Capsulation Loci of Non–Serotype b Encapsulated *Haemophilus influenzae*

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Invasive infections caused by non–type b encapsulated *Haemophilus influenzae* have increased recently. Because capsule is a major virulence factor, capsulation of 62 recently isolated non–serotype b encapsulated strains was examined. Repeated serotyping confirmed only 69.0% of isolates. The combination of slide agglutination and cap genotyping confirmed 78.9% of type a, 100% of type e, and 86.4% of type f strains. Seven nonencapsulated strains may have lost capsulation through homologous recombination. Three strains that could not be serotyped or genotyped failed to hybridize with any cap probe and are probably nontypeable *H. influenzae*. Of isolates that retained an intact cap locus, 62.2% had evidence of cap amplification. The epidemiology of non–type b encapsulated *H. influenzae* infections is complicated by the poor specificity of available serologic reagents and by spontaneous capsule loss. Recently isolated invasive non–type b encapsulated *H. influenzae* frequently have cap amplification, which may contribute to their virulence.

Encapsulated *Haemophilus influenzae* are distinguished from nontypeable strains by the presence of one of 6 structurally and serologically distinct polysaccharide capsules, referred to as serotypes a–f [1]. The capsulation (cap) locus has a common organization for all 6 capsular types and consists of 3 parts [2]. Regions 1 and 3 contain genes important in the processing and export of polysaccharide that are common to all serotypes. These regions flank the serotype-specific region 2 disaccharide subunit synthesis genes. In most invasive strains of *H. influenzae* type b (Hib), the cap locus is flanked by IS1016 insertion elements. Type-specific region 2 genes may also be flanked by repetitive sequences [3].

This organization of the cap locus predisposes to duplication or loss of the capsulation cassette via homologous recombination. Most invasive isolates of Hib contain a partial duplication of the cap locus, consisting of 1 copy of the locus that is intact and a second copy that carries a partial deletion of bexA [4]. This deletion stabilizes the duplication and results in increased capsule production and virulence. Higher-order duplications of the locus are also possible. Isolated clinical strains of Hib recently have been reported to possess up to 5 copies of the cap locus, but these strains rapidly revert in vitro to the partially duplicated genotype [5]. Organisms containing higher numbers of the cap locus are more resistant to antibody-dependent, complement-mediated bacteriolysis [6].

Hib strains spontaneously lose capsule expression both in vitro and in vivo [7]. In strains possessing a partially duplicated locus, homologous recombination between identical segments is possible, and loss of capsule expression results from deletion of the single functional copy of bexA, which is required for polysaccharide export [8]. Single-copy loci also undergo spontaneous reduction, which is hypothesized to occur by recombination of IS1016 elements or other short direct repeat regions within the cap locus [3].

Before the introduction of highly immunogenic vaccines against Hib, this serotype was the leading cause of bacterial meningitis and a common cause of other invasive infections in young children. Invasive infections caused by non–serotype b encapsulated *H. influenzae* currently are less common than were Hib infections in the prevaccine era, but sporadic invasive infections with these organisms are well recognized and are associated with significant morbidity and mortality [9]. Kroll et al. [10] described an outbreak of invasive disease due to serotype a *H. influenzae* in The Gambia in the 1980s. A proportion of these strains exhibited the partially duplicated cap locus common in Hib. The authors hypothesized that this mutation, as in Hib, might increase capsule expression and contribute to the unusual virulence of these strains. We recently reported a similar outbreak of invasive disease caused by serotype a *H. influenzae* in previously healthy young children [11].

Active surveillance studies suggest that invasive non–type b encapsulated *H. influenzae* infections have been more common in recent years. Perdue et al. [12] reported that the incidence of invasive non–serotype b encapsulated *H. influenzae* infections in Alaskan residents increased 2-fold between 1980 and...

Received 25 January 2001; revised 3 April 2001; electronically published 8 June 2001.

Financial support: Cancer Center Support (CORE grant P30 CA 21765; American Lebanese Syrian Associated Charities to E.E.A.); National Institutes of Health (AI-44167 and DCD-02873 to J.W.S. and CA-23944 to E.E.A.); American Pediatric Society (summer research fellowship to C.O.).

