a preferable methodology routinely performs in CMC hospital.

Nucleic acid detection

One ml of CSF stored at –70 °C tested for detection of *H. influenzae* in negative cultures with PCR assay. 100 µl of CSF heated in boiling water bath for 15 min and centrifuged at 10000rpm for 1min (4). Supernatant used as template in PCR assay. Hpd, outer membrane protein of Hib, were detected by PCR using specific Primers for the *hpd* gene according to previously described method (11) with some modifications. The following primers were used: 5'-CAGTAAATACACCTGTTGCCCTG-3' and 5'- GTTGTAGCAGCCATTCATCAAATA-3'. DNA amplification was carried out using 50 µl

reaction mixture containing 25mM MgCl₂, 1.5 mM dNTP, 10X PCR buffer, 5u/µl *Taq* polymerase, and 40pmol primers (Fermentase, Germany). 50ng of template DNA used for any reaction. The reaction was performed with following conditions: 94° C for 4 min, 30 cycles of 94° C 30s, 56° C for 1min, 72° C for 30 and a final extension in 72° C for 8 min. *H. influenzae* type b ATCC 9334 and *E. coli* ATCC 25922 were used as positive and negative controls, respectively.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was carried out using the disk diffusion method according to the Clinical and Laboratory Standards Institute (12-13) recommendations. An inoculum containing 10⁶ CFU/ml was inoculated on Haemophilus test medium supplemented with 15 µg/ml bovine hemin, 15 µg/ml NAD, and 15µg/ml yeast extract (Difco, BBL, USA). The antibiotic disks included ampicillin (10µg), chloramphenicol (30µg), co-trimoxazole (25µg), amikacin (30µg), gentamicin (10µg), kanamycin (30µg), tobramycin (10µg), cephalothin (30µg), ceftriaxone (30µg), ceftazidime (30µg), cefixime (5µg) and ceftizoxime (30µg). The *H. influenzae* ATCC 49247 was used as control in all experiments.

Results

From Sep 2001 to Jan 2007, 1800 samples of patients suspect to meningitis received from CMC hospital. Overall, 121 specimens (6.7%) were positive in culture. PCR assay identified 29 isolates as *H. influenzae* of which only 19 isolates were confirmed with culturing (Fig 1, 2). All of *H. influenzae* isolates identified as b serotype. Half of the patients with confirmed Hib meningitis by PCR had received prior antibiotic therapy. 60% of interviewed parents were unsure about their children prior treatment. Other common bacteria isolated from patients with culture-confirmed bacterial meningitis included *Streptococcus pneumoniae* (17.3%, n=21), *Pseudomonas aeroginsa* (10.7%, n=13), coagulase-negative *Staphylococci* and *Streptococcus agalactica*

(9%, n=11), *E. coli* and *Staphylococcus aureus* (7.4%, n=9) and other pathogens (23%, n= 28). After *Streptococcus pneumoniae* (17.3%; n=21), Hib was the most common bacterial isolated on culture.

Hib resistance to co-trimoxazol, ampicillin and chloramphenicol was 52.6%, 42.5% and 36.8%, respectively.

Resistant rate to aminoglycosides ranged from 52.6% for amikacin to 84.4% for tobramycin

and kanamycin. Resistance to all the third-generation cephalosporines was 42.2%, similarly.

Multidrug resistance (defined as being non-susceptible to three or more classes of antibiotics) was observed in 31.5% (6/19) of *H. influenzae* isolated in culturing. Antimicrobial susceptibility patterns of *H. influenzae* type b isolates are showed in Table1.

