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Determination of capsulation status in *H. influenzae* by multiplex PCR

Kevin Lee Nelson and Arnold Lee Smith *

Center for Childhood Infections, Seattle Children's Hospital Research Institute, 1900 - 9th Ave C9S-8, Seattle, WA 98101

Abstract

Since the introduction of *H. influenzae* type b conjugate vaccines there have been concerns regarding the emergence of invasive non-type b strains. Serotyping of *H. influenzae* with commercially available reagents is subjective. Definitive characterization of the capsulation status can be performed by PCR amplification of capsular genes. However, PCR amplification of the conserved export locus in the two known phylogenetic lines of type b strains, and detection of serotype conferring genes in each of the six serotypes requires multiple assays. To rapidly screen multiple isolates, we devised a multiplex method using 15 primers which produced a serotype-specific, distinct pattern of amplicons with reference encapsulated *H. influenzae*. We applied this technique to a panel of 35 clinical isolates which had been serotyped as type a, c, d, e or f by slide agglutination; 15 strains lacked capsular genes. Conversely, of 69 invasive isolates which were not serotypeable, all but 11 contained capsule genes. We conclude that this technique will be useful in screening recently isolated *H. influenzae* for capsulation status.

Introduction

H. influenzae is a ubiquitous colonizer of the human nasopharynx, with the majority of these strains being unencapsulated, i.e. nontypeable and commensals. Less frequent are six capsular types, a through f, described by Pittman on the basis of the reactivity with rabbit polyclonal antiserum (Pittman, 1931). Prior to the introduction of the type b polysaccharide-protein conjugate vaccines, over 90% of the invasive childhood infections, such as sepsis and meningitis, were due to serotype b (Slack, 1995). With the type b conjugate vaccine efficacy rate approaching 90% (Peltola, 2000) and with evidence of herd immunity against serotype b strains, there has been concern that other *H. influenzae* serotypes, or nontypeable strains may emerge as causes of invasive disease. As a result public health laboratories have sought to serotype *H. influenzae* isolated from normally sterile sites (Satola *et al.*, 2007). Comparison of the commercially available slide agglutination to genetic methods identifying the genes involved in the synthesis and export of the capsular polysaccharides had revealed both false-positive and false-negative agglutination reactions (Bokermann *et al.*, 2003; Falla *et al.*, 1993; LaClaire *et al.*, 2003; Sam *et al.*, 2005; Satola *et al.*, 2007).

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*Corresponding author, Phone: 206-884-1032, FAX: 206-884-7311, arnold.smith@seattlechildrens.org.

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The 18 kb capsulation locus of *Haemophilus influenzae* is organized into three domains with the central portion (region II) encoding serotype specific genes, flanked by regions encoding polymerization (region III) and surface expression (region I, *bexABCD*) (Kroll *et al.*, 1990; Kroll *et al.*, 1991b). There are two phylogenetic divisions of type b strains. In division I type b strains these three loci are flanked by three 771 bp insertion elements, IS1016 (Kroll *et al.*, 1991b) with the entire 18 kb capsulation locus duplicated. In the majority of strains there is a partial deletion of one IS1016-*bexA* producing a truncated region I, but an intact adjacent 18 kb capsulation locus (Hoiseth *et al.*, 1986). If the functional 18 kb locus is lost, the strain will not have capsule on the surface, but will possess regions II and III; these strains lack surface capsule and were first recognized as b-minus. They are not serotypeable, but it is not clear whether capsule expression was lost in the laboratory, or whether they possess additional virulence factors associated with the type b lineage which permitted invasive disease (Kroll *et al.*, 1993). Division II type b strains are less common and the 18 kb capsulation locus is not flanked by IS1016 (Kroll *et al.*, 1991b).

The genes encoding capsule export proteins, *bexABCD* are thought to be conserved among all encapsulated *H. influenzae* prompting PCR analysis using primers which were derived from various portions of *bexA* from the type b strain Eagan (Falla *et al.*, 1994). However a polymorphism in *bexA* in *H. influenzae* type e and type f strains precludes accurate identification of *bexA* in these serotypes with type b derived primers (Sam *et al.*, 2005). Zhou *et al.* sequenced *bexA* in the ATCC type a, b, c, d and e reference strains; a clinical type f, defined with serotyping using Difco and Denka Seiken antisera was also examined (Zhou *et al.*, 2007). They found a 3.9 to 16.4% difference in the *bexA* nucleotide sequence of the different serotypes, making the detection of *bexA* using one primer set unreliable for the definition of encapsulation status. One approach for defining the presence of *bexA* is the use of multiple primer pairs as suggested by Zhou (Zhou *et al.*, 2007). This approach will aid in determining if capsule export genes are present, however identification of the capsular serotype is still unknown. Gonin *et al.* described a two stage multiplex method for the determination of *H. influenzae* capsular serotypes: the detection of *bexABCD* locus and a type b gene was followed by touch-down PCR for the detection of a, c, d, e and f capsular serotypes (Gonin *et al.*, 2000).

