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Prevalence and Genetic Diversity of Nontypeable *Haemophilus influenzae* in the Respiratory Tract of Infants and Primary Caregivers

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Abstract

BACKGROUND—Nontypeable *Haemophilus influenzae* (NTHi) causes otitis media, sinusitis, and likely lower respiratory tract infections in children. Colonization, strain diversity, transmission, and antimicrobial susceptibility have implications for both children and their caregivers.

METHODS—For 13 months, we conducted a cross-sectional study of NTHi colonization. 273 infants and children aged 2 to 26 months old and their primary caregivers had upper respiratory tract cultures performed. NTHi isolates were characterized by multilocus sequence typing (MLST) and antibiotic resistance was examined.

RESULTS—Of the 273 infants, 44 (16.1%) were colonized with NTHi. Prevalence of NTHi varied from 14% in infants less than 6 months of age to 32% in infants 19-26 months of age (p=0.003). NTHi colonized infants were more likely to attend daycare (30% vs. 11%), have a recent respiratory infection (68% vs. 38%), recent antibiotic use (27% vs. 9%), and caregiver reported asthma (11% vs. 1%) compared with other infants (p<0.001). Of the 44 infants colonized with NTHi, we identified 33 different MLSTs. Nine (20.5%) of the 44 infant-primary caregiver dyads were colonized with NTHi and 7/9 shared identical NTHi strains. We also found beta-lactamase negative NTHi with minimum inhibitory concentrations >2 μg/mL for amoxicillin and beta-lactamase positive NTHi with minimum inhibitory concentrations >2 μg/mL for amoxicillin clavulanate.

CONCLUSIONS—We found substantial diversity by MLST analysis among NTHi isolates from this community. Infant-primary caregiver dyads usually carried the same strain of NTHi, suggesting that infant-primary caregiver transmission is occurring.

Keywords

Nontypeable *Haemophilus influenzae*; multilocus sequence typing; prevalence; diversity; transmission

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INTRODUCTION

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of respiratory tract illness. NTHi is a cause of hospitalizations secondary to pneumonia in the elderly and exacerbations of chronic obstructive pulmonary disease in persons with emphysema. In children, disease due to NTHi is observed as a frequent complication of viral respiratory tract infections, manifesting as either acute otitis media or sinusitis. Its role in lower respiratory tract infections in children is less clear as it is rarely associated with bacteremia, and it is uncommon to establish the microbiology of lower respiratory tract infections in young children.[1] Based on lung aspirates and blood cultures, Shann et al. recovered *Haemophilus influenzae*, *Streptococcus pneumoniae*, or both in approximately half of 83 children with pneumonia in Papua New Guinea. Of 32 strains of *Haemophilus influenzae* tested, 18 (56%) were non-serotypable, 8 (25%) were serotypes other than type b, and only 6 (19%) were type b.[2] Using flexible bronchoscopy and bronchoalveolar lavage, De Schutter et. al recently suggested NTHi was one of the major pathogens found in children with recurrent community-acquired pneumonia.[3]

Studies suggest that NTHi not only colonizes the respiratory tract but NTHi can be transmitted between individuals.[4-5] To further understand NTHi colonization and transmission, we performed a study with the following objectives: (1) to determine the prevalence of NTHi carriage in the respiratory tract of infants and their primary caregivers, (2) to examine the frequency of NTHi co-colonization across infant-primary caregiver dyads, and (3) to characterize the genetic diversity of NTHi isolates present in the respiratory tract of infants and their primary caregivers by multilocus sequence typing (MLST).