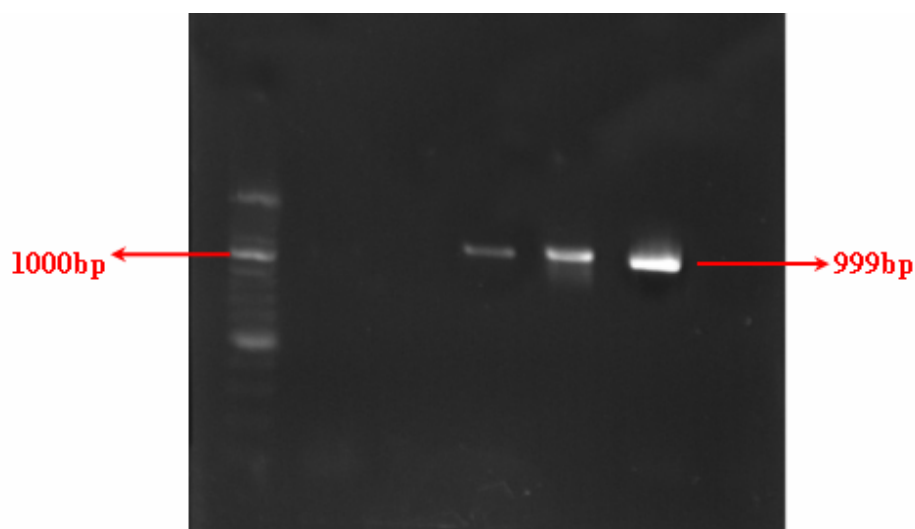


Fig. 1: Set up of PCR method. 50bp DNA ladder (Lane1), *E. coli* ATCC 25922 as control negative1 (Lane2), *S. pneumoniae* as negative control 2 (Lanes3), PCR product of 40ng of Hib DNA ATCC 9334 (Lane 4), PCR product of 50ng of Hib DNA ATCC 9334(Lane5), PCR product of 60ng of Hib DNA ATCC 9334 (Lane6).

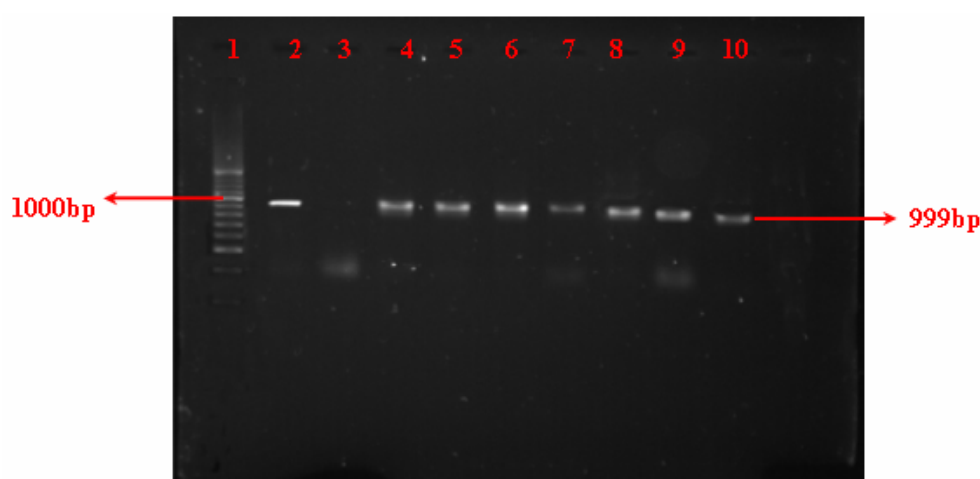


Fig. 2: *Haemophilus influenzae* type b detection in CSF samples of patients with meningitis by PCR. 50bp DNA ladder (Lane1), *Haemophilus influenzae* type b ATCC 9334 as control positive (Lane 2), *E. coli* ATCC 25922 as control negative (Lane3), CSF of patients with Hib meningitis (Lanes 4-10).

Table 1: Antimicrobial susceptibility of 19 *Haemophilus influenzae* type b isolated from Children with meningitis at CMC hospital according to CLSI guidelines.

Antibiotic	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Ampicillin	10 (52.6)	1 (5.2)	8 (42.2)
Chloramphenicol	11 (57.8)	1 (5.2)	7 (36.8)
Co-trimoxazole	9 (47.4)	0(0)	10 (52.6)
Amikacin	9 (47.4)	0(0)	10 (52.6)
Gentamicin	7 (36.8)	0(0)	12 (63.2)
Kanamycin	2 (10.4)	1 (5.2)	16 (84.4)
Tobramycin	3 (15.6)	0(0)	16 (84.4)
Cephalotin	10 (52.6)	0(0)	9 (47.4)
Ceftriaxone	11 (57.8)	0(0)	8 (42.2)
Ceftazidime	11 (57.8)	0(0)	8 (42.2)
Cefixime	11 (57.8)	0(0)	8 (42.2)
Ceftizoxime	11 (57.8)	0(0)	8 (42.2)

Discussion

According to conventional and selective culture media and biochemical testing, after *S. pneumoniae*, *H. influenzae* type b was determined as second common causative agent of bacterial meningitis (15.7%) in Iran.

