Invasive Infections with *Haemophilus influenzae* Serotype a Containing an IS1016-bexA Partial Deletion: Possible Association with Virulence

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**Background.** Recent reports of invasive *Haemophilus influenzae* non-b capsular serotypes in the era since development of conjugate vaccines have prompted concern about serotype replacement. Unusual clusters of invasive infection due to *H. influenzae* serotype a with clinical features that resemble those of infection due to *H. influenzae* serotype b have been described. A unique feature often associated with more-virulent *H. influenzae* serotype a isolates is the IS1016-bexA partial deletion, which was previously identified in the capsule locus of *H. influenzae* serotype b strains. We report the clinical, epidemiologic, and molecular genetic features of 2 cases of severe disease caused by *H. influenzae* serotype a.

**Methods.** Invasive *H. influenzae* isolates were serotyped with standard serological methods, and molecular typing was done with PCR. The capsular genotype of each isolate was characterized with PCR, partial sequencing, and Southern blot hybridization. Further strain typing was performed with pulsed-field gel electrophoresis.

**Results.** We identified 2 children with severe invasive disease due to *H. influenzae* serotype a. Both *H. influenzae* serotype a isolates contained the identical pulsed-field gel electrophoresis pattern and capsular genotype. An IS1016-bexA partial deletion in the capsule gene locus similar to that found in *H. influenzae* serotype b was identified in both isolates by means of PCR and sequencing of the IS1016-bexA junction.

**Conclusions.** We describe 2 cases of severe invasive disease due to *H. influenzae* serotype a with the putative virulence-enhancing IS1016-bexA partial deletion and duplication of the capsule locus. Our data support the hypothesis that this mutation may be associated with virulence in non-b capsular serotypes of *H. influenzae.*

Invasive disease due to *Haemophilus influenzae* in the era before the development of conjugate vaccine was most often associated with *H. influenzae* serotype b (Hib) infection [1]. A wide variety of Hib infections, many devastating, were frequently seen in older infants and toddlers aged <2 years. A smaller proportion of invasive Hib disease was seen in older children and adults. Now, more than a decade after the introduction of an Hib conjugate vaccine for the pediatric population, the incidence of invasive Hib disease has dramatically declined [2, 3], though it continues to have a small but significant presence, primarily among children who are unimmunized and among older adults with underlying diseases.

A small minority of cases of invasive *H. influenzae* disease has been associated with nontypable (noncapsulated) strains and other capsule serotypes (primarily serotypes a, e, and f) [4]. Significant declines in the rates of both invasive Hib disease and nasopharyngeal Hib carriage during the vaccine era prompted speculation that other *H. influenzae* capsular serotypes or other respiratory pathogens might fill the niche. To date, there is little evidence that significant replacement of Hib infection with infection due to non-b capsular serotypes of *H. influenzae* is occurring in children in the United States [5].

The uncommon association of non-b capsular serotypes with invasive disease in children has been attributed to a lower virulence potential in normal hosts. Invasive disease due to *H. influenzae* serotype a (Hia)
may be facilitated by the acquisition of virulence factors common to Hib, such as capsule gene duplications and an IS1016-bexA deletion in the capsule gene cluster, which may serve to stabilize capsule production [6–8]. However, invasive Hia disease has been reported in the absence of the IS1016-bexA deletion [6, 9]. We report 2 cases of invasive Hia disease that occurred within 4 days of each other in toddlers of approximately the same age whose isolates both possessed capsule gene cluster duplications with an IS1016-bexA partial capsule gene deletion that is typically seen in Hib isolates.

**CASE REPORTS**

**Patient 1.** A 14-month-old boy of Middle Eastern descent presented with a 2-day history of rhinorrhea and cough, a 1-day history of tactile fever, and swelling of his right hand. He had no significant medical or travel history. Immunizations were up-to-date. His temperature was 39.5°C. There was marked swelling of the right wrist with erythema, warmth, and tenderness. His peripheral WBC count was 18,600 cells/μL, with 62% neutrophils; his C-reactive protein level was 17.1 mg/dL. An MRI of the right hand revealed diffuse enhancement of the dorsal deep soft tissue and fascia from the distal radius to the proximal phalanges. Therapy with cefazolin and clindamycin was started and resulted in prompt improvement. Two blood samples cultured in bottles grew encapsulated *H. influenzae* that was β-lactamase–negative and was identified as serotype a. The child was discharged to home, where he continued treatment with cefuroxime axetil and had an uneventful recovery.

