# Invasive Serotype a *Haemophilus influenzae* Infections With a Virulence Genotype Resembling *Haemophilus influenzae* Type b: Emerging Pathogen in the Vaccine Era?

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**ABSTRACT.** *Objective.* ***Haemophilus influenzae* type b causes severe disease in nonimmune infants and young children; other serotypes are uncommon pathogens and thought to have low virulence. Some have hypothesized that with the virtual elimination of *H influenzae* type b, other serotypes might acquire virulence traits and emerge as important pathogens of children. We describe the clinical, epidemiologic, and molecular biologic features of 5 cases of severe disease attributable to *Haemophilus influenzae* type a.**

*Methods.* **After observing 4 cases of invasive disease caused by *H influenzae* type a, we reviewed microbiology records at 3 reference laboratories that perform all serotyping in Utah and surveillance databases. Strains of *H influenzae* type a and control strains were examined by Southern blotting with the use of the *cap* probe pUO38 and by pulsed-field gel electrophoresis. The putative virulence mutation, the IS*1016-bexA* deletion, was detected by polymerase chain reaction amplification and sequencing.**

*Results.* **During a 10-month period, we observed 5 children with severe invasive disease caused by *H influenzae* type a. No isolates of *H influenzae* type a had been submitted to the reference laboratories between 1992 and 1998. The median age of patients was 12 months (range: 6–48 months). Four of 5 had meningitis and bacteremia; 1 had purpura fulminans. Three isolates, representing 1 of 2 pulsed-field gel electrophoresis patterns, contained the IS*1016-bexA* deletion and were associated with particularly severe disease.**

*Conclusions.* **We describe an unusual cluster of severe disease caused by *H influenzae* type a that resembles the clinical and epidemiologic features of *H influenzae* type b disease. Our data support the hypothesis that the IS*1016-bexA* deletion may identify more virulent strains of *H influenzae*. *Pediatrics* 2001;108(1). URL: http://www.pediatrics.org/cgi/content/full/108/1/e18; *Haemophilus influenzae, epidemiology, virulence, serotyping, pathogenicity.***

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ABBREVIATIONS. Hib, Haemophilus influenzae serotype b; CSF, cerebrospinal fluid; PCMC, Primary Children's Medical Center; PFGE, pulsed-field gel electrophoresis.

In the 1930s, Pittman[1] described 6 serotypes of encapsulated *Haemophilus influenzae* (a–f), each with an antigenically distinct polysaccharide capsule. *H influenzae* serotype b (Hib) is highly virulent for infants and young children; non-b serotypes are rare and thought to have low virulence. Immunization with capsular polysaccharide-protein conjugate vaccines has almost eliminated invasive Hib disease in the United States and other developed countries where the vaccines are used extensively.[2,3] Some have speculated that other serotypes might acquire additional virulence traits and emerge as important pathogens.[4,5] However, surveillance in the United States, Great Britain, and Switzerland until now has not shown significant increases in infections of children with other serotypes.[5–7]

We describe 5 cases of invasive disease, including 4 cases of meningitis in young children attributable to *H influenzae* serotype a in Utah during a 10-month period. Three cases were attributable to a unique strain that possesses a deletion mutation associated with invasive strains of Hib but not normally found in noninvasive strains. Two of these cases, described below, were strikingly reminiscent of severe disease caused by Hib.

## CASE REPORTS

### Patient 1

A previously healthy 6-month-old white female infant was brought to her primary care physician on December 11, 1998, with a 1-day history of lethargy, irritability, and poor oral intake and a brief history of altered consciousness and peripheral cyanosis. She had received 3 doses of Hib-conjugate vaccine.

On admission, the patient was lethargic with poor peripheral perfusion. Blood pressure was 40/20 mm Hg, pulse was 210 beats/min, and tympanic temperature was 39.4°C. Purpura were present on her nose, ears, and legs, and petechiae was present on her face and trunk. The anterior fontanelle was soft, and meningismus could not be elicited.

