# Genotyping of *Haemophilus influenzae* type b strains and their incidence in the clinical samples isolated from Iranian patients

**Somayeh Bagherzadeh Khodashahri[1], Seyed Davar Siadat[2,*], Mohammad Rahbar[3], Meghdad Abdollahpour-Alitappeh[4], Farzam Vaziri[2], Mrjan Rahnamaye-Farzami[5], Mona Mohammadzadeh[6], Mehdi Davari[2] , Abolfazl Fateh[2], Morteza Masoumi[2]**

[1]*M.Sc. Student, Department of Microbiology, Kerman Science and Research Branch, Islamic Azad University, Kerman, Iran.*
[2]*Department of Mycobacteriology & Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran.*
[3]*Department of Microbiology, Reference Health Laboratories Research Center, Ministry of Health and Medical Education, Tehran, Iran.*
[4]*Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.*
[5]*Department of Laboratory Tecnhnology, Reference Health Laboratories Research Center, Ministry of Health and Medical Education, Tehran, Iran.*
[6]*Department of Microbiology ,Milad Hospital ,Tehran, Iran.*

## ABSTRACT

**Background and Objective:** *Haemophilus influenzae* type b (Hib) is divided into two distinct genotypes, type I and type II, based on the structure of capsular polysaccharides. The capsulation locus of *Haemophilus influenzae* type b consists of three functionally distinct regions, designated regions 1 to 3. Region III contains *hcsA* and *hcsB* genes; however, notable sequence variation in this region can be used to recognize different Hib genotypes. The purpose of this study was to investigate the prevalence and genotype of the Hib strains isolated from patients with invasive disease in Iran.

**Materials and Methods:** In the present study, 8 pairs of primers were used for identification and serotyping of encapsulated *Haemophilus influenzae* strains, as well as confirmation of species identification. Additionally, in order to identify the capsular genotypes of *Haemophilus influenzae* type b (type I and II), two additional primer pairs were used to amplify the *hcsA* gene.

**Results:** Out of 50 isolates of *H. influenzae*, four were found to be type b. Interestingly, among these 4 Hib isolates, 2 strains belonged to the type-II category.

**Conclusion:** Our study shows that the prevalence of both Hib types I and II seems to be high in Iran.

**Keywords:** Hib, genotype, *hcsA*, Iranian patients

*Corresponding author: Seyed Davar Siadat PHD.
Address: Department of  Mycobacteriology & Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran.
Tel: +98-21-66968853
Postal Address: 1316943551
E-mail: d.siadat@gmail.com

## INTRODUCTION

*Haemophilus influenzae* is a Gram-negative, coccobacillary, facultatively anaerobic bacterium belonging to the *Pasteurellaceae* family. *Haemophilus influenzae* strains, which commonly reside as commensals within the human pharynx and may cause

respiratory or invasive infections, are variable for the presence of a polysaccharide capsule (1). Isolates of *H. influenzae* can be subdivided into two major forms, encapsulated and non-encapsulated. Encapsulated strains express one of six structurally and antigenically distinct capsular polysaccharides, referred to as serotypes a through f (2). While the capsules are generally composed of a polysaccharide containing two hexose sugars as subunit carbohydrates, the type b polysaccharide capsule is the only capsular type which contains two pentose monosaccharides rather than the hexose sugars as subunit carbohydrates (3, 4). More importantly, *Haemophilus influenzae* type b (Hib) conjugate vaccines which consist of the capsular polysaccharide of Hib, polyribosyl-ribitol-phosphate (PRP), conjugated to a carrier protein are safe and highly effective in preventing invasive Hib disease (5). In the pre-vaccination era, about 90% of the invasive cases, such as meningitis and sepsis, were associated with type b strains (6). Since the introduction of the Hib conjugate vaccine in Europe and America in the 1990s, the incidence of invasive Hib disease has dramatically decreased in most countries (5).