The Journal of Infectious Diseases 2001; 184:144–9

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1990 and between 1991 and 1996. An increase in the incidence of invasive infections due to serotype f H. influenzae also has been reported over a similar time period [13]. The apparent increase in serious disease due to non–type b encapsulated H. influenzae prompted us to better characterize these bacteria. We examined the organization of the cap locus and expression of capsular polysaccharide in a large collection of recently isolated invasive non–type b encapsulated H. influenzae strains.

Materials and Methods

Bacterial strains. Since encapsulated H. influenzae may spontaneously lose capsule production, we wished to study strains that had been passaged a minimal number of times. Most isolates of non–type b encapsulated H. influenzae were obtained from the State Public Health Laboratories of Alabama, California, Colorado, Connecticut, Idaho, Iowa, Minnesota, Missouri, Oklahoma, North Carolina, Tennessee, and Utah. Strain R421 was provided by A. Smith (University of Missouri), and strain AT01 was provided by J. Kellner (Alberta Children’s Hospital, Calgary, Canada). All strains were isolated from blood or cerebrospinal fluid, and, except for strains R421, CA06, and CA08, all were isolated between 1998 and 2000. Only 1 isolate, R421, had been repeatedly passaged after definitive identification. Bacterial strains were serotyped at the time of isolation by the referring laboratories, using commercially available antisera, or were referred to the Centers for Disease Control and Prevention for serotyping. Bacteria were grown on Chocolate II Agar (Edge Biological) or in brain-heart infusion broth supplemented with 2 μg/mL of B-nicotinamide adenine dinucleotide and 10 μg/mL of hemin (sBHI).

Capsulation loci genotyping. Restriction fragment length polymorphisms (RFLPs) of the cap locus were determined by Southern blotting with probe pOU38 (provided by J. S. Kroll, Imperial College School of Medicine, London), which consists of the capsulation locus of Hib [14]. In non–type b encapsulated strains, this probe hybridizes to genes involved in capsule expression that are shared by all encapsulated strains. Hybridization of EcoRI-digested genomic DNA with the pUO38 probe yields highly conserved and distinctive patterns for each serotype [14, 15]. The presence of IS1016 elements was determined by Southern blotting, as described elsewhere [16]. DNA probes for serotype a–, e–, and f–specific capsulation genes were amplified from representative strains by polymerase chain reaction (PCR), using primers described elsewhere [17]. Genomic DNA from each bacterial strain was digested overnight with EcoRI in the supplied buffer, and restriction fragments were separated by electrophoresis through a 0.8% Tris borate–EDTA agarose gel. DNA fragments were transferred to nylon membranes and were cross-linked by exposure to UV light. Probes were labeled with fluorescein-conjugated deoxyxucleotides (Gene Images; Amersham Pharmacia Biotech) or 32P-dCTP (Random Primed DNA Labeling kit; Roche Molecular Biochemicals) and were hybridized with membranes under standard conditions. Blots were then washed at high stringency and were exposed to radiographic film.

Copy number of cap. In Hib, KpnI and Smal sites flank the cap locus, allowing the copy number of the locus to be estimated by the size of restriction fragments produced by digestion with these enzymes [5]. Earlier studies found the size of a single-copy locus to be ∼18 kb and found loci with increased copy numbers to be correspondingly larger by multiples of ∼18–23 kb [4]. The common organization of the cap loci in encapsulated H. influenzae suggested that the cap copy number in other serotypes could be identified by using the same strategy [3].