MATERIALS AND METHODS

Between January 2009 and January 2010, we conducted a cross-sectional pilot study of NTHi colonization and transmission. The study was carried out within a community of southeastern Massachusetts with a population of approximately 100,000. In this community, the citizens are 91% White, 3.3% Hispanic, 2.5% Black, and 2.2% Asian with a median income of approximately $29,000.[6]

Two hundred seventy-three infants ages 2 to 26 months old and their primary caregivers, usually the infant's mother, father or grandparent, were recruited from a large pediatric practice. The subjects were limited to a single infant per household, and enrollment was stratified by season and age of infants with two-thirds from healthy infant visits and one-third from sick infant visits. A sick infant visit was defined as an infant with any signs of respiratory illness (runny nose, earache, sinusitis, sore throat, cough, fever, or irritability). Some infants attending a healthy infant visit reported a history of a respiratory illness within the previous 30 days (see Figure, SDC 1). Each infant-primary caregiver dyad had an oropharyngeal (OP) and nasopharyngeal (NP) swab performed. Medical histories including breastfeeding, daycare attendance, presence of sibling in the home, vaccinations for the infant, and smoking by the primary caregiver were collected by questionnaires.

For identification of NTHi, OP and NP swabs were cultured on a chocolate agar plate with bacitracin and incubated at 37°C in 5% carbon dioxide overnight. Cultures were identified and confirmed to be *Haemophilus influenzae* by typical colony morphology, catalase test, and X and V factor requirements using *Haemophilus* ID Quad Plates (Remel, Lenexa, KS). Unencapsulated strains were identified by a negative result of the slide agglutination test for capsular serotype using polyvalent *Haemophilus influenzae* serotyping sera (BD Diagnostic Systems, Sparks, MD). DNA extracts from the unencapsulated strains were amplified by

polymerase chain reaction to identify strains with a 16S rDNA signature gene that matched NTHi; we did not further characterize strains when the 16S signature was discordant.[7] The two 16S primers used to amplify the DNA templates were: 16S/3' 5'-GCAGGTTCCCTACGGTTA-3' and 16S/Nor 5'-TGACATCCTAAGAAGAGC-3'. The 16S/3' primer aligned with the antisense nucleotide strand of the 16S rDNA gene, and the 16S/Nor primer aligned directly with the 16S rDNA sense strand. Three distinct colonies from each original culture plate of confirmed unencapsulated *Haemophilus* were individually grown and frozen at -80ºC for DNA extraction and further genetic analyses of the 7 housekeeping genes used to assign MLST. Antibiotic susceptibility profiles were determined by the E-test (bioMerieux, France) and nitrocefin disks (Remel, Lenexa, KS) were used to assess beta-lactamase production. Genomic DNA from each NTHi isolate was obtained with the DNeasy kit (Qiagen, Germantown, MD), and all NTHi isolates were genetically characterized by MLST. A repository MLST website (http://haemophilus.mlst.net/) provides primer sequences used in the assignment of MLST typing scheme for NTHi to sequence approximately 500 base pairs of each of 7 housekeeping genes. The genes are: adenylate kinase (*adk*), ATP synthase - subunit gamma (*atp*G), fumarate reductase - iron-sulfur subunit (*frd*B), L-fuculokinase (*fuc*K), malate dehydrogenase (*mdh*), glucose-6-phosphate isomerase (*pgi*), and recombinase A (*rec*A).[8-13]

Questionnaires and microbiologic data were analyzed using SAS software version 9.1.3 (SAS Institute, Cary, NC). Data were analyzed to describe the population and compare distributions using chi squared tests, single and multivariate logistic regression, and reported odds ratios with 95% confidence intervals.

RESULTS

Of the primary caregivers, 262/273 (96%) were parents and 205/262 (78%) of the parents were mothers. A total of 273 infants ages 2 to 26 months old and their primary caregivers each had both an OP and NP swab. Of the 273 infants, 44 (16%) were colonized with NTHi. Infants were more likely to have NTHi recovered from NP swabs, specifically 24/44 (54%) were from NP exclusively, 7/44 (16%) were from OP only, and 13/44 (30%) were from both NP and OP. Twenty-six (9.5%) of the infants' primary caregivers were colonized with NTHi. Primary caregivers were more likely to have NTHi recovered from OP swabs with 18/26 (69%) from OP only, 6/26 (23%) from NP only, and 2/26 (8%) from both NP and OP. We found the prevalence of NTHi colonization in infants to vary by age, with prevalence increasing from 14% in infants less than 6 months of age to 32% in infants 19-26 months of age (p=0.003). Infants were 2.6 times more likely to be colonized in the winter and spring than the summer and fall (p=0.01).

