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Original Research Article

Identification and genetic characterization of *cap* locus in *Haemophilus influenzae* strains isolated from suspected meningitis and pneumonia cases in Iran

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A B S T R A C T

Major virulent factor in invasive *H. influenzae* (Hi) is their capsule whose expression is dependent upon the cap locus carrying genes in three distinct regions. In this study, the *cap* loci of selected Hi strains were identified and characterized using set of primers. All the isolates were identified as Hi, capsulated or non capsulated based on ompP6, bexA and bexB primers, respectively. Hi positive isolates were subjected to serotype b specific primer set (bsc3) and later classified into type I and type II based on specific hcsA I and II primer sets. The isolates were identified as Hi type b (8), Hib –ve variant (2) and nontypeable (1). Three Hi type b strains isolated from CSF of children below one year of age were classified as type I while majority of the other Hib isolates from throat were capsular type II. Tandem repeat copies of *cap* locus were revealed in the Hi type b isolates. Additionally, an IS1016-*bexA* partial deletion in the capsule gene locus was identified by means of PCR. PRP concentrations in capsular type I strains in the Hib strains isolated from CSF of children below I year of age, appeared to be significantly greater than in capsular type II strains.

Introduction

Majority of type b Hib strains are responsible for the cause of sepsis, meningitis, epiglottitis and septic arthritis in young children, especially in underdeveloped countries where vaccination rates are low. The most important virulence factor defining pathogenic *H. influenzae* strains is its capsule, a polymer of ribose ribitol phosphate (PRP), which is also the antigen used for Hib vaccination (Jackson *et al.*, 2012).

Encapsulated Hi contain genes for the production of their respective polysaccharide capsules within the *cap* locus (29, 38), a segment of approximately 17 kb. The *cap* locus for all Hi serotypes contains the three functionally defined regions, 1, 2 and 3. Genes in regions 1 (*bex A,B,C,D)* and 3 (*hcs A,B*) are common to all 6 capsule types and are necessary for the processing and transport of the capsular material, while region 2 genes (*bcs* 1,2,3,4) are involved in

capsule biosynthesis and are unique to each of the six capsule types. Region 3 carries 2 *hcs* genes involved in the export of polysaccharide to cell surface (Satola *et al.,* 2003). Most *H. influenzae* type b isolates contain a partial tandem duplication of the *cap* b locus that includes two complete copies of regions 1 and 2, and one complete copy of region 1 and a truncated copy of region 1 with a 1.2-kb deletion within the *bex A* gene and IS1016. This duplication results in an unstable gene region, with relatively high potential for recombination events (Ueno *et al.,* 2010).

During recombination of the *cap* locus, mutants may be formed that possess a single copy of the *cap* locus containing the *bex A* mutation, which results in non-encapsulated mutants. On the other hand, such a recombination event may generate mutants possessing additional (n) copies of the *cap* locus with n-1 copies of *bex A.* Such mutants are hyper-encapsulated and have shown increased pathogenicity in infant rat model (Davis *et al.,* 2011).

Based on genetic analysis, all encapsulated Hi strains belong to one of the two major phylogenetic divisions. Hi Serotypes belonging to, division I have their *cap* locus flanked by direct repeats of insertion sequence and are frequently amplified, while division II strains, although posses the insertion element but does not appear to be physically associated with *cap* b genes. All of the serotypes c, d, and e and majority of serotype a and b, belong to division I, while all serotype f with few serotype a and b belong to division II (Schouls *et al.,* 2008). According to these reports, total amount of polysaccharide (PRP) produced by type I and type II Hib strains differ significantly.

In this study the cap loci in *H. influenzae* strains including type b, Hib negative and a Nontypeable variant was genetically characterized using PCR reactions. All Hib isolates in study were evaluated for their PRP concentrations to evaluate possible relationship between PRP concentrations and capsular genotypes.

Materials and Methods

Bacterial strains and growth conditions: Bacterial strains used in study were isolated previously from different clinical samples including CSF and throat from suspected meningitis and pneumonia cases. All samples were grown on chocolate solid media (5% sterile sheep or horse defibrinated blood), and supplemented Haemophilus test agar base (HiMedia, India). Incubation conditions included overnight incubation at 35–37ºC in 5% CO2. The colonies appearing small gram negative bacilli or cocobacilli, catalase positive were tested for their X and V factors requirement using X, V and XV factor discs (Hi Media, India).