She required intubation and mechanical ventilation and fluid and inotropic support. Empiric therapy with intravenous cefotaxime, vancomycin, and gentamicin was begun. Initial laboratory studies included a white blood cell count of 4900/mm$^3$, hematocrit of 27.5%, and a platelet count of 35 000/mm$^3$. There was evidence of disseminated intravascular coagulation with prolonged prothrombin time and partial thromboplastin time, elevated fibrin split products, and positive d-Dimer. A sample of cerebrospinal

fluid (CSF) had a white blood cell count of 738 cells/mm$^3$, 81 red blood cells/mm$^3$, protein of 243 mg/dL, and glucose of <20 mg/dL. Gram stain showed pleomorphic Gram-negative rods. Cultures of the CSF and blood grew mucoid colonies of *H influenzae* serotype a. Antimicrobial therapy was continued with ceftriaxone.

The patient had a prolonged hospital course, complicated by renal failure that required peritoneal dialysis, purpura fulminans (Fig 1), a large subdural empyema that required drainage, and persistent fever. Soft-tissue necrosis ultimately required amputation of 2 digits of her right foot and extensive debridement and skin grafting of her lower extremities. Serum quantitative immunoglobulins and total hemolytic complement levels were normal.

### Patient 2

A previously well 1-year-old white female was admitted to a referring hospital on June 31, 1999, with a 3-day history of vomiting, fever to 38.9°C, irritability, and diarrhea, followed by a generalized seizure. She had received 3 doses of Hib-CRM$_{197}$ conjugate vaccine.

On admission, she had a temperature of 39.9°C. She was toxic appearing and minimally responsive. Lumbar puncture revealed cloudy CSF with 1660 white blood cells/mm$^3$, 70 red blood cells/mm$^3$, glucose of 34 mg/dL, and protein of 300 mg/mL. Gram stain demonstrated abundant pleomorphic Gram-negative rods. Cultures of blood and CSF grew *H influenzae* serotype a. Cefotaxime and vancomycin were administered. She was transferred to Primary Children's Medical Center (PCMC) on the second hospital day because of continued fever and altered mental status.

Her hospitalization was complicated by aseptic necrosis of the right femoral head and by prolonged fever. Bilateral frontoparietal subdural fluid collections were drained on the 12th hospital day, yielding 50 mL of purulent fluid. Cultures of the empyema fluid were sterile. She was treated for 4 weeks with cefotaxime with gradual improvement. On discharge, she had evidence of decreased hearing by evoked otoacoustic emission and regression of fine and gross motor skills. Immunologic evaluation—including quantitative immunoglobulins, response to diphtheria and tetanus vaccine antigens, quantification of T- and B-cell subsets, and total hemolytic and terminal complement levels—was normal.

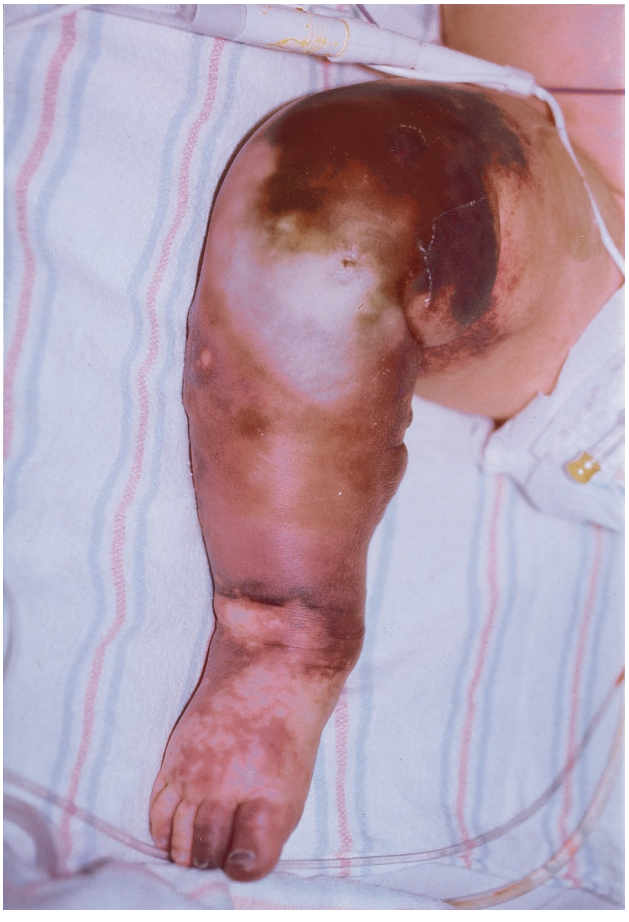

**Fig 1.** Purpura fulminans of patient 1 early in the course of *H influenzae* type a infection. Tissue necrosis led to the amputation of 2 toes, extensive debridement, and skin grafting.