Based on genetic analyses, two major phylogenetic divisions of Encapsulated *H. influenzae*, divisions I and II, have been already described (7-9). Division I consists of the great majority of serotype a and b strains and all of serotype c, d, and e strains. On the other hand, division II includes all strains with serotype f and some with serotypes a and b (7, 9, 10). It should be noted that most isolates of *H. influenzae* from clinical infections belong to clonal division I (7, 11). All encapsulated *H. influenzae* strains, whether division I or II, contain common genes for the production of their respective polysaccharide capsules (*cap* genes) found within the *cap* locus that is divided into three functionally distinct regions (9, 10). Region I contains four genes, *bexA* to *bexD*, which play a key role in capsular polysaccharide export. Region II, flanked by regions I and III, includes the genes *bcs1* to *bcs4* involved in the biosynthesis of the serotype-specific polysaccharide in Hib (9, 12, 13). Region III comprises genes referred to as *hcsA* and *hcsB*, which share significant homology with genes in a number of other encapsulated pathogens, including *Neisseria meningitidis* (*lipA* and *lipB*), *Escherichia coli* K1 and K5 (*kpsC* and *kpsS*), *Mannheimia* (*Pasteurella*) *haemolytica* (*wbrA* and *wbrB*), *P. multocida* A:1 (*phyA* and *phyB*), *P.*

*multocida* B:2 (*wcbA* and *wcbO*), *Burkholderia mallei* and *Burkholderia pseudomallei*. Collectively, the *Haemophilus influenzae* type b *hcsA* and *hcsB* gene products have complementary functions involved in the transport of capsular polysaccharide across the outer membrane and are essential for virulence. Surprisingly, the *hcsA* and *hcsB* gene products may be viable targets for antimicrobial development. In division I type b strains, these three loci are flanked by three 771 bp insertion elements, *IS*1016, whereas the capsulation locus in division II type b strains is not flanked by *IS*1016. The presence of the insertion element *IS*1016 increases the copy number of the *cap* locus. In fact, an increase in the copy number (up to 6 copies per cell) has been detected in clinical isolates (14, 15). The aim of this study was to determine the genotypes of Hib strains isolated from clinical samples, and to explore strategies to improve vaccine efficacy.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** A total of 50 clinical isolates of *H. influenzae* originated from blood, nasopharynx, eye, CSF and bronchoalveolar lavage fluid from hospitalized patients were used in the current study. All isolates were collected during the years 2010 to 2012 from Milad, Imam Khomeini and Mofid hospitals in Tehran, Iran. They were examined for morphological characters, biochemical reactions and the requirements for X and V factors (16). The samples were cultured on chocolate agar plates, and shipped to the Department of Bacteriology, Pasteur Institute of Iran, Tehran. Afterwards, the *H. influenzae* strains were cultured on chocolate agar containing 10% sterile sheep blood, and then incubated overnight at 37°C in a humidified 5% $CO_2$ incubator. The serotypes of the strains were also determined by polymerase chain reaction (PCR) as described by Falla et al. (17).

**Extraction of bacterial DNA.** After 24 hours of incubation on chocolate agar, an inoculating loop of bacteria was gently scraped from each of the chocolate agar plates and individually suspended in 150 µl of double-distilled water (DDW). The mixture was briefly mixed on a vortex mixer, placed in a boiling water bath for 7 min and then centrifuged at 12,000 × g for 5 min. A 50-µl aliquot of the supernatant was

transferred to a sterile tube and stored at −20°C until PCR testing (17).

**Control strains.** *H. influenzae* type a (ATCC 9006), *H. influenzae* type c (ATCC 9007) and non-typeable *H. influenzae* (NTHi) (ATCC 49766) were used as positive control for genotype II, positive control for genotype I and negative control, respectively in PCR assays. The control samples were obtained from the microbial collection of the Department of Bacteriology, Pasteur Institute of Iran, Tehran, Iran.