NTHi colonized infants were more likely to attend daycare (30% vs. 12%, p<0.001), have had a recent respiratory infection (68% vs. 38%, p<0.001), have recently taken an antibiotic (27% vs. 9%, p<0.001), and have primary caregiver reported asthma (11% vs. 1%, p<0.001) compared to other infants using chi-squared testing. Season, age, current respiratory infection, and primary caregiver reported asthma continued to be independent predictors of NTHi colonization for infants in multivariate analyses (Table 1).

Of the 44 infants initially colonized with NTHi, there were 28/44 (64%) that were beta-lactamase negative and 16/44 (36%) that were beta-lactamase positive. A MIC of >2 μg/mL for amoxicillin was observed in 19/44 (43%), and a MIC of >2 μg/mL for amoxicillin clavulanate was observed in 4/44 (9%) (see Figure, SDC 2).

In order to characterize the genetic diversity of NTHi present in the respiratory tract of infants and their primary caregivers, 3 colonies of NTHi from each culture positive NP or

OP swab were analyzed by MLST. In all cases, each of the 3 colonies from NP or OP swabs from the 44 culture positive infants and 26 culture positive primary caregivers were identical by MLST.

Of the 44 infants initially colonized with NTHi, 33 different sequence types based on MLST analyses were identified, and 9 of these 33 different sequence types are novel to the MLST.net database.[8] Twenty-two sequence types were found in single samples, while 11 were found in more than one sample. Nine of the 44 infants colonized with NTHi (20.5%) had a primary caregiver colonized with NTHi. Of the 9 infant-primary caregiver dyads colonized with NTHi, 7 shared identical NTHi strains as determined by MLST (Table 2).

DISCUSSION

In our study the prevalence of NTHi carriage increased with age, and our prevalence estimates are consistent with those in the published literature. For example, Faden et al. followed 200 children from birth through 2 years of age with NP cultures to determine the colonization pattern of NTHi. The acquisition rate was greatest in the first year with prevalence rates of approximately 11%, and 44% of the children were colonized on 1 or more occasions.[14] We found that daycare, a recent respiratory infection, recent antibiotic use, and primary caregiver reported asthma were predictors of NTHi carriage. Interestingly, findings by Ahrén et al. suggest that NTHi can induce an innate inflammatory response in eosinophils, thus promoting inflammation in chronic pulmonary diseases such as allergic asthma.[15] Breastfeeding could not be assessed since too few mothers in our population reported breastfeeding. Barbosa-Cesnik et al. did not find an association between NTHi carriage and breastfeeding in their study of infants in daycare despite evidence that breastfeeding is associated with higher serum concentrations of antibodies to NTHi.[16-17]

We found that 7 of 9 infant-primary caregiver dyads shared the identical strain of NTHi in our study as determined by MLST, strongly supporting the transmission of NTHi between infants and primary caregivers. Previous studies of the transmission of NTHi are limited and have focused predominantly on daycares or nursing homes.[18-19] Goetz et al. described 11 cases of pneumonia due to NTHi in a nursing home and adjacent acute care facility. At the nursing home, 8 isolates from ill individuals or their asymptomatic roommates had a single outer membrane protein profile while isolates from patients at an adjacent acute care facility all had different profiles, suggesting horizontal transmission among nursing home residents.[4] Similarly, Loos et al. used DNA restriction fingerprinting to demonstrate that identical strains were found in the nasopharynx and middle ear fluid of a child with acute otitis media; and when 3 siblings were cultured, they found similar strains of NTHi, suggesting transmission within the household setting.[20] Watanabe et al. evaluated displaced persons in evacuation camps following the 2004 Sri Lankan tsunami and found 20 individuals colonized with NTHi. Molecular analysis demonstrated 12 pulsed field patterns, and similarity in these internally displaced persons between camps is suggestive of person to person transmission.[21] One family study of NTHi carriage identified a high rate of concordance between isolates carried by two family members, proposing that transmission within families is frequent.[22] A limitation to our study was that we did not obtain data to show if colonized infants with the same MLST were relatives, lived in the same neighborhood, or attended the same daycare.