These findings were in concordance with earlier report about *H. influenzae* type b meningitis from Iran (4), but were in sharp contrast with reports from European countries and USA before introduction of vaccination program (14-15).

Interestingly, the low frequency of *H. influenzae* type b isolation in Iran is in sharp contrast with other reports from Asian countries (Pakistan (97%), Bangladesh, and India (53%) (16-18), Jordan (32%), Kuwait (45%), KSA (47%), and Libya (43%) (19-22).

Overall, more than 93% of all cases that met clinical criteria for suspected bacterial meningitis had negative cultures (4).

PCR with specific primers for direct detection of Hib were used to covering the culture negative samples. PCR results showed better identification than culture conventional method accepted as the "gold standard".

The real reason for these results is unknown; however, we estimate the actual frequency of Hib meningitis would probably be higher than the previous reports. Many factors may be playing the role in underestimation of Hib meningitis in Iran. There are many reports that widespread and misuse of antibiotics is the main reasons for underestimation of Hib meningitis throughout the world (23). Since half of the PCR-confirmed Hib meningitis cases had received antibiotics before lumbar punctures, it seems that antibiotic-induced culture negative may not have been the main reason of low frequency of Hib isolation in this study. Other factors such as improper culture of CSF samples and social and ethnic factors may play role in decreased Hib isolation in our country.

After report of ampicillin resistance among clinical isolates of *H. influenzae* in 1972 in European countries and 1974 in USA, increasing tendency to ampicillin resistance reported in many countries (24). In the USA, based on national multi-center surveillances, overall 33-39% of isolates were resistant to ampicillin. Susceptibility of *H. influenzae* strains to ampicillin varied greatly

from one country to another. Reports from Asian countries included Korea, Japan, Taiwan and Singapore were shown that susceptibility to ampicillin has increased rapidly (from 16% in Japan to 53% in Korea) (24). We found 42.5% resistance to ampicillin that was nearly similar to reports from Spanish, Korea and Taiwan and against the reports from some other countries (24-25).

Resistant rate to co-trimoxazol (42.1%) was nearly similar to China and Spanish (51.3% and 52.1% respectively), but lower than Japan and other European countries (25). Similar to other reports from western countries, amikacin and gentamicin were more effective than other aminoglycosides (25-26).

63.2% of Hib isolates were susceptible to chloramphenicol that this finding was in contrast with other reports from USA and European countries except Spain (25-26).

Chloramphenicol administration in Asian countries had not been led to side effects previously described from the USA and Europe. Thus, chloramphenicol may remain the alternative of prescription for Iranian pediatrics, too.

In developed countries, the choice drug for treatment of Hib meningitis is third-generation cephalosporins (16). The susceptibility of Hib isolates to the first and third-generation cephalosporines, similar to reports from other countries (14), was from 52.6% in cephalothin to 57.8% in ceftriaxone, respectively.

Although we found high frequency of resistance to the first line drugs of Hib infections in Iran, but the frequency of Hib isolation among children meningitis was lower than many Asian and European countries. The emergence of multidrug resistance among Hib isolates is a serious challenge for the management of Hib meningitis, which emphasizes on the fundamental role of laboratory-based surveillance for antimicrobial resistance. Regarding to importance of Hib meningitis and awareness of frequency and antimicrobial susceptibility of Hib for Iranian physicians, correct identification and evaluation of antimicrobial susceptibility of Hib in children meningitis are necessary to generate data for improvement

of therapeutic decisions and prevention strategies. As a result it was concluded that PCR method could be considered as a rapid, reliable and feasible method for the detection hib in CSF samples and may help physicians in treatment and control of Hib meningitis in Tehran, Iran.

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The authors declare that they have no conflict of interests.

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