Chromosomal DNA was prepared for pulsed-field gel electrophoresis by purification in agarose plugs. Bacteria were grown to an OD600 of 0.3 in sBHI. A 1.5-mL aliquot of cells was centrifuged briefly to pellet cells. Bacteria were washed twice in 1 mL (total volume) of 10 mM Tris (pH 8.0)/1 mM EDTA (pH 8.0)/100 mM NaCl and then were resuspended to an OD600 of 1.2. Equal volumes of bacterial suspension and 1.6% low–melting point SeaPlaque agarose (BioWhittaker Molecular Applications) were combined and were added to molds. Plugs were incubated with 100 μg/mL of Proteinase K in 50 mM Tris (pH 7.8)/50 mM EDTA (pH 8.0)/1% sarcosine at 55°C overnight, were rinsed with sterile water, and then were incubated for 30 min at 25°C with 0.05 mg/mL of phenylmethylsulfonyl fluoride in 10 mM Tris HCl (pH 7.4)/1 mM EDTA. Plugs were rinsed with sterile water, were washed 3 times with 10 mM Tris HCl (pH 7.4)/1 mM EDTA, and were stored at 4°C. Before digestion, plugs were equilibrated in 250 μL × restriction buffer at 25°C for 30 min. The buffer was replaced with 250 μL of fresh solution, and 15 U of Smal and KpnI (Life Technologies) was added. Plugs were digested at 30°C for 4 h and then at 37°C for 4 h. Fresh restriction enzymes and buffer were added, incubations were repeated, and plugs were equilibrated by incubating them for 10 min in 0.045 M Tris borate–0.001 M EDTA (0.5X TBE) buffer. Digested plugs were electrophoresed with a CHEF-DR II apparatus (Bio-Rad) in a 1% SeaKem agarose gel (BioWhittaker Molecular Applications) for 16 h at 180 V in 0.5X TBE buffer. Initial time was 1.0 s and final time was 26 s, at 12°C. Gels were transferred to nylon membranes and were hybridized with pUO38, as previously described. The relative intensity of hybridizing bands on Southern blots was determined by densitometry of autoradiographs, using the Eagle Eye II software package (Stratagene).

Presence of bexA-IS1016 deletion. The presence of the bexA-IS1016 deletion was identified by PCR by use of bexA and IS1016 primers flanking the deletion, as described elsewhere [10].

Expression of capsular polysaccharide. The expression of capsular polysaccharide was determined by slide agglutination, using serotype-specific antisera (Becton Dickinson Biosciences). In most cases, elaboration of capsule was confirmed by immunodiffusion assay. Petri plates were coated to a depth of 2 mm with 1.4% agarose in PBS with 5 mM EDTA and were allowed to harden. Vertical wells were cored in agarose at a 50-mm radius from a central well. Bacteria were grown to the stationary phase in sBHI, after which 5 μL of culture was added directly to radial wells and 5 μL of serotype a, e, or f antisera was added to the central well. Plates were incubated overnight at room temperature in a humid environment. Gels were transferred to paper and were dried under vacuum for 30 min at 50°C. Dried gels were rinsed twice with PBS and then were stained for 1 h with 0.02% Coomassie blue. Gels were destained in 7% acetic acid and 25% methanol for 1–2 h, then were washed twice for 10 min in water and were dried. Expression of capsular polysaccharide was indicated by the presence of a precipitin line between wells with reactive antiserum.
Results

Capsulation genotyping. A total of 62 bacterial strains were obtained from 12 states and 1 Canadian province. Fifty-seven strains had previously been serotyped by referring laboratories or individuals. Two of these 57 isolates (1 isolate designated as type a and 1 as type c) were actually Hib with the b(V) cap genotype (10.2-, 9.0-, 5.6-, and 4.4-kb bands) [14, 15]. Five additional strains were reported to react with a pooled antiserum against the serotype a, c, d, e, and f polysaccharides but could not be typed by the referring laboratories with antiserum against individual capsular antigens. The cap locus of 3 of these strains could be genotyped by using pUO38, 2 strains as serotype e and 1 as serotype a. Table 1 summarizes the characteristics of the 58 non–serotype b encapsulated strains evaluated.

Southern analysis using pUO38 revealed recognizable patterns of hybridization in 43 strains, including 13 of 20 serotype a, 0 of 1 serotype c, 0 of 1 serotype d, 11 of 14 serotype e, and 19 of 22 serotype f strains. Four additional type a, 1 type e, and 2 type f strains had aberrant hybridization with pUO38. Some isolates had a high frequency of spontaneous loss of capsule. On agar plates, 2 colony morphologies representing encapsulated and acapsular variants could be observed. In 3 instances, we observed that hybridization with pUO38 was lost in successive genomic DNA preparations from the same bacterial strain. All isolates that could be genotyped had RFLPs that had been described earlier except for MO03, a serotype e strain that shared a 1.8-kb restriction fragment with the conventional genotype but had 5.0- and 18-kb fragments (e[N] phenotype) rather than the expected 4.8- and 15-kb hybridizing bands. As suggested elsewhere, distinctive capsular genotypes may arise by unusual genetic rearrangements or mutations altering restriction enzyme sites [18].

