Haemophilus influenzae Prevalence, Proportion of Capsulated Strains and Antibiotic Susceptibility During Colonization and Acute Otitis Media in Children, 2019–2020

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Background: The objective of this study was to determine the prevalence, proportion of encapsulated strains and antibiotic susceptibility of *Haemophilus influenzae* isolated from young children.

Methods: Children, 6 months to 30 months old, were prospectively enrolled from September 2019 to September 2020 at Rochester, NY, pediatric clinics. *H. influenzae* isolates from nasopharynx (NP) at healthy visits and disease isolates from NP and middle ear fluid (MEF) at onset of acute otitis media (AOM) were characterized by capsular typing, β-lactamase production and antibiotic susceptibility.

Results: Samples from 565 healthy visits and 130 AOM visits were collected. *H. influenzae* was detected 5.9% and 27% in the NP from healthy and AOM visits, respectively. In the MEF, *H. influenzae* was isolated in 43% of samples. Eight percent of *H. influenzae* isolates were encapsulated, 88% type *f*. Overall 39.7% of isolates were β-lactamase producing; 43% for MEF isolates. Ampicillin, trimethoprim/sulfamethoxazole, erythromycin and clarithromycin nonsusceptibility were found in more than 25% of isolates. None of the encapsulated *H. influenzae* isolates were positive for β-lactamase production or ampicillin nonsusceptibility. 9.2% of isolates were β-lactamase negative, ampicillin resistant (β-lactamase negative, ampicillin resistant + β-lactamase negative, ampicillin intermediate).

Conclusions: The prevalence of *H. influenzae* in the NP of young children is very low at times of health, but *H. influenzae* is highly prevalent in MEF at onset of AOM. Nontypeable *H. influenzae* accounts for >90% of all *H. influenzae* isolates. Type *f* predominated among encapsulated strains. β-lactamase production and antibiotic nonsusceptibility among *H. influenzae* strains isolated from the NP and MEF are common.

Key Words: *Haemophilus influenzae*, acute otitis media, colonization, antibiotic susceptibility

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Haemophilus influenzae is a pathogen able to cause a wide spectrum of diseases in children, ranging from respiratory tract infections to invasive disease.[1] The species *H. influenzae* comprises 6 capsular types (types *a–f*) and noncapsule types, commonly referred to as nontypeable *H. influenzae* (NTHi).[2] Introduction of *H. influenzae* type *b* (Hib) conjugate vaccine dramatically reduced the incidence of invasive and noninvasive diseases caused by type *b* in children. Although NTHi currently accounts for most of the childhood diseases caused by *H. influenzae* in Hib vaccinated children. Emergence of other encapsulated *H. influenzae* types causing invasive diseases, especially *H. influenzae* type *a* (Hia), has been noted in the United States.[3]

Acute otitis media (AOM) and recurrent AOM[1,4–7] are common diagnoses in children and a common reason for antibiotic prescription.[8,9] NTHi and *Streptococcus pneumoniae* are the 2 main bacterial otopathogens responsible for AOM. Pneumococcal conjugate vaccines have had a major impact on reducing the incidence of AOM caused by *S. pneumoniae*, especially strains expressing vaccine serotypes.[10] Consequently, NTHi has emerged as the most common bacterial pathogen causing AOM in children.[6] Currently, there is no licensed vaccine in the United States that targets NTHi causing AOM[4,11] or other diseases caused by NTHi such as conjunctivitis,[12] chronic obstructive pulmonary disease[13] and invasive disease.[14] Determining evolving antibiotic susceptibility of *H. influenzae* is important to guide appropriate antibiotic selection.

In the present study, the prevalence of *H. influenzae* in the nasopharynx (NP) at times of health and onset of AOM as well as in the middle ear fluid (MEF) during was investigated in young children in the Rochester, NY, area. Capsule types and antibiotic susceptibility among the *H. influenzae* strains isolated were determined.

MATERIAL AND METHOD

Study Population

Children enrolled in this study were part of an ongoing prospective, longitudinal study of NP colonization and AOM in young children, funded in part by the Centers for Disease Control and Prevention from September 2019 to September 2020. Children were enrolled at 6–30 months of age from 2 pediatric clinical practices within the Rochester, NY, area. Written informed consent was obtained from parents before enrollment in the study as approved by the Rochester Regional Health Institutional Review Board.