**Patient 2.** A 30-month-old African American boy presented to the same hospital 4 days after patient 1 with a 5-day history of fever and lip ulcers and a 3-day history of decreased activity and neck motion. He had no significant medical history, but he had not been immunized. He was alert and irritable, but consolable. His temperature was 38.7°C. He had photophobia and neck rigidity. There was severely decreased range of motion and tenderness of the right hip. His peripheral WBC count was 10,100 cells/μL, with 78% neutrophils; his C-reactive protein level was 23.4 mg/dL. CSF analysis revealed a WBC count of 283 cells/μL, with 77% neutrophils, a glucose level of 49 mg/dL, and a protein level of 57 mg/dL. Arthrocentesis of the right hip revealed a WBC count of 60,875 cells/μL with a predominance of neutrophils. The Gram stain of his CSF revealed many gram-negative coccobacilli, and the cultures of blood, CSF, and synovial fluid grew encapsulated *H. influenzae* that was β-lactamase–negative and was identified as serotype a. The child was treated with cefotaxime. Despite an uneventful initial recovery, he was later noted to have significant cognitive and motor developmental deficits and profound bilateral sensorineural hearing loss.

An epidemiologic investigation of contacts within both families was undertaken to identify any common source of possible infection, such as a day care facility, playground, or other location, but no link was found.

**METHODS**

**Bacterial strains and serotyping.** *H. influenzae* type a strains GA41512 and GA41513 were isolated from patients 1 and 2, respectively, and collected as part of the Active Bacterial Core surveillance of the Centers for Disease Control and Prevention–funded Georgia Emerging Infections Program. Additional Hia strains GA04774, GA11151, and GA18491 were part of the Georgia Active Bacterial Core surveillance’s collection, and Hia strain ATCC 9006 was obtained from the American Type Culture Collection (Manassas, VA). Hib strain 1007 [10] was included in the analysis for comparison. Bacteria were grown on chocolate II solid media or in brain-heart infusion broth, supplemented with 10 μg/mL hemin and 2 μg/mL nicotinamide adenine dinucleotide (Becton Dickinson Microbiology Systems). Standard slide agglutination capsule serological testing was performed as described by the manufacturer of the antiserum (Bacto-DiFCO Diagnostic Systems) and PCR molecular capsule typing was performed as described by Falla et al. [11].

**Southern blot hybridization.** Capsular genotyping of Hia strains was performed with Southern blot hybridization using the cap probe pUO38 [12, 13]. *H. influenzae* chromosomal DNA was isolated by the method described by Moxon et al. [14]. Ten micromgs of chromosomal DNA were digested with restriction enzyme EcoRI (New England Biolabs) overnight at 37°C, separated in 0.7% agarose (Bio-Rad) for 20 h at 430 V, then loaded on a 1% SeaKem (FMC Bioproducts) gel with a pulse time of 1–25 s.

**Identification and characterization of the IS1016-bexA partial deletion.** The area of the IS1016-bexA partial deletion was amplified from genomic DNA with the primers I5lout and bexB [15]. Amplicons were subcloned into pCR4-TOPO (Invitrogen) and sequenced by Lark Technologies.

**Pulsed-field gel electrophoresis (PFGE).** Cells were lysed with proteinase K and underwent PFGE. Lysis was halted with phenylmethanesulfonyl fluoride. Agarose plugs were digested at 25°C with *Smal* for 18 h, then loaded on a 1% SeaKem agarose gel (Cambrex) in 0.5% Tris-borate-EDTA. Electrophoresis was performed with a Chef mapper (Bio-Rad Laboratories) for 20 h at 4°C on a ramp at a 120° angle with a ramped pulse time of 1–25 s.
RESULTS