PCR Assay

DNA template was prepared using Genomic DNA purification kit (Thermos Scientific, Lithuania) according to manufacturers' instruction. All purified samples were stored at -20°C until use.

PCR primers used in study are mentioned in Table 1. Primer sets HI4-HI5 ($ompP_6$), HI – HII (bexA), bexB1F-bexB. 1R (bexB) and b1 and b2 (*bcs 3*) were used for differentiation of *H. influenzae* capsulated, non capsulated and type b specific capsule strains, respectively. The PCR reactions with HI-HII and b1-b2 primers were further confirmed by third internal primer H1-3 and bI3 in combination with one of the first round primer pairs, respectively.

Additionally, two set of primers targeting *hcsA* region in cap loci were used to determine capsular genotypes I and II in the isolates (Schouls *et al.,* 2008).

To determine the presence of tandem repeat copies of the *cap* locus and *bex A* deletions in the tested Hib isolates, genotype specific primers namely BexB, ORF6 and ISLOUT were used in two combinations as mentioned by Leaves *et al.,* (1995).

PCR conditions for the first three set of primers as listed in Table 1, were: 30 s at 94°C, 1 min at 60°C, 1 min at 72°C, for 30 cycles, 10 min at 72°C, and 4°C hold. While for the later two set of primers 35 cycles were used with increased denaturing time (72°C for 2 min). The PCR conditions used for the remaining primer sets were as reported by their mentioned references.

PCR control included *Streptococcus pneumoniae* strains as negative control while *H. influenzae* ATCC 10211 was used as positive control. The sizes of the amplified products were compared with those of a positive control amplified product and a 1 kb molecular mass marker ladder.

PRP Concentrations

PRP produced by the Hib isolates in study was estimated using modified Bial assay (Ashwell *et al.,* 1957) with D-ribose (Sigma) and purified PRP (NIBSC, UK) as standards. Ten ml samples of overnight grown cultures of selected Hib isolates were centrifuged at 6000 g, 4C for 40 min. To supernatant fluid 10% CTAB (Sigma Chemicals, USA) added and kept in shaking at room temperature for 1 hr, and later centrifuged for 20 min at 5000 g. The pellet was solubilized in 4 M NaCl and 95% ethanol and kept for 1 hr at room temperature with shaking. The pellet was once more collected with centrifugation as above, and dissolved in 5 ml deionized water. A conversion factor in which 1 mg of ribose corresponded to 2.55 mg of PRP based on the PRP structural formula reported by Crisel *et al.* (1975) was used for calculating the amount of PRP produced by individual strains.

Results and Discussion

The cap loci in all the tested Hi isolates were studied using different set of primers targeting different regions of a *cap* locus in *H. influenzae*. As Table 2 indicates, the isolates were identified as Hi by ompP6 primer set producing an amplicon of 273bp in all of the tested isolates. The capsule specific primers targeting *bex A* (HI I- HI 2) differentiated between capsulated and non-capsulated *H. influenzae* strains. All isolates giving products with the bexA primers were confirmed by second round of PCR using internal primers HI-3 with one of the first round primers. The DNA of the isolates giving product with the first pair of primers was able to amplify a DNA band of approximately 181 bp by this set of primers (Fig. 1).

The primer pair bexB (bexB1F- bexB1-R) was also used for distinguishing *H. influenzae* strains containing a complete or partial capsule locus from those completely lacking the capsule locus. On the basis of these primers we were able to distinguish between capsulated and noncapsulated, Hib -ve and non typeable strains of Hi, respectively. Three of the isolate failed to produce product with the *bex*A primers responsible for the transport of capsule and was thus considered non-capsulated. Among the 3 *bex A* negative isolates, two were able to give products with primer bexB and were considered Hib-ve variants. One of the isolate was negative with both primer sets and was considered nontypeable strain.

The *H. influenzae* strains (capsulated or uncapsulated) with or without products were further analyzed with serotype b specific primers. The primers b1 and b2 were able to amplify a DNA fragment of approximately 480bp in all serotype b isolates including the Hib positive control (*H. influenzae* b ATCC 10211). Apart from serotype b strains, the two Hib negative strains which gave no amplification with the primer pair HI-1and HI-2, were also positive with primer set b1-b2. The Nontypeable strain was unable to produce amplicon with any of the primers sets used, except for ompP6 primer sets. The specificity of selected primers appeared to be highly specific for *H. influenzae* since there was no DNA amplified from other bacteria used in this study.