Sample Processing

NP wash samples (instilling and withdrawing ~2 mL of saline in each nostril with bulb syringe) were collected during health visits of children at 6, 9, 12, 15, 18, 24 and 30–36 months of age. Clinical diagnosis of AOM of children with AOM symptoms between 6 and 36 months of age was made by validated otoscopist clinicians and then confirmed based on tympanocentesis with collection of MEF. NP wash samples were also obtained at AOM visits. Standard microbiology processing and identification techniques were used in detecting *H. influenzae*, *S. pneumoniae* and *Moraxella catarrhalis*.[15] *H. influenzae* isolates were confirmed using HAEMOPHILUS ID QUAD (Remel, Lenexa, KS). The number of culture-positive cases was used to calculate the prevalence of *H. influenzae* in this study. Stocks of all strains were maintained in brain heart infusion media with 20% glycerol and stored at −80 °C until further testing. *H. influenzae* isolates from healthy

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and AOM visits were tested for antibiotic susceptibility and typed by polymerase chain reaction (PCR). In cases where *H. influenzae* was isolated from both ears, only 1 ear sample was tested because previous results have shown that the same strain is isolated from both ears.[16,17] PCR testing was performed on culture-negative MEF samples that were stored in TRI-reagent (Sigma-Aldrich, MO) after microbiologic processing. A multiplex PCR procedure was used for the simultaneous detection of *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and *Alloiococcus otitidis* using primers after DNA extraction from MEF.[18]

DNA Preparation and PCR

The *H. influenzae* isolates were cultured on Chocolate II Agar (BectonDickinson, MD) plates overnight at 37 °C, 5% CO_2 and then suspended in 500 µL of phosphate-buffered saline. The suspension was centrifuged at 12,000 revolutions per minute for 5 minutes, and the supernatant discarded. DNA was extracted from the pellet using PureLink Genomic DNA Mini kit (Invitrogen, CA). Six different encapsulated *H. influenzae* strains (American Type Culture Collection number: A:9006, B: 9795, C:9007, D:9008, E: 8142, F: 9833) were used as control. Reference strains were kindly provided by Dr. Dayle Danies, Old Dominion University.

Six primers set specific for capsular types *a–f* were modified from Falla et al[19] based on the references (Table, Supplement Digital Content 1, http://links.lww.com/INF/E386). Primer sequence homology was confirmed using nucleotide-nucleotide BLAST against available *H. influenzae* sequences at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) that had both query coverage and percent identity higher than 90%. Detailed primer sequences and references are described in Table (Supplement Digital Content 1, http://links.lww.com/INF/E386). Multiplex PCR Primer sets (Set1: Type A, C, E, Set2: Type B, D, F) were optimized using Multiple Primer Analyzer software (Thermo Scientific Web Tools available at https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html). Two primary multiplex PCRs were carried out for each isolate. The PCR reaction, 30 µL, contained 1× TaqMaster Mix (ACCURIS, NJ), 1 µM (each) oligonucleotide primers (Integrated DNA Technologies, IA) and 1 µL of template DNA. Annealing temperature was set at 53 °C for 35 cycles. PCR product was checked on a 1% agarose gel and compared with positive control. Confirmation of the primary product was performed by a seminested PCR for 25 cycles under the same conditions except annealing at 55 °C and 0.5 µL of first PCR product as the template. PCR negative samples were recorded as NTHi.

Antibiotic Susceptibility

β-lactamase production of *H. influenzae* was determined using Cefinase-Disc (BectonDickinson). Antibiotic susceptibility of *H. influenzae* isolates to 13 different antibiotics (ampicillin, trimethoprim/sulfamethoxazole, cefaclor, cefuroxime, cefprozil, cefdinir, cefixime, cefpodoxime, ceftriaxone, erythromycin, azithromycin, clarithromycin, amoxicillin-clavulanate) was determined with the Sensi-Disc (BectonDickinson) by agar disc diffusion test using media recommended by Clinical and Laboratory Standard Institute (CLSI).[15] *H. influenzae* isolates were grown on Chocolate II Agar (BectonDickinson) overnight at 37 °C, 5% CO_2. The inoculum was suspended in phosphate-buffered saline and adjusted to 0.15–0.20 OD600 (equivalent to 0.5 on the McFarland scale). *Haemophilus* test medium agar was prepared with Difco Mueller-Hinton broth (BectonDickinson), Bacto Agar (BectonDickinson), Yeast Extract (BectonDickinson), Hemin (BectonDickinson, NJ) and nicotinamide-adenine-dinucleotide (Roche, Mannheim, Germany). The adjusted inoculum was plated on *Haemophilus* test medium agar within 15 minutes from adjusting OD_{600} inoculums by a sterile swab and then each antibiotic disc was placed. The inoculated plates were incubated for 16–18 hours at 37 °C, 5% CO_2. The interpretive zone sizes were measured. The isolates were classified as susceptible, intermediate or resistant based on the size of the interpretive zone by referring to current CLSI breakpoints for *Haemophilus*.[20] Erythromycin disc breakpoints are not provided in CLSI-2018 M100 guidelines for *H. influenzae.* Therefore, we used arbitrary cut off values (zone diameter: ≤15 mm = R; ≥21 mm = S). Although there is no uniform definition,[21] β-lactamase positive and ampicillin resistant (AR), β-lactamase negative and ampicillin sensitive (AS) and β-lactamase negative, AR (BLNAR) phenotypes were defined by the susceptibility of β-lactamase and AR according to the CLSI definition. For AR, we differentiated ampicillin-intermediate strains (zone diameter: 19–21 mm) as low-BLNAR and ampicillin-resistant strains (zone diameter: ≤18 mm) as BLNAR as described in previous studies.[22,23] β-lactamase–positive amoxicillin-clavulanate resistant (BLPACR) strains were defined as amoxicillin-clavulanate–resistant (zone diameter ≤19 mm) that excluded intermediate resistant isolates.[21]