## METHODS

### Surveillance

We reviewed surveillance data from the Utah Department of Health from 1991 to 1999. We reviewed laboratory records at the 3 reference laboratories that perform virtually all reference microbiology services for Utah and the region, PCMC, Associated Regional and University Pathologists Inc, and the Utah Department of Health state laboratory. Strains of *H influenzae* isolated from sterile body sites at PCMC or University Hospital or strains from sterile sites referred to the microbiology laboratories at PCMC or Associated Regional and University Pathologists Inc were screened with commercial polyvalent (types a, c–f) and monovalent type b antisera for *H influenzae* type b capsule. All non-type b encapsulated strains were sent to the Utah Department of Health microbiology laboratory where definitive serotyping was performed with monovalent antisera for *H influenzae* types a through f.

### Bacterial Strains

*H influenzae* type a strains 1 through 5 were isolated from the patients. *H influenzae* type b strain 11201 was supplied by Dr. Judy Daly (PCMC, Salt Lake City, UT). *H influenzae* type a strain ATCC 9006 was obtained from the American Type Culture Collection (Manassas, VA). 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, Cockeysville, MD).

### Capsular Typing of *Haemophilus* Strains

Genotyping of *Haemophilus* strains was performed by Southern blot analysis using the *cap* probe pUO38 (provided by Dr. S. Kroll, Imperial College of Science, Technology, and Medicine, London, UK).[8] Genomic DNA was prepared by proteinase K digestion, digested with *Eco*RI endonuclease, and subjected to agarose gel electrophoresis on a 0.8% Tris-acetate gel.[9] Restriction fragments were transferred to nylon membranes (Hybond-N+; Amersham Life Science Limited, Arlington Heights, IL) and cross-linked by exposure to ultraviolet light. Membranes were prehybridized, then hybridized with fluorescein-dUTP-labeled pUO38 (Gene Images labeling and detection systems; Amersham). After washing at high stringency, blots were incubated with detection reagent and exposed to radiographic film.

### Amplification of IS1016-bexA Deletion

The IS*1016-bexA* deletion was amplified from genomic DNA with the use of a sense IS*1016* (5′ATTAGCAAGTATGCTAGTCTAT 3′) and antisense *bexA* (5′ CAATGATTCGCGTAAATAATGT 3′) primers.[10] Amplification reactions were performed in a 75-μL reaction mixture consisting of 300 ng of genomic DNA, 1.5 mM $MgCl_2$, 2 mM deoxynucleotides, 50 pmol of each primer, and 4 units of high-fidelity Bio-X-Act DNA polymerase (ISC Bioexpress, Kaysville, UT), in the manufacturer's buffer. Reaction conditions consisted of denaturation at 95°C for 1 minute, annealing at 42°C for 1.5 minutes, and extension at 72 ° for 2 minutes. Thirty-five rounds of amplification were performed. The predicted 362-bp amplification products were purified from agarose gels or cloned directly into pBSKII phagemid vectors (Stratagene, La Jolla CA). The cloned amplification products were sequenced with the use of an ABI2000 automated sequencer (Perkin-Elmer Corporation, Norwalk, CT). Products from a minimum of 2 independent preparations of genomic DNA and amplification reactions were sequenced.

### Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed with the use of the CDC/PulseNet protocol for Gram-positive cocci. Cells were lysed with a combination of proteinase K and lysozyme. The plugs were digested at 25°C with *Sma*I for 4 hours.