In another PCR reaction, we used primers targeting *hcsA* genes from region 3 of the cap loci responsible for the transport of capsular polysaccharide across the outer membrane. The hcsA I and hcsA II primer allowed discrimination between type I and II *H. influenzae* type b isolates used in study. According to results, among the 10 tested Hi isolates, four belonged to type I while 6 of the isolates belonged to type II. All type I isolate yielded DNA fragment of approximately 450bp while type II isolates yielded amplicon of 817bp. Both Hib negative variant in study yielded 450bp product with hcsA I primer only and were thus shown to be of type I (Fig. 2).

Finally, the capsular genotypes of Hi isolates were determined using three primers ORF6, ISLOUT and bexB in two combinations. As depicted in Table 3, all Hib isolates except for NM21, yielded 3 Kb amplicon with ORF6 and bexB primer set. These results were indicative of the presence of tandem repeat copies of cap b in these isolates. In contrast, the Hib negative strain did not show any amplification with this set of primer indicating the absence of intact bexA. With primer set bexB and ISLOUT all Hib isolates amplified both 1.5 kb and 300bp products while the two Hib –ve isolates yielded only a single product of 300bp (Fig. 3).

Table 4 shows the amount of PRP produced by type I and type II Hi isolates in study. According to Bial reaction, highest amount of PRP was produced by Hib isolates NM3 (209 mg/L) and NM2a (175 mg/L), respectively. Interestingly both these isolates belonged to type I and were isolated from CSF samples of children below I year of age. Least amount of PRP was detected in isolate NM6, a type II Hib strain isolated from throat sample of a 6 years old child. Comparatively, type I capsular types produced significantly higher amounts of PRP than type II capsular types Hi isolates.

Haemophilus influenzae (Hi) is a human pathogen responsible for a variety of respiratory infections. Hi pathogenicity varies depending on the presence or absence of capsule and the specific capsule type (Munson *et al.,* 1989). Among different serotypes, Hi type b (Hib) is the leading cause of invasive Hi in children younger than 5 years in unvaccinated populations. However, in last decade the incidence has significantly decreased in many developed countries due to widespread of the Hib conjugate vaccine (Peltola, 2000; Sande and Gwaltney, 2004). While other serotypes of Hi mainly non typeable (NTHi) are becoming more common cause of invasive disease in all age groups (Hussein and Shafran, 2000).

The capsule genes of *H. influenzae* responsible for Polysaccharide production are extensively characterized, especially in Hi type b strains.

Table.1 List of primers and their sequences used in PCR assay

No	Gene	Primer name	Primer Sequence	Product size (bp)	Reference
1	*ompP$_6$*	HI-4 F HI-5 R	ACT TTT GGC GGT TAC TCT GT TGT GCC TAA TTT ACC AGC AT	273	Van ketel *et al.* (1990)
2	*bex A*	HI-1 HI-2	CGT TTG TAT GAT GTT GAT CCA GAC T TGT CCA TGT CTT CAA AAT GAT G	343	Falla *et al.* (1994)
3	*bex A-HI-3*	HI-3	TGA TGA GGT GAT TGC AGT AGG	181	Falla *et al.* (1994)
4	*bex B*	bexB.1F bexB.1R	GGT GAT TAA CGC GTT GCT TAT GCG TTG TGC CTG TGC TGG AAG GTT ATG	567	Davis *et al.* (2011)
5	*bcs 3*	b1 b2	GCG AAA GTG AAC TCT TAT CTC TC GCT TAC GCT TCT ATC TCG GTG AA	480	Falla *et al.* (1994)
6	*bcs 3-b3*	b3	ACC ATG AGA AAG TGT TAG CG	370	Falla *et al.* (1994)
7	*hcs A I*	hcsA 1 hcsA 1	GTA CTT GTC ATT GAC CAA ACT TT GGT ATA TTG AAA GTA TGC TGC AT	450	Schouls *et al.* (2008)
8	*hcs A II*	hcsA 1I hcsA 1I	TGC TTG TCA TCG ATC AAA ACT AAA GAA AGG GGT GCA A	817	Schouls *et al.* (2008)
9	*Orf6-bexB*	ORF6 bexB	GTT ATT ACT TGC GTG ATC GT GGC GAT ACA GTG GTTACT TA	3000	Leaves *et al.* (1995)
10	*Islout-bex B*	ISLOUT bexB	GAG CAG CGG CTG ATT AC GGC GAT ACA GTG GTTACT TA	300/1500	Leaves *et al.* (1995)