To our knowledge, this is the first report to show 3 distinct colonies from either NP or OP samples with consistent identity when analyzed by MLST. Other investigators have used diagnostic techniques different from ours and have demonstrated findings both similar and dissimilar from ours. Lebon et al. found a similar rate of *Haemophilus influenzae* colonization of 23/511 (4.5%) from maternal infant pairs, however by pulsed field gel

electrophoresis (PFGE) they found that only 3 of 23 maternal infant pairs were colonized with a genotypically indistinguishable strain.[5] Farjo et al. demonstrated 64% of children attending daycare were colonized with *Haemophilus influenzae* with wide variation in rates of colonization (0 to 95%) within individual daycare centers, yet 37% of children were colonized with 2 or more genetically distinct *Haemophilus influenzae* with evidence of sharing the same strain in 13 of 15 daycare centers. These *Haemophilus influenzae* isolates obtained by throat culture were genetically typed by enterobacterial repetitive intergenic consensus polymerase chain reaction as the initial screen to identify unique strains within each child and then PFGE was used to investigate the genetic diversity of strains between children.[18] Again, these results likely reflect differences between PFGE and MLST for identifying genetic differences and similarities.

PFGE was typically the method of choice before the development of MLST for epidemiologic studies of diverse bacterial pathogens. PFGE typing is based on the restriction of genomic DNA with infrequently cutting restriction enzymes, generating large fragments of DNA (>20 kilobases) distinguishable after electrophoresis. The band patterns so obtained are the “bar code” for a given isolate. MLST is a DNA sequencing-based technique of approximately 500 base pairs from 7 housekeeping genes distributed across the genomic map. Housekeeping genes encode proteins essential in the bacterial cell physiology and evolve at a constant or neutral rate. As such, MLST is a typing method well suited to identifying differences between 2 isolates. PFGE, on the other hand, has less precision because the location of the cut sites in the genome is unknown; these might fall within a gene evolving at a neutral rate or within a fast evolving gene due to selective pressure. However, PFGE might allow the detection of quickly evolving, emerging epidemic strains or clones. Recent studies are implementing the application of both MLST and PFGE with other polymerase chain reaction-based typing techniques in order to optimize the accuracy of isolate identification.[23-24]

We found that each individual carried a single strain of NTHi based on MLST analyses of 3 isolates despite substantial diversity of NTHi strains within this community. Among 44 children colonized with NTHi, 33 different MLST sequence types were identified and approximately a third of these different sequence types are novel. Although the MLST.net *Haemophilus influenzae* database currently has 1412 isolates representing 796 MLSTs, it is a continuously growing collection and we are just beginning to learn about the extent of its diversity.[9] Our study demonstrates that approximately 20% of the infant-primary caregiver dyads were colonized with NTHi, and most of these shared identical NTHi strains as determined by MLST. This finding gives preliminary support for using MLST, which was developed to characterize phylogenetic relationships within a bacterial species, as a tool for further studies of NTHi transmission. The potential advantages of MLST are its standardized approach, the availability of a global strain network (MLST.net), and the ability to analyze strain relationships (i.e. single and double locus mutants). A longitudinal, prospective study with multiple respiratory samples from infants, their primary caregivers, and their households would allow us to fully demonstrate transmission patterns over time.