**Identification of *H. influenzae* species and capsular typing of encapsulated *H. influenzae* by PCR.** To confirm species identification, PCR amplification was performed to detect the *omp6* gene

from genomic DNA (18). Additionally, the PCR amplification was further carried out using the primer pair specific to *bexA* genes (HI-1 and HI-2) to distinguish encapsulated *H. influenzae* strains from NTHi strains. For capsular typing of encapsulated *H. influenzae*, six primer sets specific for capsule types a through f were used for all strains, which recognize sequences in capsule-specific genes located within region 2 of the *cap* locus (17). Isolates containing both the *bexA* gene and one of the cap-specific genes are designated as the specific capsule type. In contrast, isolates lacking both *bexA* and any of the *cap* genes are considered as NTHi, and those containing a *cap* gene but not *bexA* are designated as capsule-deficient *H. influenzae* type b or Hib⁻ strains (19) (All primers are listed in Table 1).

Table 1. The primer sets used in this study

| Primer name | Primer sequence (5' to 3') | Target | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| F1-(F) | AACTTTTGGCGGTTACTCTG | *Omp6* | 351 | 18 |
| R1-(R) | CTAACACTGCACGACGGTTT | | | |
| HI-1 (F) | CGTTTGTATGATGTTGATCCAGAC | *bexA* | 343 | 17 |
| HI-2 (R) | TGTCCATGTCTTCAAAATGATG | | | |
| a1 | CTACTCATTGCAGCATTTGC | **a** | 250 | 17 |
| a2 | GAATATGACCTGATCTTCTG | | | |
| b1 | GCGAAAGTGAACTCTTATCTCTC | **b** | 480 | 17 |
| b2 | GCTTACGCTTCTATCTCGGTGAA | | | |
| c1 | TCTGTGTAGATGATGGTTCA | **c** | 250 | 17 |
| c2 | CAGAGGCAAGCTATTAGTGA | | | |
| d1 | TGATGACCGATACAACCTGT | **d** | 150 | 17 |
| d2 | TCCACTCTTCAAACCATTCT | | | |
| e1 | GGTAACGAATGTAGTGGTAG | **e** | 1350 | 17 |
| e2 | GCTTTACTGTATAAGTCTAG | | | |
| f1 | GCTACTATCAAGTCCAAATC | **f** | 450 | 17 |
| f2 | CGCAATTATGGAAGAAAGCT | | | |
| HiHcsA12667F-I | GTACTTGTCATTGACCAAACTTT | *hcsA-I* | 450 | 23 |
| HiHcsA13116R-I | GGTATATTGAAAGTATGCTGCAT | | | |
| HiHcsA12668F-II | TGCTTGTCATCGATCAAA | *hcsA-II* | 817 | 23 |
| HiHcsA13484R-II | ACTAAAGAAAGGGGTGCAA | | | |

**Capsular genotyping of Hib strains.** To determine the capsular genotypes, or phylogenetic relationships, of the Hib strains, PCR amplification was performed with two sets of primers designed by Leo Schouls et al (18). In this regard, two oligonucleotide primer

pairs HiHcsA12667F-I and HiHcsA13116R-I, and HiHcsA12668F-II and HiHcsA13484R-II were used to detect capsular genotypes I and II of *H. influenzae* type b, respectively. The primers HiHcsA12667F-I and HiHcsA13116R-I permitted the amplification

of a 450-bp DNA fragment, whereas another set of primers, HiHcsA12668F-II and HiHcsA13484R-II, allowed the amplification of an 817-bp fragment (Table 1).

To discriminate between the two capsular genotypes, type I and type II, two separate PCR amplifications were performed for each *H. influenzae* type b strain. The PCR reactions were carried out in a final volume of 25 µL containing 2.5 µl of 10× PCR buffer, 1.5 µl of 25 mM MgCl$_2$, 0.2 µL of 5 U/µL Taq DNA polymerase (Genet Bio, Korea), 0.5 µl of each 10 µM primer, 0.5 µL of 10 mM dNTP (Genet Bio Company, Korea), 17.3 µl of double-distilled water and 2 µL of genomic DNA. PCR conditions were as follows: an initial denaturation at 95°C for 15 min, 30 cycles of denaturation at 95°C for 30s; annealing at 52°C for 1 min; elongation at 72°C for 1min, and a final extension step at 72°C for 7 min.