Slide agglutination serotyping of strains GA41512 and GA41513, performed in the hospital laboratory and again in our laboratory, detected serotype a. Molecular capsule typing with cap a– and bexA-specific primers also confirmed that both clinical isolates were serotype a. Next, samples of genomic DNA from both isolates were amplified using PCR with paired primers that corresponded to sequences in IS1016 and bexB of the capsule gene cluster. The target sequence of this PCR method contains a portion of bexB, all of bexA, and a portion of IS1016. The expected result for H. influenzae strains with a partially duplicated cap locus, such as that found in most Hib strains belonging to phylogenetic Division I, is a 1.5 kilobase (kb) amplification product containing an intact bexA gene and a 300-bp amplification product containing a 1.2-kb deletion in bexA and IS1016 (figure 1). The 1.5-kb PCR product, which includes an intact bexA gene, and the 300-bp IS1016–bexA deletion product were present in both Hia isolates from the patients we describe and in an Hib strain (1007), but not in 2 other Hia strains belonging to the a(T) genotype that were included for comparison (figure 1). Sequencing of the PCR products confirmed the presence of an IS1016–bexA deletion that was identical in size and location to that seen in Hib. However, the nucleotide sequence near the site of the IS1016–bexA deletion (GenBank accession number DQ086152) contained 4-bp differences from the Hib sequence that had been previously reported in several invasive serotype a strains from The Gambia, West Africa, and in a nasopharyngeal isolate from Kenya, East Africa [7]. The 4-bp changes were not noted in a cluster of invasive Hia isolates from Utah that had an otherwise identical IS1016–bexA deletion [6].

Southern blot hybridization of chromosomal DNA restriction fragment–length polymorphisms with a pUO38 probe showed the capsular genotype to be a(N) for Hia strains GA41512 and GA41513 (figure 2B, lanes 3 and 4). Note the reduction in size of the 6.8-kb EcoRI fragment found in the more common a(T) capsule genotype (figure 2A, top, and figure 2B, lane 2) to a 5.6-kb fragment in the a(N) genotype, which results from the partial IS1016–bexA deletion (figure 2A, asterisk, and figure 2B, lanes 3 and 4). The a(N) genotype has been reported previously in Hia strains that contain the partial deletion [6,7]. Additional Southern blot hybridization with an IS1016 probe confirmed the association of IS1016 with the cap locus in strains GA41512 and GA41513, as well as the presence of an IS1016–bexA deletion (data not shown).

GA41512 and GA41513 had indistinguishable SmaI-restriction patterns on PFGE, and they differed significantly from Hib, the control Hia strain ATCC 9006, and other clinical Hia isolates (figure 2C). The PFGE patterns for strains GA41512 and GA41513 also differed somewhat from the Hia strains isolated in a study in Utah and described by Adderson et al. [6]. PFGE was not performed on the invasive Hia strains from The Gambia and from Kenya [7]. However, the Hia isolates reported from the 2 locations in Africa were from distinct clones, despite having identical capsular genotypes and DNA sequences near the site of the IS1016–bexA deletion.

DISCUSSION

We have described 2 cases of serious invasive infection with H. influenzae serotype a in young children who presented with infections reminiscent of invasive infection with Hib. The isolates were found to be identical according to PFGE typing, despite the absence of an epidemiologic link, and they shared several important characteristics of the Hib capsule locus organization.

Epidemiology. Invasive disease due to encapsulated H. influenzae in US children has declined dramatically since the licensure of the Hib conjugate vaccine in 1987 for use in children aged >18 months, and later, in 1990, for use in infants aged ≥2 months [2,3]. Owing to the decreased prevalence of nasopharyngeal carriage associated with the use of the conjugate vaccine and the resulting decreased overall risk of exposure to Hib, the incidence of invasive disease in older children and, fortunately, in unimmunized infants and adults, has decreased as well. Sporadic reports of invasive disease due to non-serotype b encapsulated isolates has sparked concern by many clinicians that serotype replacement may be occurring [6,7,16–18].