As expected, about a third of the infants had NTHi that was beta-lactamase positive. However, we also found beta-lactamase negative NTHi with elevated minimum inhibitory concentrations for amoxicillin and beta-lactamase positive NTHi with high minimum inhibitory concentrations for amoxicillin clavulanate. We sequenced the penicillin binding protein 3X gene in these isolates and found the N526K mutation as previously reported by Shuel et al. (data not shown).[25-26] While our isolates do not necessarily represent disease causing strains of NTHi, they suggest that NTHi populations colonizing the nasopharynx contain antimicrobial resistance genes for both beta-lactamase and penicillin binding protein mediated resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Predictors of Nontypeable *Haemophilus influenzae* (NTHi) Colonization in Infants

	Total		NTHi-		NTHi+			
Infant Information	**No.**	**(%)**	**No.**	**(%)**	**No.**	**(%)**	***P***	**Multivariate Logistic Regression Odds Ratio Adjusted (95% Confidence Intervals)**
Total	273		229		44			
Season								
Winter	103	(38)	85	(37)	18	(41)	0.052	
Spring	76	(28)	58	(25)	18	(41)		
Summer	47	(17)	42	(18)	5	(11)		
Fall	47	(17)	44	(19)	3	(7)		
Season								
Winter/Spring	179	(66)	143	(62)	36	(82)	0.01	**2.59** (1.04-6.49)
Summer/Fall	94	(34)	86	(38)	8	(18)		reference
Age by months								
0 - 6	98	(36)	92	(40)	6	(14)	0.004	reference
7 - 12	68	(25)	56	(24)	12	(27)		**3.68 (1.20-11.33)**
13 - 18	45	(16)	33	(14)	12	(27)		**5.23 (1.63-16.81)**
19 - 24	62	(23)	48	(21)	14	(32)		**4.74 (1.53-14.64)**
Sex								
Male	140	(51)	120	(52)	20	(45)	0.39	
Female	133	(49)	109	(48)	24	(55)		
Race								
White	194	(71)	166	(72)	28	(64)	0.5	
Black	30	(11)	23	(10)	7	(16)		
Hispanic	33	(12)	26	(11)	1	(2)		
Other	16	(6)	14	(6)	2	(5)		
Respiratory illness in past 30 days for infant								
No	156	(57)	142	(62)	14	(32)	0.0002	reference
Yes	117	(43)	87	(38)	30	(68)		**2.7 (1.25-5.83)**
Antibiotic in past 30 days								
No	240	(88)	208	(91)	32	(73)	0.0007	
Yes	33	(12)	21	(9)	12	(27)		

Infant Information	Total No.	(%)	NTHi- No.	(%)	NTHi+ No.	(%)	*P*	Multivariate Logistic Regression Odds Ratio Adjusted (95% Confidence Intervals)
Current breastfeeding								
No	248	(91)	207	(90)	41	(93)	0.55	
Yes	25	(9)	22	(10)	3	(7)		
Current daycare								
No	233	(85)	202	(88)	31	(70)	0.002	reference
Yes	40	(15)	27	(12)	13	(30)		**2.97 (1.21-7.31)**
Primary caregiver reported asthma								
No	265	(97)	226	(99)	39	(89)		reference
Yes	8	(3)	3	(1)	5	(11)	0.0003	**7.34 (1.54-34.84)**
Caregiver NTHi+								
No	247		212	(93)	35	(80)	0.0007	reference
Yes	26		17	(7)	9	(20)		**6.18 (2.07-18.46)**
Caregiver with recent respiratory illness								
No	208	(76)	180	(79)	28	(64)	0.03	
Yes	65	(24)	49	(21)	16	(36)		

Table 2

MLST (Multilocus Sequence Type) and Beta-lactamase Production of Nasopharyngeal (NP) and Oropharyngeal (OP) Isolates of 9 Infant-Primary Caregiver Dyads

		NP Isolate		OP Isolate	
Family	**Person**	**MLST**	**β-lactam Production**	**MLST**	**β-lactam Production**
A	Infant	14	-		
A	Caregiver			14	-
B	Infant	103	+	103	+
B	Caregiver	103	+		
C	Infant	602	+		
C	Caregiver			602	+
D	Infant	422	+	422	+
D	Caregiver	422	+	422	+
E	Infant			14	-
E	Caregiver	395	+		
F	Infant	531	+	531	+
F	Caregiver			531	+
G	Infant	165	+		
G	Caregiver			1008	-
H	Infant	156	+		
H	Caregiver	156	+		
I	Infant	103	+		
I	Caregiver	103	+		