Presence of the bexA-IS1016 deletion. In total, as assessed by a PCR assay, 10 strains were positive for the bexA-IS1016 deletion. In most cases, serotype a strains with this deletion had the a(N) genotype [10]. However, 1 strain, CA05, had the a(N) genotype, although attempts to amplify genomic DNA spanning the typical deletion were repeatedly unsuccessful. It is possible that this failure is related to inhibitors of the amplification reaction, to an atypical cap structure resulting in primer mismatch, or to an unusual deletion.

Serotype-specific probes. Each of the 11 strains with a pattern of hybridization with pUO38 that was indicative of a type e strain hybridized with a type e–specific probe. Of 19 genotypically confirmed serotype f strains, all hybridized with the type f probe, and 14 of 15 serotype a strains hybridized with the serotype a–specific probe. Only 1 strain with aberrant or no hybridization with pUO38 hybridized with a serotype-specific probe. The exception was strain UT06, which demonstrated a single, ~7-kb band with pUO38 and also hybridized with the type a probe. DNA from this strain did not hybridize with IS1016, and no capsule elaboration was detected serologically. No serotype-specific probes hybridized with DNA from another serotype.

IS1016 probe. Twelve serotype e strains and all serotype f strains hybridized with the IS1016 probe. Seventeen of 20 type a strains hybridized with IS1016, including 3 strains with aberrant or no hybridization with pUO38, which suggests that these strains had deleted or were in the process of deleting the cap locus by recombination among IS1016 elements. DNA from 5 strains (AT01, CA06, UT06, OK04, and WI02) failed to hy-

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NOTE. Data are no. of strains, unless otherwise indicated.

a As determined by Southern blotting.
b As determined by polymerase chain reaction.
c As determined by slide agglutination or immunodiffusion assay.
Figure 1. Copy number of capsulation genes. Shown are restriction fragments of Smal- and KpnI-digested chromosomal DNA separated by pulsed-field gel electrophoresis and hybridized with pUO38. Pattern A consists of 1 restriction fragment of 36–40 kb, and pattern C consists of 1 fragment of ~62 kb. Pattern B consists of 2 restriction fragments of ~20 and 36 kb. The relative intensity of hybridization suggests that 2, 3, and 4 copies of the cap locus are present in isolates with patterns A, B, and C, respectively.

bridize with IS1016. It is likely that CA06, OK04, and WI02, which also failed to hybridize with pUO38 and to express capsule, are actually nontypeable strains (i.e., bacteria that never were encapsulated and produced false-positive agglutination reactions at the time of initial serotyping). In support of this hypothesis, Smal and ApaI restriction digest patterns of these strains do not resemble the relatively limited number of polymorphisms exhibited by the other invasive serotype a and e strains (data not shown). Or, a recombination event between repetitive elements outside the cap locus may have resulted in excision of both capsular synthesis genes and IS1016 elements.

Elaboration of capsular polysaccharide. Of 22 strains designated serotype f by reference laboratories, 19 (86.3%) expressed sufficient capsular polysaccharide to be detected by slide agglutination and/or immunodiffusion; in contrast, only 14 serotype a strains (70.0%) and only 8 serotype e strains (57.1%) had detectable capsule by the same assay. One strain, AT01, expressed serotype a capsule, but failed to hybridize with any cap probe. Although we carefully selected encapsulated colonies for these studies, we suspect that the capsular locus of this strain was deleted shortly after inoculation into broth culture for the preparation of genomic DNA. Alternatively, a false-positive reaction may have resulted from autoagglutination or a nonspecific interaction. This strain, however, failed to agglutinate in PBS or with anti–type f and anti–type e sera, making this explanation less likely. Capsular polysaccharide could be detected by both immunodiffusion and slide agglutination in most strains; 8 strains, however, were positive by slide agglutination only.

Copy number of encapsulation genes. Hybridization of pUO38 with Smal/KpnI-digested DNA from 30 of 37 invasive H. influenzae strains tested identified a single restriction fragment of ~18, 36, or 62 kb. The mean relative intensities of hybridization of these bands were 1.11, 1.92, and 3.90, respectively, suggesting that a total of 1, 2, or 4 copies of the locus were present.

One serotype a strain (UT05) demonstrated 2 bands (32 and 42 kb) of approximately equivalent intensity. Five serotype e strains and 1 serotype f strain had identical RFLP patterns with 32- and 18-kb bands, suggesting the presence of 3 copies of the cap locus (figure 1).

