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Invasive Disease Due to Nontypeable *Haemophilus influenzae* among Children in Arkansas

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In this study, we reviewed cases of invasive disease due to nontypeable *Haemophilus influenzae* among children hospitalized at Arkansas Children's Hospital from 1993 to 2001. A total of 28 cases were examined, including 21 associated with bacteremia and 4 associated with meningitis. Of the patients examined, 86% were ≤ 4 years of age, and 68% had underlying medical conditions. Characterization of the bacterial isolates by multilocus sequence type genotyping revealed significant overall genetic diversity, similar to the diversity in the general population structure for nontypeable *H. influenzae*. However, four separate pairs of isolates were closely related genetically, a relationship confirmed by pulsed-field gel electrophoresis and Southern hybridization studies using probes for the major *H. influenzae* adhesin genes. These results suggest that selected strains of nontypeable *H. influenzae* may have more invasive potential, especially in young children and patients with underlying medical conditions. At this point, the specific factors that contribute to enhanced virulence remain unclear.

Nontypeable *Haemophilus influenzae* (NTHi) is present in the nasopharynx in approximately 50% of young children and is a common cause of localized respiratory tract disease, including sinusitis, otitis media, bronchitis, and pneumonia (12). Since the implementation of routine immunization against *H. influenzae* type b (Hib) just over a decade ago, nontypeable strains have taken on greater relative importance as a cause of bacteremia, meningitis, and other forms of invasive disease (27). NTHi is rarely a cause of invasive disease in healthy older children or adults.

Isolates of NTHi are genetically distinct from Hib strains but share a common ancestry (32, 48). Examination of the population structure of NTHi reveals significant genetic heterogeneity and a lack of clonality, while isolates of Hib can be categorized as a limited number of clones (33). Nevertheless, current evidence suggests that nontypeable and typeable strains most likely originated from a common nonencapsulated ancestor (46). Interestingly, some isolates of NTHi possess a partial capsule locus, suggesting that they are more closely related to encapsulated strains (46, 48).

Although NTHi strains lack a polysaccharide capsule, they possess a number of adhesive factors that promote colonization, a prerequisite for disease. For NTHi, at least four major adhesin families have been identified, including the HMW1/HMW2 proteins, Hia, Hap, and pili. Based on analysis of diverse collections of NTHi, approximately 75% of strains express HMW1/HMW2-like proteins, and most of the remaining strains express Hia (1, 2, 46). HMW1 and HMW2 are high-molecular-weight proteins that share 71% identity and 80%

similarity overall and have broad adhesive potential, with differing specificities (1, 9). Hia is a high-molecular-weight autotransporter protein that shares sequence homology and functional similarity with Hsf, the major nonpilus adhesin in Hib (2, 44). Hap is another autotransporter protein and was first discovered based on its ability to facilitate adherence and low-level invasion in assays with cultured epithelial cells (21, 45). More-recent observations have established that Hap mediates adherence to extracellular matrix proteins and formation of microcolonies as well (16, 22). Hemagglutinating pili are phase-variable structures that promote adherence to oropharyngeal cells and have been implicated in colonization in the monkey model of nasopharyngeal colonization (30, 51).

The purpose of this study was to explore the epidemiology of invasive disease due to NTHi among children in Arkansas and to characterize the genetic relatedness of invasive isolates. We found that nearly 90% of cases in our series occurred either in children younger than 4 years of age or in patients with underlying disease. Interestingly, four pairs of isolates were identical by multilocus sequence-type (MLST) genotyping and had similar pulsed-field gel electrophoresis and Southern hybridization patterns, suggesting that selected strains of NTHi may be more likely to produce invasive disease.

MATERIALS AND METHODS

Invasive NTHi infection. A retrospective review of the medical and microbiological records at Arkansas Children's Hospital was conducted to identify patients with invasive disease due to nontypeable *H. influenzae* from January 1993 through April 2001. Data were collected from the medical records, including age, sex, race, underlying medical conditions, and body site from which the isolate was recovered. The diagnoses of ventriculoperitoneal shunt (VPS) infection, bacteremia, and peritonitis were assigned on the basis of positive cultures from the appropriate body fluids. The diagnosis of pneumonia was assigned on the basis of the presence of bacteremia in association with an infiltrate on chest radio-

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graph, cough or respiratory distress, and fever. One exception was a patient who lacked bacteremia but had an infiltrate and an effusion on chest radiograph and a pleural fluid culture positive for NTHi. Finally, one patient was diagnosed with a brain abscess when purulent fluid from the abscess grew NTHi. The Human Research Advisory Committee at the University of Arkansas for Medical Sciences approved this study.