The Active Bacterial Core surveillance program collected and analyzed microbiologic data from 1998 to 2002 from 9 participating sites of population-based surveillance across the United States with an approximate population of 35 million. Cases of H. influenzae isolated from a normally sterile body site in pa-
Figure 2. A, Chromosomal organization of cap loci from Haemophilus influenzae serotype a strains with a(T) and a(N) genotypes. Each locus contains directly repeated duplications of cap genes from regions I, II, and III and is flanked by copies of IS1016 elements (black boxes). Regions I and III are shared among all encapsulated Hia strains. Region II contains serotype-specific genes. The Hia a(T) genotype contains 2 complete copies of the cap genes. The Hia a(N) genotype contains a partially duplicated cap locus with a 1.2-kilobase (kb) deletion between IS1016 and bexA in region I. Top line, EcoRI restriction map with fragment sizes indicated in kb. *Reduction of the size of the 6.8-kb EcoRI fragment to 5.6 kb because of the IS1016-bexA deletion in the a(N) strains. B, Southern blot hybridization of EcoRI-digested DNA using pUO38 as a probe from Hia strains GA41512 (lane 3), GA41513 (lane 4), the a(T) capsular genotype GA18491 (lane 2), as well as a Digoxigenin-labeled DNA molecular weight marker II (Roche Diagnostics) (lane 1). Molecular size markers are noted at the left, and capsular genotype band sizes are noted on the right. *Reduction of the size of 6.8-kb EcoRI fragment to 5.6 kb because of the IS1016-bexA deletion in the a(N) strains (lane 3 and lane 4). C, PFGE of SmaI-digested chromosomal DNA including S. aureus as a size marker (lane 1 and lane 10), Hib strain 1007 (lane 2), and Hia strains 9006 (lane 3), GA41512 (lane 4 and lane 5), GA41513 (lane 6), GA04774 (lane 7), GA11151 (lane 8) and GA18491 (lane 9). The gel was stained with ethidium bromide and photographed under ultraviolet light.

tients of all ages were included [5, 19]. Nearly 70% of 1743 invasive isolates were nontypable H. influenzae; 18% were serotype f, 5.5% were serotype b, 5.2% were serotype e, and 1% were serotype a. However, almost 40% cases of invasive Hia disease occurred in children aged <2 years, whereas <20% of cases of disease were due to other capsule serotypes. In the Active Bacterial Core surveillance’s program in Georgia, no other cases of invasive Hia disease in any age group had been detected in the previous 6 years of continuous active surveillance (M.M.F., unpublished data).

In some populations, such as Native Americans, in whom the incidence of disease due to H. influenzae is very high, the
peak Hib disease incidence occurs in a younger age group (<6 months old) and may likely be related to earlier or more abundant exposure to the pathogen within the specific community [1]. A recent population-based study by Millar et al. [20] that characterized invasive disease due to *H. influenzae* within the Navajo and White Mountain Apache populations reported an overall annual incidence of Hia as high as 20.2 cases per 100,000 population among children <5 years of age. The authors looked for evidence of serotype replacement and found 76 incident Hia cases in children <5 years of age during the period from 1988 to 2003, which indicated that Hia was the most frequent non–serotype b isolate causing invasive disease in that cohort (it accounted for 34% of all cases of invasive *H. influenzae* disease). Although the overall rate of Hia disease was quite high in this population, no increase in the rate of Hia disease was noted in the post–conjugate vaccine era. This contrasts with data from a Brazilian cohort in which there was a small increase in the incidence of Hia meningitis in children aged <2 years after the introduction of the conjugate vaccine in 1999 [21].

Among adults with invasive disease due to encapsulated *H. influenzae*, serotype f is now the most common capsule type, and disease incidence appears to have increased modestly in adults since the introduction of the conjugate Hib vaccine for infants in the United States [5, 22]. No such increase in the incidence of disease due to serotype f was noted in children old enough to receive the vaccine. Among Alaskan residents aged ≥10 years, the incidence of invasive disease due to non–b isolates (serotypes a, c, e, f, and nontypable isolates) increased from 0.5 to 1.1 per 100,000 population per year (*P* = .01) after the introduction of the Hib conjugate vaccine for infants [18]. Although no significant increase in the incidence of invasive non–serotype b *H. influenzae* disease was noted among Alaskan children <10 years old, a recent report of a cluster of 5 cases of Hia disease in Alaska Native infants from a remote region of Alaska raises new concerns [9]. Because of the small increases in rates of non–serotype b encapsulated *H. influenzae* disease noted in some populations and recent reports of case clusters of Hia disease in infants and young children, close monitoring of serious *H. influenzae* disease and accurate tracking of the capsular serotype distribution is warranted.

**Pathogenesis.** The dominance of Hib as a cause of serious pediatric *H. influenzae* disease is most often attributed to unique characteristics of the serotype b capsule [1, 23]. Cases of serious invasive Hia disease in infants and young toddlers with clinical presentations that are similar to those previously seen with Hib disease may suggest increased susceptibility of the host, a shift in the virulence of Hia, and/or increased exposure of infants and toddlers to Hia strains.