We recognized that published primers for *bexA* and those for the individual serotype specific capsular genes yielded amplicons of different size and sought to develop a multiplex screening method for the determination of *H. influenzae* encapsulation status and serotype. We then verified this screening method with reference *H. influenzae* and screened a collection of invasive *H. influenzae* that had serologically been identified as non serotype b, either unencapsulated or a serotype other than b. The PCR conditions to optimize the multiplex assay were as recommended (Edwards *et al.*, 1994) recognizing that the electrophoretic mobility of amplicons may be distorted by interaction among DNA fragments and polymerase.

Materials and Methods

H. influenzae strains

Reference *H. influenzae* serotypes were obtained from the American Type Culture Collection: Serotype a (ATCC 9006), a division II type b (ATCC 9795) and Eagan, a division I type b (Kroll *et al.*, 1991a), type c (ATCC 9007), type d (ATCC 9008), type e (ATCC 8142) and type f (ATCC 9833) were stored at -80°C in sterile skim milk. Unencapsulated strains for whom the complete genomic sequence is available were Rd KW20 (Wilcox *et al.*, 1975), R2866, an invasive nontypeable strain (Nizet *et al.*, 1996), 86-028NP (Harrison *et al.*, 2005) strain R2846 and 3224A. The complete genomic sequence of these latter three strains is available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=9621. *H. influenzae* strain S2, a

bexA-minus derivative of strain Eagan (Hoiseth *et al.*, 1985) was also used. Three thousand and six *H. influenzae* strains isolated from the blood or CSF of children between two months and 18 years of age hospitalized at Children's Hospital in Boston, MA (1970–1978), or Children's Hospital Regional Medical Center, Seattle, WA (1978–1994) were examined for capsular serotype by slide agglutination using Bacto *H. influenzae* antisera (Burroughs-Wellcome Diagnostic, Research Triangle Park, NC) or Difco Laboratories anti-*H. influenzae* antisera (Difco, Detroit, MI) at the time of primary isolation. Serotype assignment was performed in the hospital clinical bacteriology laboratory, and repeated in the research laboratory of one of the authors (ALS) prior to storage. Sixty-nine invasive isolates from this collection were identified as serologically nontypeable as there was no agglutination with polyvalent, or serotype-specific *H. influenzae* antisera.

In addition we randomly selected 35 non-invasive strains from our collection which were identified as a serotype other than type b using the same antisera described above. Nineteen of these strains were collected from the nasopharynx of well children in a study which sought to define the prevalence of antibiotic resistance; six strains were from the sputum of patients with Cystic Fibrosis experiencing a pulmonary exacerbation, two were from conjunctival exudate and six were from blood cultures.

PCR typing

The strains were inoculated on chocolate agar plates and incubated overnight at 37°C in 5% CO₂. The colonies were harvested and DNA isolated using the Qiagen DNAeasy kit (Cat # 69504) and stored at –20°C until tested. The stored DNA preparations were used as a template in PCR reactions targeting all six capsular types and the *bexA* export genes using the primers in table 1 which were purchased from Integrated DNA Technologies, Coralville, IA. DNA harvested from strains Eagan, Rd KW20 and R2866 were included in each assay of experimental strains. DNA polymerase was obtained from Bioline USA Inc., Randolph, MA (Biolase Polymerase, Cat # BIO-21042). The PCR amplification was performed in a total volume of 25 µl with 1µl template, in a BIOLASE DNA Polymerase (Bioline, London, UK) based reaction. Each reaction included 2.5 µl 10xNH₄ Reaction Buffer, 1 µl 50 mM MgCl₂, 2 µl 2.5 mM dNTP and 0.2 µl of polymerase from the BIOLASE kit. For the multiplex assay 1µl of each primer from a 20 µM stock was added to each reaction with water added making the final volume with the primers 25 µl. An initial melting temperature of 94 °C was used for 2 minutes, followed by cycles of 30 secs at 94 °C, 30 secs at 55 °C, and 45 secs at 72 °C for 40 cycles and 2 min at 72 °C. The PCR products were analyzed on a 1.8% MetaPhor agarose gel (Cambrex Biosciences, Charles City, IA), run at 50 v for 3 hrs with an EC-154 power supply (EC-Apparatus Corp, Milford, MA) containing 0.1% ethidium bromide (Sigma, St. Louis, MO) and viewed with FluorChem 8900 illuminator (Alpha Innotech, San Leandro, CA). The molecular weight standards were Hyperladder I (200 to 10,000 bp) and Hyperladder IV (100 to 1,000 bp) and obtained from Biocompare Corporation (South San Francisco, CA).