Discussion

Most clinical microbiology laboratories currently identify the capsular serotype of encapsulated H. influenzae by slide agglutination. A striking finding of this study is that we were able to confirm the initial serotyping in only 75.8% of strains in this collection by this method. The use of capsular genotyping or PCR amplification of serotype-specific genes has been suggested as an alternative to serotyping [14, 17]. However, with our collection of isolates, these strategies apparently were successful in only 75.8% and 77.6% of isolates, respectively.

We cannot exclude the possibility that there were errors in the initial identification or subsequent handling of these strains; in many cases, however, the loss of genes responsible for capsule production is responsible for this phenomenon. With some strains, visual inspection of cultures on agar plates readily demonstrated acapsular variants, and in several cases, genomic DNA prepared from serial cultures demonstrated the loss of the cap locus by Southern hybridization. The rapid loss of capsule by some encapsulated H. influenzae strains has obvious consequences for disease surveillance. Bacteria that are not ex-
pediently serotyped upon primary isolation may be incorrectly designated as nontypeable strains.

One study of the genetic relationships between Hib and nontypeable *H. influenzae* suggested that nontypeable strains are not simply variants of encapsulated strains [19]. However, another study showed that >30% of a group of pharyngeal isolates of nontypeable *H. influenzae* had evidence of cap sequences and/or IS1016, suggesting that a subgroup of nontypeable strains is derived from an encapsulated precursor [16]. It is possible, therefore, that invasive infections caused by “nontypeable” *H. influenzae* may in fact be due to undetected encapsulated bacteria. This may be a particular problem with serotype e strains. Among strains possessing apparently intact cap loci, only 72.7% of serotype e expressed sufficient capsular polysaccharide to be detected by slide agglutination, compared with 85.7% of serotype a and 86.3% of serotype f strains. Serotype f is regarded as the most common cause of non–type b invasive infection [13]. It is possible that the perceived frequency of these infections may be affected by a reduced likelihood of capsular loss by serotype f strains or increased sensitivity of type f serologic reagents.

As was observed in studies of the mechanism of loss of encapsulation in Hib, the most common explanation for capsular loss of other serotypes appears to be recombination events between IS1016 elements flanking the cap locus or between other repetitive elements [4, 7, 8]. However, in 5 strains with typical cap genotypes, capsule could not be detected by slide agglutination or immunodiffusion, which suggests that current serologic reagents may be relatively insensitive. Alternatively, some non–type b encapsulated *H. influenzae* may down-regulate capsule expression or fail to express capsule because of point mutations affecting gene function. In considering the possibility of down-regulation of encapsulation, it is noteworthy that in Hib, encapsulation promotes survival in the bloodstream, whereas loss of capsule expression enhances the ability of bacteria to adhere to and invade human epithelial cells [20].

It has been suggested that the presence of the *bexA*-IS1016 deletion in serotype a strains may confer increased virulence on these strains [10]. Because of confidentiality of patient information, we were able to obtain clinical information related to only a small number of the isolates in our collection. Two patients with infections caused by serotype a strains with the *bexA*-IS1016 deletion had severe clinical courses, whereas 3 patients with infections caused by strains without the deletion had milder disease (authors’ unpublished data). One infection caused by a serotype e strain with the *bexA*-IS1016 deletion was uncomplicated. This mutation occurs in a small minority of invasive non-b encapsulated *H. influenzae* strains; therefore, its presence is not obligatory for invasion. The potential role of the mutation in increasing the virulence of certain strains will require further study.

By analogy to Hib, increased cap copy number and/or amount of capsular polysaccharide may contribute to the virulence of invasive non–type b encapsulated *H. influenzae* [5]. Other investigators have demonstrated that serotype a strains usually contain 2 intact tandem repeats of the cap locus, whereas type c and d strains contain a single copy [18]. Here, we show that serotype f *H. influenzae* is also likely to maintain 2 intact or partially duplicated tandem repeats, whereas serotype e strains may have single or duplicated loci. In our collection of isolates, larger numbers of cap amplification may reflect the selective advantage of large amounts of capsular polysaccharide in the bloodstream or cerebrospinal fluid. Further studies of the effects of variation in capsule expression will provide insights into the pathogenesis of invasive infections caused by non–type b *H. influenzae*.

References