Table.2 PCR products amplified from *H. influenzae* isolates with different set of primers

No	**Bacterial Isolates**	**Source of isolates**	**Hi Type**	**PCR Primers and product size (bp)**			
				ompP$_6$ 273	**bexA 343**	**bexB 567**	**bcs3 480**
1	NM2	throat, 4 yrs old	b	+	+	+	+
2	NM3	CSF, 9 month old	b	+	+	+	+
3	NM6	throat,6 yrs old	b	+	+	+	+
4	NM7	CSF,6 months old	b	+	+	+	+
5	NM2a	CSF,2 months old	b	+	+	+	+
6	NM16	Throat, 1 yr old	b	+	+	+	+
7	NM21	Throat,3 yrs old	b	+	+	+	+
8	Hib	ATCC 10211	b	+	+	+	+
9	Hib-ve	Throat,3 yrs old	Hib-	+	-	+	+
10	Hib-ve	Serum, 1 yr old	Hib-	+	-	+	+
11	NT	Throat,15 yrs old	NT	+	-	-	-
12	*S. pneumoniae*	Local isolate	-	-	-	-	-

Table.3 Characterization of cap loci in *H. influenzae* isolates using different primer pairs

No	Hi isolates	PCR primers and product size (bp)				
		hcsA I bexB	hcsAII	ORF6 and	bexB and ISLOUT	
		450	817	3000	1500	300
1	NM2	-	+	+	+	+
2	NM3	+	-	+	+	+
3	NM6	-	+	+	+	+
4	NM7	-	+	+	+	+
5	NM2a	+	-	+	+	+
6	NM16	-	+	+	+	+
7	NM21	-	+	-	+	+
8	Hib ATCC 10211	-	+	+	+	+
9	Hib-ve	+	-	-		+
10	Hib-ve	+	-	-	-	+

Table.4 PRP production by selected Hib isolates

No	Hi isolates	PRP concentrations mg/L
1	NM2	104
2	NM3	209
3	NM6	88
4	NM7	94
5	NM2a	185
6	NM16	120
7	NM21	98
8	Hib ATCC 10211	110

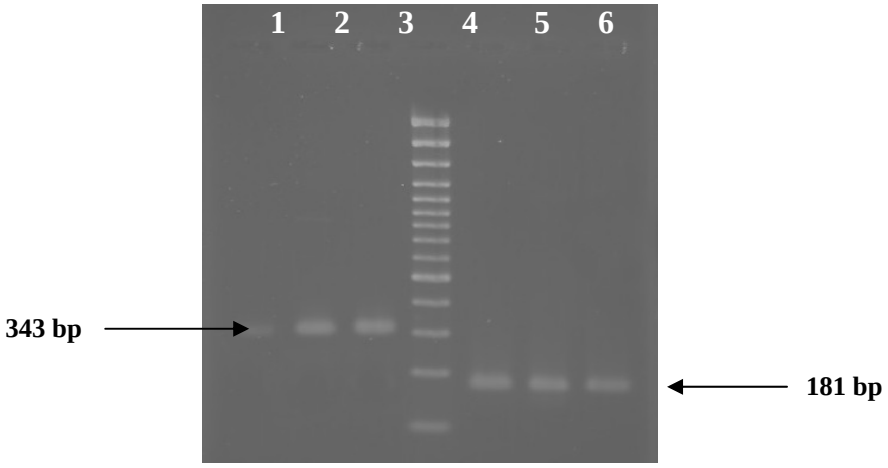

Fig.1 Agarose gel electrophoresis of PCR products from Hi strains with capsular specific primers (HI-1 and HI-2) Lane 1-3: encapsulated strains yielding a fragment of 343 bp with bexA primers. Lane 4: molecular weight marker (100bp ladder), lane 5-7: Hi positive samples with internal primers H2-H3 yielding 181 bp DNA fragments.