Results

We first verified that the primer sets for *bexA* (Falla *et al.*, 1994) yielded the predicted amplicon with the type b strain Eagan, and the ATCC serotypes a, b, c, d, e, and f (data not shown) and the serotype of each of the reference strains was confirmed by PCR using the primers of Falla (Falla *et al.*, 1994) (data not shown). When we examined the reference strains in a multiplex format using 15 different primers, each strain yielded a distinct amplicon pattern (figure 1). Since there was a serotype specific pattern of amplicons we reasoned that the pattern could be used for screening clinical isolates. It should be emphasized that the amplicon **pattern** correlated with the serotype of each reference strain. To confirm that the fragments comprising the pattern are due to priming from the capsule gene cluster we purified and sequenced each

of the fragments in figure 1. Table 2 depicts the relative mobility of the fragments and the regions of homology found by sequencing: 21 of the 24 PCR products contained sequence which was identical to portions of the capsule cluster in the NCBI database. The 250 bp fragment seen with DNA from the ATCC reference type c strain, the 140 bp sequence from the ATCC reference type d strain and the 400 bp fragment from the ATCC type f strain did not match any *Pasteurellae* sequences in the NCBI database.

The primer set 1 of van Ketel (van Ketel *et al.*, 1990) for *bexA* yields a 343 bp amplicon with strain ATCC 9795, while we found a 550 bp product using, *bexAF* and *bexAR* primers (table 1) derived from the published sequence of strain Eagan (Kroll *et al.*, 1988a); both of these amplicons approximate the size predicted from direct analysis of the sequence. We used two primer sets for *bexA* as previous work (Kroll *et al.*, 1990) found sequence divergence in *bexA* between the two phylogenetic divisions of type b strains. When both of the primer sets for *bexA* are used together with the type b strain Eagan (division I) and the ATCC 9795 type b strain (division II) both strains contain an amplicons of approximately 380, 480, and 620 bp; however strain Eagan produces an additional 550 and 1150 bp amplicon (figure 1). With the *bexA*⁻ derivative of strain Eagan, strain S2 only the ~480 bp amplicon is produced (figure 1).

In this multiplex assay the fragments generated in the multiplex assay do not exactly correlate with the predicted products when a single pair of primers is used. For example in figure 1, excision, purification and re-assay of the fragments generated in the multiplex assay with strain Eagan showed that the fragment migrating at 1150 bp contains an amplicon of that size as well as a 620 bp fragment and a 380 bp fragment. The reason for the formation of apparent concatamers of these genes is unclear. Thus this assay is intended to permit screening of a large number of samples of *H. influenzae* isolates for the presence or absence of capsulation based on amplicon pattern, not for characterization of a cap locus.

We prepared DNA from 69 invasive strains which did not agglutinate with *H. influenzae* typing sera and were identified in a clinical and research lab as nontypeable, and analyzed them using the above multiplex conditions. Of the 69 invasive, serologically nontypeable isolates we found that 52 yielded an amplicon pattern which was typical of one of the two reference type b strains. Three isolates had a pattern consistent with the absence of *bexA* but possessing a gene encoding the type b capsule, isolates defined as b⁻ (Kroll *et al.*, 1989). One isolate had a type a amplicon pattern, while two were consistent with type f. The remaining 12 isolates did not yield an amplicon. This data shows that the prior reliance of serotyping can result in misidentification, suggesting that nontypeable *H. influenzae* might not have been prevalent before the widespread introduction of the type b conjugate vaccine.