**Gel electrophoresis of PCR products.** 6 µL of PCR products mixed with 2 µl of loading buffer were loaded into individual wells of 1% agarose gel, and electrophoresis was performed at 100 V for 45 min. Further, the sizes of the amplicons were measured by comparison with 3µl of the 100-bp DNA ladder (Pars Tous Biotechnology Company, Iran). The resulting PCR products were stained with ethidium bromide for 20 min and visualized under UV illumination. To confirm whether the amplified bands corresponded to *hcsA* type I or *hcsA* type II fragments, one of the PCR products from each gene fragment were randomly sequenced (TAG Copenhagen, Copenhagen, Denmark).

**RESULTS**

**Preparation of samples.** A total of 50 isolates of *H. influenzae,* obtained from nasopharynx (n=39), CSF (n=2), blood (n=3) eyes (n=5) and bronchoalveolar lavage fluid (n=1), were examined (Table 2). All *H. influenzae* isolates were initially confirmed by culture and biochemical tests. The strains were then further characterized by PCR amplification of the *omp6* gene to confirm the *H. influenzae* species. Moreover, the primer specific for the *bexA* gene was used to identify encapsulated strains of *H. influenzae*. In PCR assays, 4 (8%) of the 50 strains were able to amplify the *bexA* gene (a fragment of 343 bp in length). Capsular types (a-f) were determined for each of the 4 strains using specific primer pairs (Table 1). Out of 4 isolates of *H. influenzae*, 4 were typed as b, successfully amplifying the *bcs3* gene (a fragment of 480 bp in length). Four strains of *H. influenzae* type b examined had been isolated from blood (2 patients) and CSF (2 patients) specimens. In addition, two samples had been obtained from the infants at the ages of 3 months (blood) and 1 year (CSF), but the age-specific data were not available about the two other samples (Table 2).

**Capsular genotyping of Hib strains.** As mentioned previously, *H. influenzae* type b strains are known to belong to both phylogenetic groups, clonal divisions I and II. In the present study, to determine which clinically isolated Hib strains belong to which phylogenetic groups, specific primers to the *hcsA* type I and *hcsA* type II genes were used to identify genotype I and II Hib strains, respectively. However, it is found that among 4 *H. influenzae* strains examined two strains belong to type I (Fig. 1), and the two other strains belong to type II (Fig. 2).

**Table 2.** Characteristics of isolates used in this study

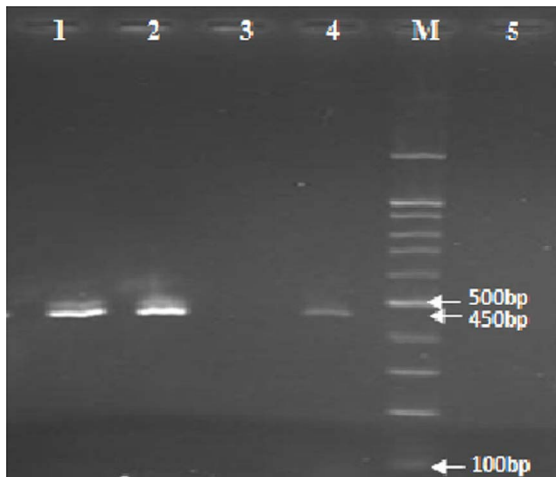| Number of strains | P6 (*Haemophilus influenzae*) | bexA (type or nontype) | Serotype (a-f) | Genotype (I or II) |
|---|---|---|---|---|
| Nasopharynx 39 | + | - | No | **No** |
| CSF 2 | + | 2 (+) | 2 (b) | **2 (I)** |
| Blood 3 | + | 2 (+), 1(-) | 2( b),1(nontype) | **2 (II)** |
| Eye 5 | + | - | No | **No** |
| *BAL 1 | + | - | No | **No** |
| **Total 50** | **50 +** | **4(type), 46(nontype)** | **4(b), 0(non b)** | **2(I), 2(II)** |