The gel was made of Seakem Gold Agarose (1%) and electrophoresed on a CHEF mapper (Bio-Rad Laboratories, Richmond, CA) for 18.5 hours using 120-degree angle ramp, ranging from 2.16 seconds to 54.17 seconds, at 6V/cm. The gel was stained with ethidium bromide and photographed under ultraviolet light. The PFGE patterns were compared with the use of Molecular Analyst Fingerprinting Plus software (Bio-Rad) by the Dice coefficient and unweighted pair group method.

## RESULTS

No cases of invasive disease caused by *H influenzae* type a were reported in Utah between January 1991 and November 1998. Four cases initially were identified during a 10-month period between December 1998 and October 1999. Clinical and epidemiologic characteristics are summarized in Table 1. The children ranged in age from 6 to 13 months. All had bacteremia and meningitis. Three of 4 had complications similar to those seen with invasive Hib disease, including prolonged fever, subdural empyema, and aseptic necrosis of the hip. All 4 had normal total hemolytic complement activity and normal quantitative immunoglobulins. There was no historical or hematologic evidence to suggest hyposplenism, a risk factor for severe disease with *H influenzae*.

A review of laboratory records revealed a single additional case of *H influenzae* type a disease, also summarized in Table 1. A 4-year-old boy, the youngest of 11 siblings, was admitted to the Burn Trauma Unit at University Hospital on September 18, 1999, with a flash burn to the face. Chest radiograph and oropharyngeal examination were normal. He was intubated because of concern over facial swelling. On the day after admission, he developed a fever to 38.5°C, and new lower lobe infiltrates were noted on chest radiographs. Gram-stained secretions from the endotracheal tube showed numerous polymorphonuclear cells, no squamous cells, and numerous Gram-negative coccobacillary organisms. The specimen grew *H influenzae* type a in pure culture.

Two of the infants with meningitis (patients 1 and 2) resided in the same county (Utah County) but in different towns. They attended separate child care centers. Patients 3 and 4 resided in Salt Lake County. Patient 3 lived in a polygamous family group with 10 other children who were younger than 10 years in 2 related households but did not attend child care. Three of 4 had been vaccinated with 3 doses of Hib-conjugate vaccines.

Hybridization of genomic DNA with pUO38 showed the genotype to be consistent with the serotype for the *H influenzae* type b and the type a isolates (Fig 2). Serotype a strains from patients 1, 2, and 5 exhibited the previously described a(N) Cap genotype.[10] In this Cap restriction fragment polymorphism, there is a 1.2-kB reduction in the size of 1 of the probe-positive *Eco*RI fragments, suggesting that these strains have the IS*1016-bexA* deletion described in both invasive type a and type b strains.[10,11]

To confirm the presence of the IS*1016-bexA* deletion, genomic DNA was amplified with primers corresponding to IS*1016* and *bexA*. A 362-bp fragment was amplified from genomic DNA of each *Haemophilus* strain. Sequencing of these amplification products confirmed the previously described IS*1016-bexA*

**TABLE 1.** Clinical and Epidemiologic Characteristics

| Patient Number | PFGE Pattern | Age/Gender | Onset | Site(s) | County | Vaccines | Household Size | Risk Factors | Complications | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | I | 6 mo/F | 12/21/98 | CSF, blood | Utah | Hib × 3 | 8 | Child care | Renal failure, purpura fulminans, brain abcess, subdural empyema | Hearing loss, developmental delay |
| 2 | I | 12 mo/F | 06/29/99 | CSF, blood | Utah | Hib × 3 | 4 | Child care | Aseptic necrosis of hip, subdural empyema, prolonged fever | Hearing loss, loss of developmental milestones |
| 3 | II | 7 mo/F | 10/4/99 | CSF, blood | Salt Lake | None | 14 | Crowding | Subdural effusion, prolonged fever | Recovered |
| 4 | II | 13 mo/M | 10/23/99 | CSF, blood | Salt Lake | Hib × 3 | 4 | None | | Recovered |
| 5 | I | 4 y/M | 09/18/99 | Sputum | Washington | Unknown | 13 | Crowding | None | Recovered |