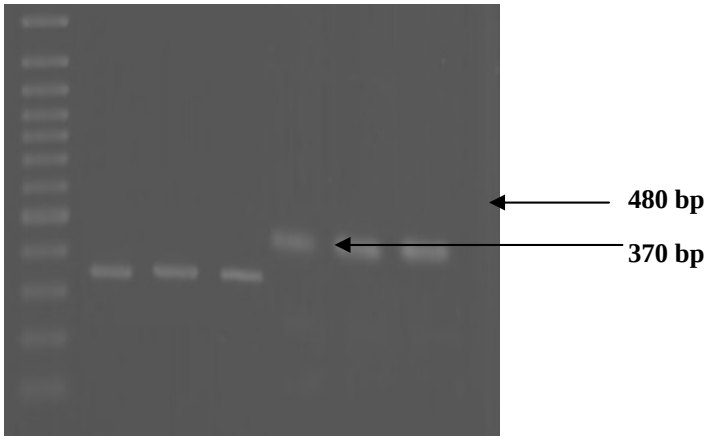

Fig.2 Agarose gel electrophoresis of PCR products from Hi strains with capsular type b specific primers (*bcs 3*). Lane 1: DNA molecular weight marker; Lane 2, 3 and 4: serotype b strains yielding a DNA fragment of 370bp with internal primers b2 and b3. Lane 5, 6 and 7: serotype b strains yielding a DNA fragment of 480 bp with primers b1 and b2.

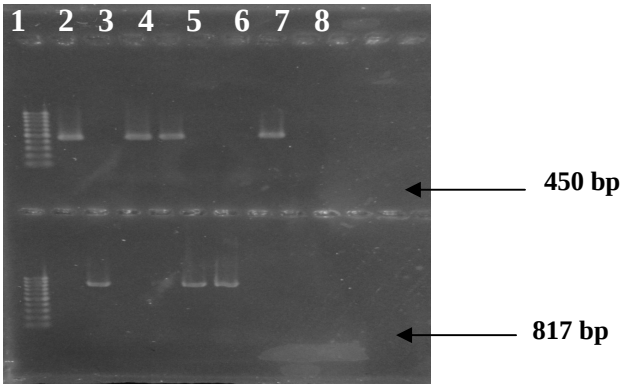

Fig.3 PCR products of *H. influenzae* isolates with primer pairs hcsAI and hcsAII. Upper portion: hcsAI primer (450bp) and Lower portion: hcsAII primers (817bp): Lane 1: 1 Kb DNA Ladder, lane 2-8: *H. influenzae* NM3, NM6, Hib-ve 1, Hib –ve 2, NM2, NM 7, and NM 2a.

According to reports, changes of the genes in the capsular gene cluster locus of *H. influenzae* type b strains might lead to an altered capsule and thus the vaccine induced immunity may not provide optimal protection (Schouls *et al.,* 2008; Ueno *et al.,* 2010).

In this study we selected set of primers targeting specific sequences from the three regions of *cap* locus, in order to analyze the organization of genes responsible for polysaccharide production and export in the selected local Hi isolates.

In a previous study, we isolated and identified different serotypes of Hi from suspected cases of meningitis and pneumonia based on serotype specific primers targeting *bex A* and capsule specific genes by PCR (Mojgani *et al.,* 2011). However, as reported by Davis and his colleagues (2011), using bexA primers alone might fail to identify capsule-deficient variants because the 5 primer of bexA primer pair targets the deleted region of *bex A.* Based on these reports, we used bexB primers in addition to bexA which could distinguish between true nontypeable Hi strains from those containing complete or partial *cap* locus. According to these reports, *bex B* which is located within region I of the *cap* locus could serve as a more reliable marker for the capsule locus than *bex A*, based on the observations that region I genes are present across all capsular types and that there are no reports of *bex B* partial deletions analogous to those observed in *bex A* (Davis *et al.,* 2011; Kroll *et al.,* 1991).

In a study, Abdulhasan and his colleagues (2013) were able to determine type I and II capsular genotypes of *H. influenzae* type b in children with meningitis by targeting hcsA I and hcsA II type primers in a PCR reaction. According to their reports, majority of children below four years were infected with genotype I of *H. influenzae* type b. Similar reports were also made by other researchers who showed that vast proportion of Hib belong to capsular type I. According to these reports, type I had a thinner, dense and compact capsular layer with twice the amount of capsular polysaccharide than type II (Schouls *et al.,* 2008). According to these reports, higher amount of capsular polysaccharide produced by type I requires higher amount of antibody titer to eliminate type I in comparison with type II. This may be attributed to the lack of bactericidal activity against this bacterium in children less than 1 year of age. In accordance with these reports we also observed higher PRP production in *H. influenzae* type b isolates NM 3 and NM 2a which both belonged to type I, compared to type II strains.