**Immune defects.** As has been documented by others, immunodeficiencies may play a significant role in the pathogenesis of invasive disease due to Hia, as well as other non-b capsular serotypes, in adult and pediatric populations [1, 24, 25]. Additionally, the pediatric age group historically at highest risk for invasive *H. influenzae* disease is one in which there is well-documented immaturity of the B cell immune response to polysaccharide antigens [1]. On the basis of our 2 cases of Hia infection and a review of published reports of invasive Hia disease, it remains unclear whether there are other underlying diseases that may predispose people to Hia infection.

**Increased virulence and molecular genetic analysis.** Increased virulence of the organism may also play a central role in invasive Hia disease pathogenesis, as suggested by several studies that have compared the virulence factors of Hia and Hib [6–8]. Among encapsulated *H. influenzae* serotypes, Hib and Hia have the most closely related capsules; each contains ribitol 5-phosphate and possesses near-complete identity with 1 of 4 genes in the capsule-specific Region II of the capsule gene cluster [26, 27]. Hia strains usually contain 2 intact tandem repeats of the cap locus [15]. However, several investigators have now documented the presence of the duplicated cap locus with a partial deletion of both bexA and the adjacent IS1016 in 1 of the copies of the capsule genes among a number of genetically diverse Hia isolates, an organization previously seen only in the Hib cap locus [6–8]. This finding has been noted most often in Hia strains with the a(N) capsule genotype, as in the strains reported here. Kroll et al. [7] have proposed that the finding represents an infrequent recombination event between naturally transformable Hib and Hia strains that may enhance the fitness and virulence of Hia strains. The IS1016-bexA partial deletion has also been identified in a small number of invasive isolates of *H. influenzae* serotypes e and f [8].

The 2 isolates associated with serious invasive *H. influenzae* disease reported here possessed the duplicated cap locus with a partial deletion of 1 copy of the IS1016-bexA segment, typical of Hib. The DNA sequence near the site of the deletion was identical to that of previous Hia isolates from Africa [7] and slightly different from that of isolates from a cluster of invasive Hia infections in Utah [6]. Furthermore, PFGE patterns differed between our isolates and those from Utah, and the isolates from Africa also showed genetic diversity, suggesting that the capsule gene deletions had arisen from independent recombination events rather than through the dissemination of a single Hia clone.

**Potential for Hia exposure.** In a highly immunized population with a reduced prevalence of nasopharyngeal carriage of Hib, the risk of exposure to Hib has declined, which has provided significant herd immunity. However, the effect of the Hib vaccine on the risk of Hia exposure is unknown. Because of the recent experience of replacement of pneumococcal vaccine serotypes with non-vaccine pneumococcal serotypes in the nasopharynxes of infants after the introduction of a 7-valent pneumococcal vaccine, one might speculate that the prevalence...
of carriage of non-serotype b *H. influenzae* isolates might increase as these serotypes fill the Hib ecological niche. Unfortunately, most carriage studies done after the introduction of the Hib conjugate vaccine reported only on its effects on Hib carriage and not its effects on the carriage of non-b capsular serotypes [28]. In a recently reported cluster of Hib cases among Alaskan Native infants, Hib carriage was identified in 1 (6%) of 17 close contacts of 1 patient and in 4 (29%) of 14 close contacts of another patient with recurrent invasive Hia infections [9]. However, these carriage rates occurred in a population with increased risk of *H. influenzae* infections and among close contacts of infants with recurrent Hia disease, and therefore may not be representative of the background rate of Hib carriage in the United States.

**CONCLUSION**

The introduction of a Hib conjugate vaccine for use in infants in the United States resulted in dramatic declines in the incidence of invasive *H. influenzae* serotype b disease in infants but has not resulted in substantial replacement of Hib by other capsule serotypes, to date. Unique features of the Hib capsule likely contributed to its predominance as a pediatric pathogen. The association of serious, invasive Hib disease with isolation of Hia strains with a duplicated cap locus and IS1016-bexA partial deletion that is typical of the Hib capsule locus raises concern about the potential for increased virulence of such Hia strains. The cases of invasive Hia disease reported here and other recently reported case clusters of Hia disease emphasize the need for continued close monitoring of invasive *H. influenzae* disease in infants and young children and accurate tracking of capsular serotype distribution.

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