Strain isolation and growth. All isolates recovered during the study period were identified by the clinical microbiology laboratory at Arkansas Children's Hospital, confirmed by the Arkansas State Health Department, and then stored at -70°C in skim milk. Organisms were recovered from direct specimens (for example, cerebrospinal fluid, blood, etc.) by being plated on chocolate agar. After 18 to 24 h of incubation, suspected *H. influenzae* organisms were subcultured on a *Haemophilus* Identification II triplate (REMEL, Lenexa, Kan.) to assess the need for hemin (X factor) and NAD (V factor). Serotyping was performed by the Arkansas State Health Department using latex agglutination.

MLST genotyping. Isolates were examined for genetic relatedness by comparing partial gene sequences of phosphoglucosyltransferase (*pgi*), adenylate kinase (*adk*), malate dehydrogenase (*mdh*), L-fuculokinase (*fucK*), and recombination protein *recA* (*recA*) (15). Coding sequences (406 to 479 bp) were amplified from genomic DNA or directly from single bacterial colonies in a reaction mixture containing 50 pmol each of sense and antisense primers, 2.6 U of Expand High Fidelity Enzyme Mix (Roche Molecular Biochemicals, Mannheim, Germany), 200 μM (each) deoxynucleotide triphosphates, and 2.5 mM MgCl_2 in the buffer supplied by the manufacturer. Reaction conditions included denaturation at 94°C for 1 min, annealing at 42 to 53°C for 1 min, and extension at 72°C for 30 s, for a total of 35 cycles. Sense and antisense strands of amplification products were directly sequenced.

PFGE. To confirm the genetic relatedness of *H. influenzae* isolates with identical MLST genotypes, restriction digest patterns produced by digestion of chromosomal DNA with *SmaI* were compared. High-molecular-weight genomic DNA was prepared and digested with *SmaI* as previously described (34). Restriction fragments were separated on a 1% Tris-borate-EDTA (TBE) agarose gel with a CHEF-mapper apparatus (Bio-Rad, Hercules, Calif.) programmed to separate fragments between 20 and 500 kb, stained for 45 min with 1.5 mg of ethidium bromide/ml in TBE, and visualized by UV light.

Southern hybridization. Isolates were analyzed for the presence of specific virulence sequences by Southern hybridization with probes for *hap* (encoding the Hap adhesin), *hia* (encoding the Hia adhesin), *hmwA* (encoding HMW1/HMW2 adhesins), *hifABCDE* (encoding hemagglutinating pilus proteins and assembly machinery), and *cap* (encoding capsule).

Control strains included nontypeable *H. influenzae* strain 12, from which the *hmw1* and *hmw2* genes were originally cloned; nontypeable *H. influenzae* strain 11, from which *hia* was originally isolated; and Hib strain Eagan, which contains the *capB* locus and an intact pilus gene cluster. Strain 12 lacks *hia* and pilus genes, strain 11 lacks *hmw* genes and pilus genes, and strain Eagan lacks *hmw* genes (25). All three strains contain *hap*.

Chromosomal DNA was extracted from colonies on chocolate agar with the use of the Wizard Genomic DNA Purification kit (Promega, Madison, Wis.) and was digested overnight with *EcoRI*. The resulting DNA fragments were separated by agarose electrophoresis with 0.7% agarose gels and were transferred to nitrocellulose filters by the method of Smith and Summers (42). After cross-linking with UV light, filters were prehybridized for 1 h at 42°C with hybridization solution (Amersham Pharmacia, Piscataway, N.J.). Subsequently, they were hybridized overnight at 42°C with probes that were labeled with horseradish peroxidase by using DNA labeling reagent (Amersham) according to instructions from the manufacturer. Immediately after hybridization, they were washed twice with 0.4% (wt/vol) sodium dodecyl sulfate- $0.5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C , each time for 10 min. Next, they were washed twice with $20 \times \text{SSC}$ at room temperature, each time for 5 min. Ultimately, they were developed with ECL reagents (Amersham) and exposed to Classic Blue Sensitive film (Molecular Technologies, St. Louis, Mo.).