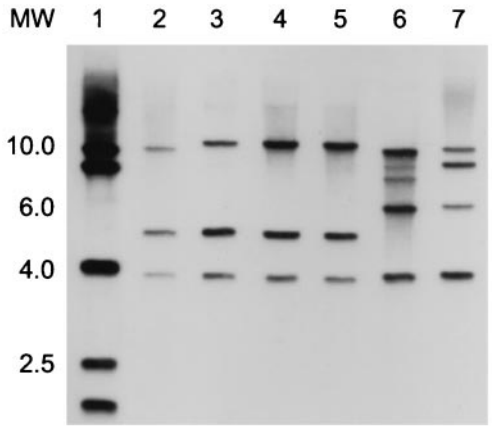


**Fig 2.** Genotyping of *Haemophilus* strains. Autoradiograph of Southern hybridization of *Eco*RI-digested DNA from type b strain Hib 11201 (lane 1) and type a strains ATCC 9006 (lane 2), Pt 1 (lane 3), Pt 2 (lane 4), Pt 5 (lane 5), Pt 3 (lane 6), and Pt 4 (lane 7) hybridized with photometrically labeled pUO38. Molecular size markers are noted at left. Strains 1, 2, and 5 have the previously described a(N) capsular genotype; strains 3 and 4 have the more common a(T) genotype.

deletion (Fig 3).[10] In contrast to the nucleotide sequence of previously reported invasive type a strains, the sequence of this region of the genome is identical to that of Hib strains 7004 and 11201. Previously reported invasive type a strains from Gambia had 3 to 4 base differences from the 7004 sequence that are shared by family B type b strains.[10]

Three strains (patients 1, 2, and 5) had indistinguishable digest patterns (pattern I) on PFGE (Fig 4). They differed significantly from Hib and from a control strain of *H influenzae* type a (ATCC strain 9006; not shown). Strains from patients 3 and 4 were different from pattern I but differed from one another by 3 bands, indicating that they were closely related.[12] Of interest, PFGE patterns from 2 isolates obtained from patient 3, 1 from blood and 1 from CSF, differed by 2 bands.

## DISCUSSION

Serotype a *H influenzae* is a rare cause of invasive disease.[13] In 1 large population-based study, serotype b accounted for 383 of 408 cases (94%), nontypable strains caused an additional 24 cases, and no disease caused by serotype a was noted.[14] In another population-based study in 1986, 1324 of 1872 isolates (71%) from invasive disease were type b; 14 of 1872 (0.7%) were type a.[6] Invasive disease caused by nonserotype b encapsulated organisms occurred almost exclusively in persons older than 5 years. Thus, the clustering of 4 cases of invasive disease and 1 of pneumonia caused by *H influenzae* serotype a during a 10-month period is unexpected on the basis of active and passive surveillance from a variety of sites.

In contrast to recently described *H influenzae* type f infections,[15] the illness that we observed to be caused by *H influenzae* type a is strikingly reminiscent of invasive disease caused by Hib, resulting in severe disease in seemingly normal young children who are between 6 months and 2 years of age. Because anti-*Haemophilus* antibody elicited by the current vaccines is directed against capsular polysaccharide, these vaccines would not protect against serotype a disease. Thus, the possibility of the emergence of a new, virulent strain of *H influenzae* with the virulence characteristics of Hib are of concern.

Two distinct strains seem to be circulating in Utah. The 3 isolates with PFGE pattern I (patients 1, 2, and 5) seem to represent a unique clone with similar PFGE patterns and identical nucleotide sequences in the IS*1016-bexA* deletion region. The meningitis and sepsis caused by this organism was striking in its severity. The isolates from patients 3 and 4 seem to be related closely by the criteria proposed by Tenover et al.[12] They lack the IS*1016-bexA* deletion.

The small number of cases precluded a case-control study to confirm risk factors, but the apparent risk factors resemble those for invasive Hib disease: young age and exposure to other children in child care settings or crowded households.