In summary, the capsule locus of *H. influenzae* could be best characterized using combination of primers which not only could differentiate typeable strains from true non typeable strains but could also genetically characterize the *cap* locus in these isolates. The information collected through this study, might further provide an insight in the prevalence of *H. influenzae* non type b strains in the country and categorize their virulence properties based on their capsular genotype.

References

Abdulhasan, G.A., Sabbah, M.A., Jasem, K.A., Hassani, H.H. 2013. Identification and typing of *H. influenzae* in Iraqi children diagnosed with meningitis. *Int. J. Microbiol. Res.,* 5: 417–423.

Ashwell, G. 1957. In: Methods in enzymology, Vol. 3, Colwick, S.P., Kaplan, N.O. (eds), Academic Press

Inc., New York. Pp. 87–90.

Crisel, R.M., Baker, R.S., Dorman, D.E. 1975. Capsular polymer of *H. influenzae*, type b. I. Structural characterization of the capsular polymer of strain Eagan. *J. Biol. Chem.,* 250: 4926–4930.

Davis, S.G., Sandstedt, S.A., Patel, M., Marrs, C.F., Gilsdorf, J.R. 2011. Use of bexB to detect the capsule locus in *H. influenzae*. *J. clin. Microbiol.,* 49: 2594–2601.

Falla, T.J., Crook, D.W.M., Brophy, L.N., Maskel, D., Kroll, J.S., Moxon, E.R. 1994. PCR for capsular typing of *H. influenzae*. *J. Clin. Microbiol.,* 32: 2382–2386.

Hussein, A.S., Shafran, S.D. 2000. Acute bacterial meningitis in adults: a 12-year review. *Medicine (Baltimore).,* 79: 360–368.

Kroll, J.S., Loynds, B.M., Moxon, E.R. 1991. The *H. influenzae* capsulation gene cluster: a compound transposon. *Mol. Microbiol.,* 5: 1549–1560.

Leaves, N.I., Falla, T.J., Crook, D.W. 1995. The elucidation of novel capsular genotypes of *H. influenzae* type b with the polymerase chain reaction. *J. Med. Microbiol.,* 43: 120–124.

Mojgani, N., Rahbar, M., Taqizadeh, M., Ashtiani, M.P., Mohammadzadeh, M. 2011. Biotyping, capsular typing and antibiotic resistance pattern of *H. influenzae* b strains in Iran. *Jpn. J. Infect. Dis.,* 64: 66–68.

Munson, R.S., Kabeer, M.H., Lenoir, A.A., Granoff, D.M. 1989. Epidemiology and prospects for prevention of disease due to *H. influenzae* in developing countries. *Rev. Infect Dis.,* 11: 588–S597.

Peltola, H. 2000. Worldwide *H. influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. *Clin. Microbiol. Rev.,* 13: 302–317.

Sande, M.A., Gwaltney, J. 2004. Acute community-acquired bacterial sinusitis: continuing challenges and current management. *Clin. Infect. Dis.,* 39: S151–S158.

Satola, S.W., Schirmer, P.L., Farley, M.M. 2003. Complete sequence of the cap locus of *H. influenzae* serotype b and nonencapsulated b capsule-negative variants. *Infect. Immun.,* 71: 3639–3644.

Schouls, L., van der Heidal, H., Witteveen, S., Zomer, B., van der Ende, A., Burger, M., Schot, C. 2008. Two variants among *Haemophilus influenzae* serotype b strains with distinct *bcs4*, *hcsA* and *hcsB* genes display differences in expression of the polysaccharide capsule. *BMC Microbiol.,* 8: 35–46.

Ueno, K., Nishi, J., Imuta, N., Tokuda, K., Kawano, Y. 2010. Presence of multiple copies of capsulation loci in invasive *H. influenzae* type b strains in Japan before introduction of the Hib conjugate vaccine. *Microbiol. Immunol.,* 54: 160–163.

van Ketel, R.J., De Wever, B., van Alphen, L. 1990. Detection of *H. influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *J. Med. Microbiol.,* 33: 271–276.