A 3.8-kb *Eco47III-EcoNI* intragenic fragment of *hmw1A* from nontypeable *H. influenzae* strain 12 was used as a probe for *hmw* genes (1). This probe hybridizes with both the *hmw1A* and the *hmw2A* genes. A 1.6-kb *SspI-StyI* intragenic fragment of *hia* from nontypeable *H. influenzae* strain 11 was used as a probe for *hia* homologs (2). A 2.2-kb *BsbMI* intragenic fragment of *hap* from nontypeable *H. influenzae* strain N187 was used as a probe for *hap* (45). Fragments corresponding to the intact *hifA*, *hifB*, *hifC*, *hifD*, and *hifE* genes from Hib strain Eagan were used as probes for the pilus gene cluster. *hifA*, *hifB*, *hifC*, and *hifD* were purified as *EcoRI-BamHI* fragments from pJS201, pJS202, pGK101, and pMMB91-*hifE*, respectively (47). *hifC* was amplified by PCR from strain Eagan chromosomal DNA, gel purified, and used directly as a probe. Plasmid pUO38 was used as a probe for capsule sequences and the *IS1016* element

TABLE 1. Clinical characteristics of previously healthy children with invasive NTHi disease in Arkansas from 1993 to 2001

Strain no.	Patient		Infection
	Age	Sex	
784	6 wk	Female	Bacteremia, meningitis
772	3 mo	Female	Bacteremia
774	4 mo	Male	Bacteremia, pneumonia
765	19 mo	Male	Bacteremia
776	3 y	Male	Bacteremia
789	3 y	Male	Bacteremia, pneumonia
794	4 y	Male	Brain abscess
777	8 y	Female	Bacteremia
775	15 y	Male	Lung abscess (smoker)

associated with encapsulated strains of *H. influenzae*. This plasmid is a derivative of pBR322 and contains a complete set of the *cap* genes, including one copy of *IS1016*, from a phylogenetic division I Hib strain (26). Of note, the *capB* locus includes genes shared among all encapsulated strains of *H. influenzae*. Results with pUO38 confirmed the absence of patterns of hybridization characteristic of *H. influenzae* serotypes a to f, providing additional evidence that all strains were nontypeable.

Statistics. Statistical analyses were performed with SPSS for Windows (SPSS, Inc., Chicago, Ill.). Chi-square analysis was used to evaluate significant relationships within the study population.

RESULTS

Epidemiology and clinical characteristics of patients. During the study period, a total of 33 invasive isolates of *H. influenzae* were identified. Thirty of the 33 isolates were nontypeable strains, one was a type b strain, one was a type f strain, and one was a type e strain. Medical records of 28 of 30 cases of invasive NTHi infection were available for review.

Patients with invasive nontypeable *H. influenzae* disease ranged in age from 1 day to 15 years, with a median of 17 months. Twenty-four (86%) of the patients were ≤ 4 years of age, and three were less than 3 months, including two who were premature infants less than 24 h old. Only two patients were older than 6 years of age (8 and 15 years). Sixteen (57%) of the patients were male, and 16 (57%) were Caucasian. Tables 1 and 2 summarize the clinical characteristics of the study population.

Twenty-one (75%) patients had bacteremia, four (14%) had VPS infections, one had peritonitis, one had a lung abscess, and one had a brain abscess. Eight (38%) of the patients with bacteremia also presented with respiratory distress and chest radiographs displaying infiltrates consistent with the diagnosis of pneumonia. One patient with bacteremia had a cerebrospinal fluid (CSF) leukocyte count of 1,337, with normal CSF protein and glucose, and a negative CSF gram stain. Although these CSF indices were consistent with a diagnosis of meningitis, the CSF culture remained negative.