Most virulent encapsulated *Haemophilus*, including *H influenzae* type a, belong to a single large electrophoretic type, division I.[16] In most division I isolates of Hib, the genes that are responsible for capsule synthesis and secretion, the Cap locus, are partially duplicated, with each segment flanked by the insertion element IS*1016*.[17] The majority of virulent Hib strains have an 1198-bp terminal deletion in 1 duplicated segment that removes a portion of IS*1016* and *bexA*. This deletion promotes gene amplification, resulting in a dramatic increase in capsule production that is likely to contribute to the virulence of these strains.[11] Kroll et al[10] described a similar mutation in 3 serotype a isolates from an outbreak in Gambia and a Kenyan isolate and postulated that this polymorphism may have resulted from genetic transfer from a serotype b strain. *Haemophilus* species are naturally transformable, and the Cap locus is flanked by transposable elements, features that would facilitate such genetic exchange. Although IS*1016-bexA* deletion is not requisite for invasive disease, our data support the hypothesis that the IS*1016-bexA* deletion is a marker for recombinant events and may identify unusually virulent serotype a strains. The *bexA* sequences of our isolates differ from those reported previously, suggesting a novel genetic event rather than the global spread of the originally described strains.

How the IS*1016-bexA* mutation may contribute to virulence of serotype a strains is not certain. Increased capsule may limit opsonophagocytic killing, but in serotype b strains, noncapsular factors also are critical.[18] It is possible that the IS*1016-bexA* deletion may be linked to other genes that are important in colonization, invasion or other virulence genes. Additional dissection of the virulence mechanisms of invasive *Haemophilus* is needed.

## CONCLUSION

We observed an unusual increase in invasive disease caused by *H influenzae* type a; 2 distinct clones seem to have been circulating. Whether either clone has the potential to spread and become established remains to be seen. The ability of a strain to emerge