We also used the multiplex assay to examine randomly selected isolates serologically identified as encapsulated, but not serotype b (table 3). We found that all four isolates serotyped as type a, and isolated from the nasopharynx did not yield amplicons in the multiplex assay. Similarly, both isolates identified as serotype c, one from blood and one from nasopharynx also failed to yield detectable amplicons. Of the 11 isolates identified as serotype d, only those isolated from the eye (n = 2) and throat (n = 5) had an amplicon pattern consistent with type d. All four blood isolates initially identified as type d in a state public health laboratory in 2005 were nontypeable on multiplex PCR assay. Only three of the eight isolates reacting with type e antisera, and all from sputum, had a type e pattern on multiplex assay, while the remainder did not yield an amplicon. All four nasopharyngeal isolates serotyped as type f were confirmed with the multiplex assay; one of two blood isolates and three of four sputum isolates were genetically confirmed as type f. The amplicon pattern illustrating some of these findings is depicted in figure 2, while table 4 depicts the clinical focus and the results from serotyping and multiplex screening.

Discussion

Difficulties in interpretation of slide agglutination tests with *H. influenzae* producing false-positive results was reported by Shively et al (Shively *et al.*, 1981), but was not confirmed by Himmelreich (Himmelreich, 1985). This laboratory to laboratory difference appears to be due to interpretation of a positive agglutination reaction between the bacterial suspension and antisera, as amplification of the agglutination reaction using antibody coated latex particles yields congruent results (Himmelreich, 1985). Because of concerns regarding the efficacy of the type b conjugate vaccines, and to address the potential for serotype replacement the Active Bacterial Core Surveillance of the Emerging Infections Program Network (<http://www.cdc.gov/ncidod/dbmd/abcs/reports.htm>) of the CDC compared the results of serotyping performed by state laboratories with those obtained by PCR performed at CDC with 141 invasive *H. influenzae* isolates (LaClaire *et al.*, 2003). They found discrepancies in 56 isolates: of these 54 which were identified as a specific serotype by slide agglutination were unencapsulated by PCR. More recently Satola et al (Satola *et al.*, 2007) surveyed 360 invasive *H. influenzae* collected between 1989 and 1998 comparing slide agglutination with PCR. They found of 168 serologically nontypeable strains, eight contained portions of the capsule gene cluster; of 192 which were serotypeable on slide agglutination, 48 were genetically unencapsulated. We confirmed these observations and found misidentification errors when antisera is used for serotyping; genetically nontypeable isolates were classified as serotypes other than type b, and strains classified as possessing a capsule, lacked genes for encapsulation.

The capsule export gene common to all encapsulated *H. influenzae*, *bexA* consists of a 654 bp orf (Kroll *et al.*, 1988b) which is located in the *bexABCD* gene cluster (Kroll *et al.*, 1990). Expression of serotype-specific capsule on the bacterial surface requires a ~ 18 kb segment of DNA containing a central serotype specific region (region II) flanked by genes not specific for each serotype, but encoding export and polymerization proteins. One copy of an intact 18 kb locus is required for expression of surface capsular carbohydrate, but up to six tandem loci have been described in a single strain (Cerquetti *et al.*, 2006).

Using this multiplex assay with 15 primers designed to identify *bexA* in the two phylogenetic classes of type b, and serotype specific primers for types a, b, c, d, e and f we found that a distinct amplicon pattern was generated with each reference strain, the presence of a unique pattern allowed presumptive classification of clinical *H. influenzae* isolates. This technique does not permit genetic characterization of the cap locus, but will indicate the presence or absence of genes responsible for capsulation.

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Figure 1. Agarose gel electrophoresis of reference *H. influenzae*

Lane 1, 100 bp standards (Hyperladder I); lane 2, strain R2866; lane 3, strain ATCC 9006 (type a); lane 4, strain Eagan (type b); lane 5, strain ATCC 9795 (type b); lane 6, strain S2 (b⁻ derivative of Eagan); lane 7, strain ATCC 9007 (type c); lane 8, strain ATCC 9008 (type d); lane 9, strain ATCC 8142 (type e); lane 10, strain ATCC 9833 (type f); lane 11, strain R2846 and lane 12, 1 kb standards (Hyperladder IV).

Figure 2. Multiplex analysis of selected *H. influenzae**

Lane 1, 100 bp standard (Hyperladder I); lane 2, R1775; lane 3, R3735; lane 4, R2978; lane 5, C188; lane 6, R1735; lane 7, R730; lane 8, R751; lane 9, R1048; lane 10, R1612; lane 11, C858; lane 12, R2778; and lane 13, 1 kb standard (Hyperladder IV).