As shown in Table 1, nine patients had no chronic underlying medical condition. Among these patients, seven had bacteremia, one had a lung abscess, and one had a brain abscess. Additionally, one of the bacteremic patients was a 6-week-old infant who had spinal fluid indices indicating meningitis but whose CSF culture was negative. Two of the previously healthy bacteremic patients had radiological evidence of pneumonia

TABLE 2. Clinical characteristics of children with underlying medical conditions and invasive NTHi disease in Arkansas from 1993 to 2001

Strain no.	Patient		Medical condition	Infection
	Age	Sex		
785	1 day	Male	Prematurity	Bacteremia
802	1 day	Male	Prematurity	Bacteremia
783	3 mo	Male	Chronic lung disease of prematurity	Bacteremia, pneumonia
773	3 mo	Male	Seizures	Bacteremia
788	6 mo	Female	Chronic lung disease of prematurity	Bacteremia
790	8 mo	Male	VPS	Meningitis
767	10 mo	Female	Myotonic dystrophy	Bacteremia, pneumonia
778	13 mo	Male	Chronic lung disease of prematurity	Bacteremia, pneumonia
781	14 mo	Female	Chronic lung disease of prematurity	Bacteremia, pneumonia
786	14 mo	Female	Chronic renal failure	Peritonitis
796	16 mo	Female	VPS	Meningitis
803	18 mo	Male	Cerebral Palsy, seizures	Bacteremia
769	19 mo	Female	Trisomy 21, leukemia	Bacteremia
801	20 mo	Male	Mechanical ventilation	Bacteremia, pneumonia
800	2 yr	Male	Mechanical ventilation	Bacteremia, pneumonia
787	3 yr	Female	Leukemia	Bacteremia
795	3 yr	Female	Leukemia	Bacteremia
798	3 yr	Female	VPS	Meningitis
797	5 yr	Male	VPS	Meningitis

and respiratory distress at presentation. Six (67%) of the previously healthy patients were less than 4 years of age.

As shown in Table 2, nineteen (68%) of the patients had underlying medical conditions, including chronic lung disease, hydrocephalus, prematurity, and illnesses requiring immunosuppressive therapy. Six of these patients had clinical evidence of pneumonia, including three patients with a history of chronic lung disease. All four of the patients with infected CSF had ventriculoperitoneal shunts.

None of the patients in the study died during hospitalization. Three of the patients with VPS infections required replacement of their shunts. No other significant complications were noted as a direct consequence of infection.

Genetic evaluation of isolates. In order to assess the genetic relatedness of the 28 NTHi isolates associated with invasive disease, MLST genotyping was performed. Overall the 28 isolates were moderately diverse, consistent with the heterogeneity in the general population structure for NTHi (data not shown). However, four pairs of isolates, namely, strains 773 and 796, strains 769 and 795, strains 788 and 800, and strains 767 and 794, shared identical *pgi*, *adh*, *mdh*, *fucK*, and *recA* alleles. Nucleotide sequences of these alleles are available from the GenBank database under accession numbers AY245376 to AY245415. As shown in Fig. 1, PFGE confirmed the genetic similarity of these pairs of strains. Strain 788 shared an identical restriction fragment pattern with strain 800, and strain 767 differed from strain 794 by a single band. Strains 773 and 796 and strains 769 and 795 each differed by 2 or 3 bands. There were no epidemiologic relationships between patients infected with genetically related strains and no temporal or geographic clustering of a particular clonotype of bacteria.

Presence of adhesin and capsulation genes. As shown in Table 3, Southern hybridization results with the major adhesin genes were consistent with the MLST genotyping and PFGE studies and confirmed relatedness of three of the four pairs of related strains, including strains 773 and 796, strains 769 and 795, and strains 767 and 794. Overall, 8 isolates (29%) had