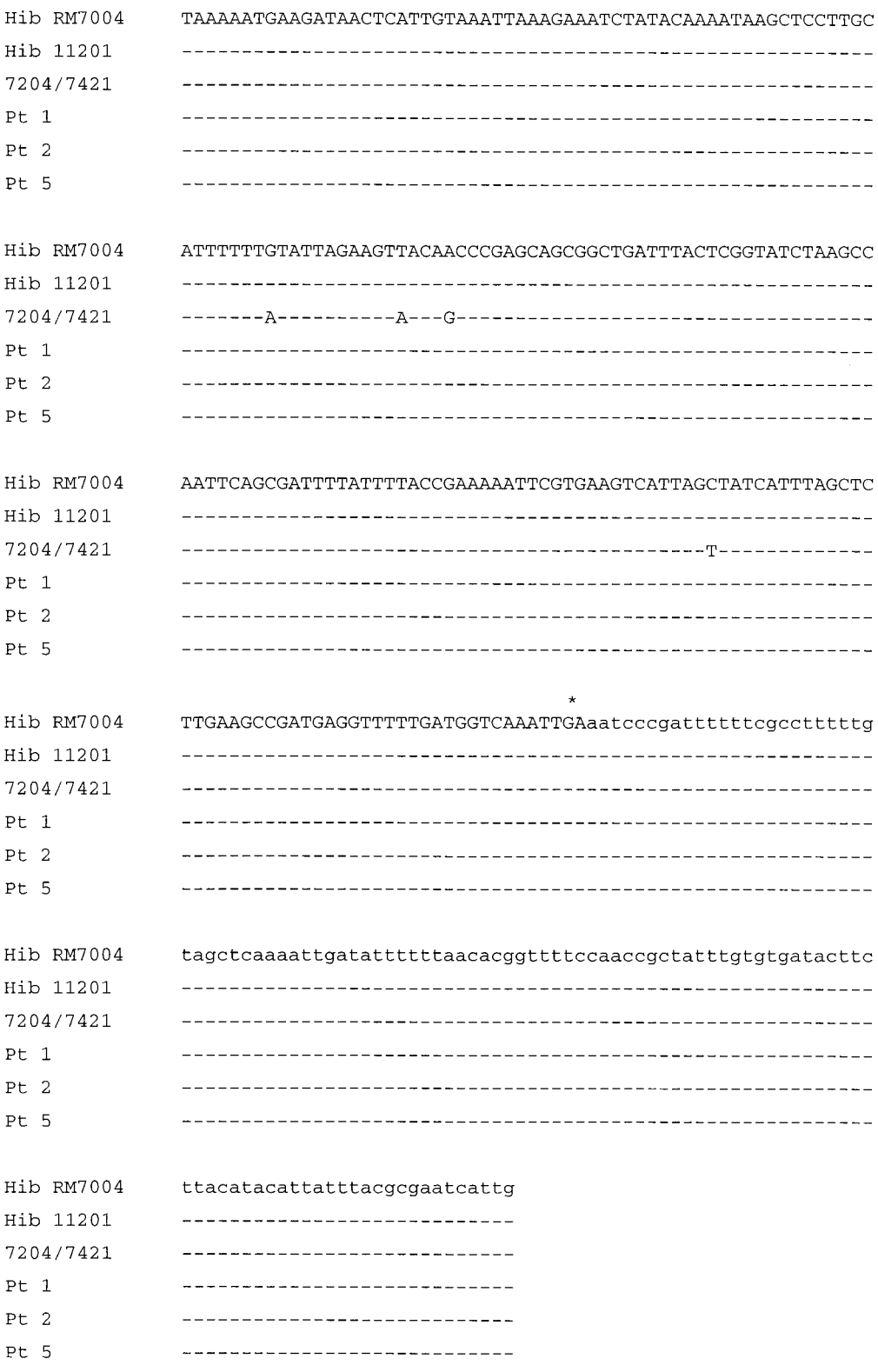


**Fig 3.** Nucleotide sequences of the IS*1016-bexA* deletion region of type b strain Hib 11201 and type a strains 1, 2, and 5. Sequences are compared with those of the previously reported type b strain RM7004 and type a 7204 and 7421 strains.[3] Sequence derived from IS*1016* is in capitals, sequence derived from *bexA* is in lower case, and an asterisk indicates the fusion site.

as an important pathogen may depend not only on virulence but also on the ability to establish colonization and spread among susceptible children. Active surveillance and seroepidemiologic studies are being initiated in Utah.

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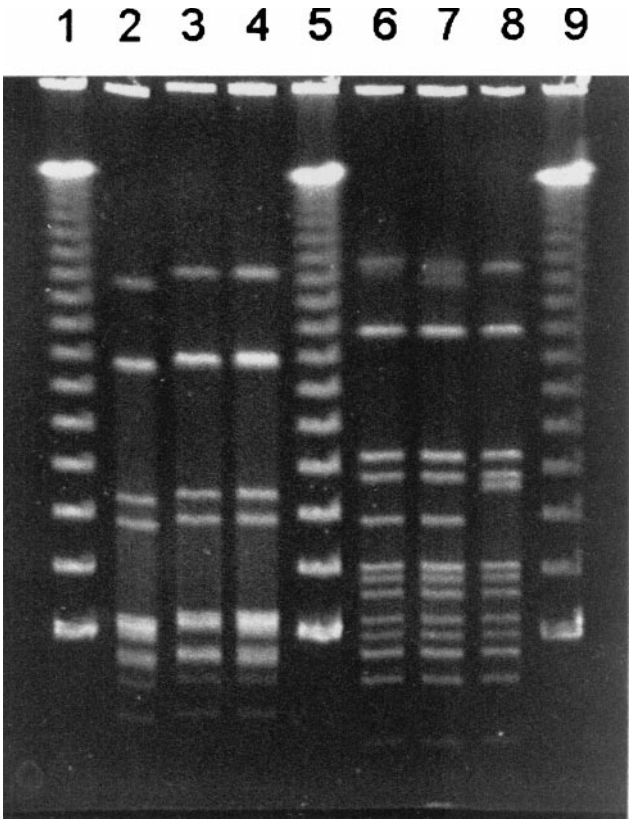


**Fig 4.** PFGE of chromosomal DNA. Lane 1 contains 47.5-kb λ marker; lane 2, *H influenzae* type a Pt 5; lane 3, *H influenzae* type a Pt 2; lane 4, *H influenzae* type a Pt 1; lane 5, λ marker; lane 6 *H influenzae* type a Pt 3 (CSF isolate); lane 7, *H influenzae* type a Pt 3 (blood isolate); lane 8, *H influenzae* type a Pt 4; lane 9, 47.5-kb λ marker.

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