* Bronchoalveolar Lavage

**Fig. 1.** PCR amplification of the *hcsA* type I gene: the amplified DNA fragment was 450 bp in length, lane 1 and 2: clinical isolates of *H. influenzae* type b containing *hcsA* type I, Lane 3: clinical isolate of *H. influenzae* type b lacking *hcsA* type I, Lane 4: positive control containing *H. influenzae* type c (ATCC 9007), lane 5: negative control containing *H. influenzae* type a (ATCC 9006), M: Marker 100-bp.
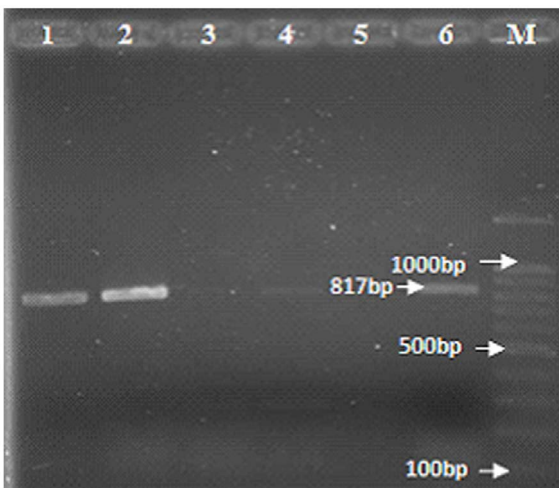


**Fig. 2.** PCR amplification of the *hcsA* type II gene: the amplified DNA fragment was 817 bp in length, lane 1 and 2: clinical isolates of *H. influenzae* type b containing *hcsA* type II, Lane 3 and 4: clinical isolates of *H. influenzae* type b lacking *hcsA* type II, Lane 5: negative control containing *H. influenzae* type c (ATCC 9007), lane 6: positive control containing *H. influenzae* type a (ATCC 9006), M: Marker 100-bp.

**Sequencing and BLAST homology search.** Two randomly selected PCR products of *hcsA* type I and *hcsA* type II genes were sequenced, confirming the identity of *H. influenzae* type b. After sequencing of PCR products, the newly determined sequences were analyzed using the Chromas software. In parallel, the nucleotide sequences of *hcsA* type I and *hcsA* type II were compared and aligned with those deposited in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/). The nucleotide sequence data of *hcsA* type I and *hcsA* type II reported in this paper have been submitted to GenBank, and assigned the accession numbers KF263489 and KF263490 respectively.

## DISCUSSION

*Haemophilus influenzae* is an environmentally sensitive and fastidious bacterium, making it difficult to isolate the pathogen from human clinical samples in microbiology laboratories. The diagnosis of bacterial meningitis is based on cerebrospinal fluid biochemical findings as well as clinical features. However, there is no specific treatment proven to be effective for bacterial meningitis, and patients are randomly assigned to the treatment regimens, often resulting in misdiagnosis and improper treatment. Hence, the isolation of even a single colony of *H. influenzae* from suspected cases will be valuable. *H. influenzae* type b remains one of the most common causes of bacterial meningitis in Iran as reported by the Iranian Ministry of Health. In Tehran (2005), among 32 CSF isolates of *H. influenzae* with positive cultrure, 5 strains(15.6%) were Hib (20). In another study in Tehran the rate of transmission of *H. influenzae* type b in children aged ≤5 was reported as 7.8% in 2007 (21) and recently in 2014, one study reported a 28% prevalence of *H. influenzae* among children ≤6 years old (22). Therefore, vaccination against *H. influenzae* type b will be included in the national immunisation program schedule to help control disease spread. In this light, the identification of *H. influenzae* serotypes is essential for vaccination strategies, supplied through domestic production or imports.

In this regard, two sets of primers were used to amplify the *hcsA* gene that could successfully discriminate between two capsular genotypes, type I and II. In 2008, Schools et al. revealed that the type I isolates carry more surface-bound capsular

polysaccharides than type II isolates. The lower capsule production in type II strains may have resulted in a selective disadvantage, explaining the rapid disappearance of type II strains from the Dutch Hib population. Type II strains were isolated from 0–4 year old, non-vaccinated patients, suggesting that only type II strains are able to cause invasive disease in hosts who have not yet mounted an antibody response against the Hib capsule (23). Moreover, their results revealed that Hib strains isolated from patients in different geographic regions of the world show a considerably different distribution of the type I and II strains; In other words, the proportion of type II strains among the strains from the various global regions varied from 47% for strains from the African continent, 4% for Asian strains, 8% for Australia and 43% for Cuba. Furthermore, none of the Hib isolates from patients in other parts of Europe (Finland & Switzerland) belonged to the type II strains (23). On the other hands, the highest proportion of type II strains (73%) was found among Hib strains isolated from patients in the United States (23).