1 2 3 4 5 6 7 8 9 10

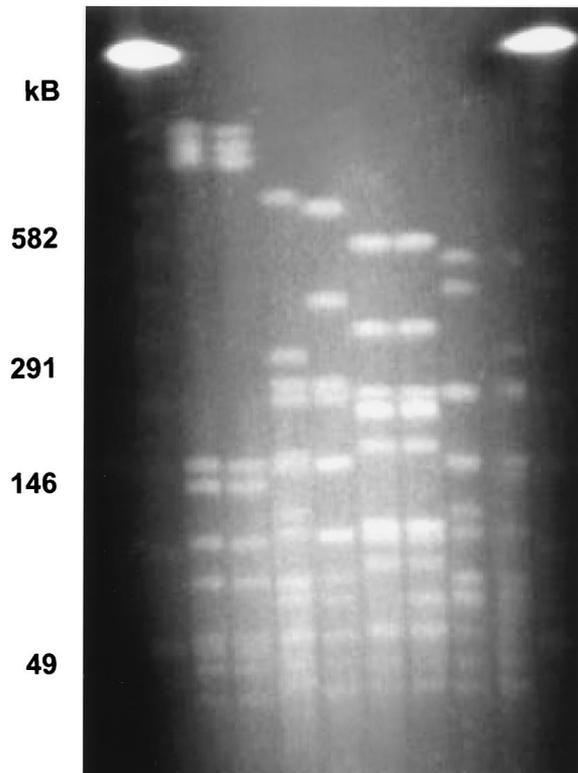


FIG. 1. Restriction digest patterns of invasive isolates of nontypeable *H. influenzae*. High-molecular-weight DNA from four pairs of nontypeable *H. influenzae* isolates that shared identical alleles at five loci tested was digested with *Sma*I, and restriction fragments were separated by PFGE. Lambda ladder molecular weight standards (lanes 1 and 10) and restriction fragments of strains 800 (lane 2) 788 (lane 3), 773 (lane 4), 796 (lane 5), 767 (lane 6), 794 (lane 7), 769 (lane 8), and 795 (lane 9) are shown. Strains 800 and 788, 773 and 796, 767 and 794, and 769 and 795 share identical or highly similar restriction digest patterns.

TABLE 3. Southern hybridization results with probes for the major adhesin genes

Strain no.	<i>hap</i>	<i>hmw1A/hmw2A</i>	<i>hia</i>	<i>hifA</i>	<i>hifB</i>	<i>hifC</i>	<i>hifD</i>	<i>hifE</i>	pUO38
773	Yes	Yes							Yes
796	Yes	Yes							
785	Yes	Yes							
797	Yes	Yes							Yes
769	Yes	Yes		Yes	Yes	Yes	Yes	Yes	
795	Yes	Yes		Yes	Yes	Yes	Yes	Yes	
775	Yes	Yes							Yes
788	Yes	Yes							
800	Yes		Yes						
802	Yes	Yes							
767	Yes								
794	Yes								
765	Yes	Yes							
774	Yes	Yes							
783	Yes	Yes							
790	Yes	Yes							
776	Yes	Yes	Yes						Yes
789	Yes		Yes	Yes	Yes	Yes	Yes	Yes	
778	Yes	Yes		Yes	Yes	Yes	Yes	Yes	
777	Yes		Yes						Yes
781	Yes	Yes							
784	Yes								
798	Yes	Yes							
801	Yes			Yes	Yes	Yes	Yes	Yes	
772	Yes		Yes						
786	Yes		Yes						
787	Yes		Yes						
803	Yes		Yes						

sequences that hybridized with the *hia* gene, and 17 isolates (60%) had sequences that hybridized with the *hmwA* gene. One isolate hybridized with both *hia* and *hmwA*, and only four isolates hybridized with neither *hia* nor *hmwA*. Five (18%) isolates had sequences that hybridized with *hifABCDE*. Interestingly, all isolates contained sequences homologous to the *hap* gene, consistent with other studies suggesting that this gene is ubiquitous among *H. influenzae* organisms (36).

Five (18%) of the 28 isolates hybridized with pUO38, suggesting that these strains may have contained a *cap* locus or the associated *IS1016* insertional element at some point in time (Table 3).

DISCUSSION

The dramatic decline in invasive disease due to Hib since the introduction of Hib conjugate vaccines has been well documented (3, 5, 17, 19, 23, 31, 39). Indeed, during the 8 years of our investigation, there was only one episode of invasive Hib infection at our institution. The available literature provides no evidence for a concomitant increase in invasive disease due to nontypeable *H. influenzae* (8, 38, 41). Nevertheless, serious NTHi disease is now more important in relative terms, and in areas where the Hib vaccine is universally available, nontypeable strains are now more likely to be the causative agents of invasive *Haemophilus* disease. Accordingly, it would be beneficial to identify the patient populations at high risk for these infections and to characterize the bacterial factors that contribute to invasive disease.