*Source, serotype by slide agglutination and multiplex characterization are depicted in table 3

Table 1

Target Primer		Sequence	ref
Gene	name		
<i>bexA</i>	bexAF*	5'-ATGAATCGCGTAAATAATGTATGTAAGAAG-3'	this lab
<i>bexA</i>	bexAR*	5'-TTGCGTCTCGTTGTAGTATTGATAC-3'	this lab
<i>bexA</i>	HI-1**	5'-CGTTTGATGATGTTGATCCAGA-3'	(18)
<i>bexA</i>	HI-2	5'-TGCCATGTCTTCAAAATGATG-3'	(18)
<i>acs3</i>	a2	5'-GAATATGACCTGATCTTCTG-3'	(2)
<i>acs3</i>	a3	5'-AGTGGACTATTCTGTTACAC-3'	(2)
<i>bcs3</i>	b1	5'-GCGAAAGTGAACCTTATCTCTC-3'	(2)
<i>bcs3</i>	b2	5'-GCTTACGCTTCTATCTGGTGAA-3'	(2)
region II	c1	5'-TCTGTGTAGATGATGGTTCA-3'	(2)
region II	c2	5'-CAGAGGGCAAGCTATTAGTGA-3'	(2)
region II	d1	5'-TGATGACCGATACAACCTGT-3'	(2)
region II	d2	5'-TCCACTCTTCAAACCATTTCT-3'	(2)
region II	d3	5'-CTCTTCTTAGTGCTGAATTA-3'	(2)
region II	f1	5'-GCTACTATCAAGTCCAAATC-3'	(2)
region II	f2	5'-CGCAATTAGGAAGAAAGCT-3'	(2)

* From the *bexA* sequence of Strain Eagan.

** Primer is one base shorter at the 3' end than that published

Table 2

Identity of DNA fragments generated in multiplex assay seen in figure 2

Strain	Lane	Relative size (bp)	Sequence identity Serotype	Reference Serotype
ATCC 9006	3	180	serotype a specific	a
		340	<i>bexA</i>	
ATCC 9795	4	380	<i>bexA</i>	b
		480	serotype b specific	
		620	<i>bexA</i>	
Eagan	5	380	<i>bexA</i>	b
		480	serotype b specific	
		550	<i>bexA</i> -serotype c hybrid	
		620	<i>bexA</i>	
		1150	<i>bexA</i> doublet	
S2	6	480	serotype b specific	b ⁻
ATCC 9007	7	250	unknown	c
		380	<i>bexA</i>	
		550	<i>bexA</i> -serotype c hybrid	
		620	<i>bexA</i>	
		1150	<i>bexA</i> doublet	
ATCC 9008	8	140	unknown	d
		380	<i>bexA</i>	
		550	<i>bexA</i> -serotype c hybrid	
		620	<i>bexA</i>	
ATCC 8142	9	320	<i>bexA</i>	e
		360	<i>bexA</i> -serotype d hybrid	
ATCC 9833	10	320	<i>bexA</i>	f
		400	unknown	

Table 3

Examination of non-serotype b strains by multiplex PCR

Serotype Classification*	Number	source	Multiplex pattern
a	4	nasopharynx	4 NTHi
c	1	blood	1 NTHi
c	1	nasopharynx	1 NTHi
d	2	eye	2 d
d	5	throat	5 d
d	4	blood	4 NTHi
e	5	throat	5 NTHi
e	3	sputum	3 e
f	2	blood	1 f, 1 NTHi
f	4	sputum	3 f, 1 NTHi
f	4	nasopharynx	4 f

* Slide agglutination was performed on initial recover in the clinical laboratory and repeated before store in the research lab of one of us (ALS).

Table 4

Representative strains in which the serotype determined by slide agglutination yielded a result different from that determined by multiplex PCR (figure 2).

Accession #	focus	serotype	multiplex type
R1775	CSF	b	nontypeable
R3753	blood	nontypeable	d
R2978	sputum	nontypeable	f
C188	blood	c	nontypeable
R1735	middle ear	c	nontypeable
R730	nasopharynx	a	nontypeable
R751	nasopharynx	e	nontypeable
R1048	sputum	a	nontypeable
R1612	CSF	b	b ⁻
C858	CSF	nontypeable	b
R2778	CSF	nontypeable	b