In the present study, 4 Hib strains isolated from Iranian patients were examined. The phenotypic and genotypic characteristics of Hib strains suggested that 2 Hib strains belonged to type I, and 2 other Hib strains were type II. It is important to note that 50% of Hib strains examined in this study belonged to the type-II category. However, this result is inconsistent with the results reported by other investigators carried out in many parts of the world, especially in Asia, because type II and type I Hib strains are completely dominant in the United States and in other countries in which the study was conducted, respectively. More importantly, these results demonstrate that the distribution pattern of Hib strains from Iran is closely similar to those from African countries and Cuba. To develop a broadly protective Hib vaccine, it would be better to use the native strains of type I PRP, because a recent study by Schouls et al. strongly demonstrated that type I strains produce PRP twice as much as type II stains. Notably, the purified PRPs should be used in the development of the Hib vaccine; this leads to reduce the time and cost for vaccine development and increase the extraction yield of PRPs. Sukupolvi-Petty *et al.* demonstrated that the products of the *hcsA* and *hcsB* genes facilitate transport of capsular polysaccharides across the outer membrane. Inactivation of *hcsA* alone resulted in accumulation of polysaccharides in the periplasm and a partial decrease in surface-associated polysaccharides,

whereas inactivation of *hcsB* alone, or both *hcsA* and *hcsB* resulted in accumulation of polysaccharides in the periplasm as well as complete loss of surface associated polysaccharides. It is therefore feasible that alterations in the *hcsA* and *hcsB* genes would influence the degree of capsular polysaccharide export to the surface of the Hib cells, or the efficiency of retaining the exported polysaccharides on the surface (12). Meanwhile, after analyzing the complete sequences of the type I and type II *Cap* b loci, they showed that the variation between the capsular gene cluster sequences of type I and type II strains was predominantly found in the *hcsA* and *hcsB* genes. The nucleotide sequences of *bex*, *bcs1* and *bcs2* of type I and type II were identical (23). However, in another study, Kroll and Moxon compared capsule production in strains containing one or two copies of the *cap* b locus and observed a gene dosage effect, with twice as much polysaccharide capsule associated with two copies of the *cap* b locus. Interestingly, despite differences in capsule production, all strains were able to produce sustained bacteremia and meningitis in the infant rat model system, suggesting that only one copy of the *cap* b locus is sufficient for virulence *in vivo*. These findings suggest that a certain threshold level of polysaccharide capsule on the bacterial surface is required for intravascular survival and virulence of *H. influenzae*. However, despite the presence of less polysaccharide capsule on the surface of type II Hib strains, it seems that these strains can cause long-term meningitis in humans.

## CONCLUSIONS

In summary, there are two different types of *Haemophilus influenzae* type b strains, which differ in coding sequences of genes involved in the synthesis of a polysaccharide capsule, especially the *hcsA* gene. This gene plays an important role in the transport of capsular polysaccharide to the cell surface. Importantly, any change or mutation in the sequence of this gene can affect on the amount of capsular polysaccharide present on the Hib surface as well as pathogenicity of *H. influenzae* in humans. In this study, it is found that the incidence and prevalence rates of both Hib types are significantly high in Iran. However, type I and type II Hib strains showed significant differences in their genotype (sequence diversity in the capsule-producing genes) and phenotype (the amount of capsular

antigens) as well as pathogenicity. It seems that if type I Hib strains are used to produce the Hib vaccine, this vaccine can not only cover type II Hib strains but also induce a strong protection against *H. influenzae* type b infection, offering potential advantages such as economic benefits and high-quality products.

## ACKNOWLEDGMENTS

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