In our population of children with invasive NTHi disease,

the most striking observation was that almost all were younger than 4 years of age. This finding is consistent with studies from the United Kingdom. Falla et al. identified 24 children in Oxford, England, with serious NTHi disease during the period from 1985 to 1991 and found that 83% of these children were less than 3 years old (14). Heath et al. noted that the mean age of children in the United Kingdom with invasive NTHi disease from 1992 to 1998 was 9.2 months (median, 19.6 months) (19). The predominance of disease among young children may reflect the high prevalence of nasopharyngeal colonization with NTHi and the high incidence of viral respiratory infection (and therefore increased mucosal inflammation and the potential for bacterial invasion) in this age group (12). It has also been reported that levels of immunoglobulin G (IgG), IgM, and IgA antibodies to NTHi are relatively low until 2 years of age, reaching adult levels by 4 years of age (13).

Over two-thirds (68%) of our patients with invasive NTHi disease had underlying medical problems. By comparison, Heath et al. reported that 41% of the children identified during their 6-year study had an underlying medical condition (19). Perhaps most striking was a report by Gilsdorf, who described nine patients with invasive NTHi disease between 1982 and 1986 and found that all but one (89%) had underlying illness (18). All three studies defined underlying medical problems in a similar fashion.

NTHi meningitis is an uncommon diagnosis in the pediatric population, and an appreciable fraction of cases involve immunocompetent children (3, 7, 11). Only a few cases in children with VPS have been previously reported (20, 28, 29, 37, 43). Heath et al. noted that four of the patients in their prospective study in the United Kingdom had a history of VPS, although it was not clear whether these four patients had meningitis or some other invasive NTHi disease (19). In the study by Falla et al., one of five patients with meningitis due to NTHi had pre-existing VPS (14). In the present study, four of five patients with meningitis had a VPS in place when the diagnosis was established. Although our numbers are small, they suggest that there may be an association between the presence of a VPS and the development of NTHi meningitis. The mechanism responsible for this association remains unclear.

Neonatal invasive disease due to NTHi has been well described (4, 24, 50). Such cases are often diagnosed within the first 1 to 2 days of life, and prematurity is a recognized risk factor. Heath et al. reported that 29% of the invasive NTHi disease in their study was in the neonatal population and the majority of cases occurred in premature infants within the first 7 days of life (19). Most of these infants were diagnosed with septicemia. Falla et al. reported that 10 of 24 patients (42%) in their study were neonates, all preterm (14). In the present study, there were only two neonates, both of whom were bacteremic with respiratory distress and radiographic findings suggestive of pneumonia. This is a much smaller percentage (7%) of the overall population than was noted in other studies.

Overall, the strains of NTHi isolated over the review period appeared to be quite diverse, a finding consistent with the general heterogeneity of the NTHi population structure (10, 32). On the other hand, based on MLST genotyping, PFGE, and Southern hybridization studies using probes for the major *H. influenzae* adhesin genes, 8 of 28 isolates were genetically

similar to another strain. Of note, based on work by Musser et al., the overall population structure of NTHi is believed to be nonclonal (32). Other studies have also confirmed the genetic diversity of NTHi strains (6, 35, 40). In this context, it is remarkable that almost a third of the isolates in our small collection of epidemiologically unrelated strains were found to be closely related to at least one other strain in the collection. These results suggest that selected strains of NTHi may have more invasive potential, especially in young children and patients with underlying medical conditions.

In considering the specific bacterial factors that contribute to enhanced virulence, we found no evidence for disproportionate representation of HMW1/HMW2, Hia, or pilus adhesion genes compared with larger collections of NTHi strains from diverse clinical sites (46). All strains had sequences homologous to *hap*, but this gene appears to be ubiquitous among *H. influenzae* strains, suggesting a fundamental role in colonization, an essential step in both asymptomatic infection and invasive disease. A recent study by Vitovski et al. revealed that invasive isolates of NTHi exhibit increased IgA1 protease activity compared to isolates from asymptomatic carriers (49). The level of IgA1 protease activity was not assayed in the current study but may be one factor that contributes to NTHi invasive disease.

The success of the Hib immunization program in this country has placed more importance on invasive non-type b disease, caused most commonly by NTHi strains. The findings of the present study underscore the importance of age (younger than 4 years) and underlying medical conditions, such as prematurity, lung disease, or the presence of a VPS, in contributing to NTHi invasive disease in children. It appears that selected strains of NTHi may be more likely to produce invasive disease, and future studies should address the specific factors that contribute to enhanced virulence.

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