Statistical Analysis

GraphPad Prism 8.2.1 (CA) was used for all statistical analyses. Nominal variables were compared using Fisher exact tests except gender and antibiotic history that were tested by Pearson χ^2 tests. Continuous variables were compared using student *t* test for 2 independent groups.

RESULTS

A total of 611 healthy visits and 130 AOM visits (Table 1) occurred among the 334 study children from September 2019 to September 2020. Two-hundred forty-four (73%) children were Caucasian, 11 (3%) African American, 13 (4%) Hispanic and 66 (20%) mixed/other race. Forty-two percent of the children were female. No significant racial or gender differences in *H. influenzae* detection during healthy colonization or AOM were identified. Median age at time of AOM visit was 16 months.

The prevalence of *H. influenzae* in the NP at healthy visits was 5.9%. At onset of AOM, *H. influenzae* was isolated in 27% of the NP samples, significantly higher compared with healthy visits (Table 1, $P < 0.0001$). β-lactamase positivity was 42% among *H. influenzae* isolates collected at healthy visits and 34% when isolates were collected at onset of AOM ($P = 0.63$, not significant). Overall, 39.7% of isolates were β-lactamase producing. *H. influenzae* was isolated from MEF in 43% of AOM cases. At onset of AOM, the detection of *H. influenzae* from the NP was 27% of cases, significantly lower than the isolation rate from MEF ($P = 0.03$). In the MEF, 43% of all AOM cases caused by *H. influenzae* were β-lactamase producing (Table 1). Thirty-three MEF samples were culture negative. In addition to the *H. influenzae* culture-positive AOM cases (43%), *H. influenzae* DNA was detected by PCR in 21% of the culture-negative MEF samples. *H. influenzae* DNA was detected by PCR in 21% of the culture-negative MEF samples.

The frequency of β-lactamase–producing *H. influenzae* strains occurring among children with risk factors, that is, day-care center attendance, number of siblings and antibiotic prescription history, was analyzed. Antibiotic prescription history in the 30 days before collection of a *H. influenzae* isolate was identified as a risk factor for β-lactamase producing (52.3%) versus not producing (34.2%) *H. influenzae* (odds ratio 2.1 [95% CI, 1.0–4.4]).

TABLE 1. Prevalence of *Haemophilus influenzae* Detection and β-Lactamase Production From NP of Healthy and AOM Visits Along With *H. influenzae* Detection in MEF During AOM of Children Isolated in September 2019 to September 2020

	NP			MEFs		
	Healthy (n = 611)	AOM (n = 130)	*P* Healthy vs. AOM	Total Taps (n = 104)	Total AOM (n = 70)	*P* AOM NP vs. MEF (Total AOM)
H. influenzae, n (%)	36 (5.9)	35 (27)	**<0.0001**	42 (40)	30 (43)	**0.03**
H. influenzae β-lactamase production, n (%)	15 (42)	12 (34)	0.63	18 (43)	13 (43)	0.45

The prevalence of *H. influenzae* was based on the isolation by culture method. The PCR results on culture negative cases were not included in the data tabulated. $P < 0.05$ was considered as significant in bold.

H. influenzae isolates were tested for capsular type. Eight of 101 *H. influenzae* isolates were encapsulated, and of these, 1 isolate was type *e* (isolated from NP during AOM) and 7 isolates were type *f* (4 from NP during AOM, 2 from MEF, 1 from a healthy visit). β-lactamase–producing strains and nonsusceptibility to ampicillin were not observed in encapsulated isolates (Table 2); therefore, β-lactamase–producing strains ($P = 0.02$) and nonsusceptibility to ampicillin ($P = 0.02$) among encapsulated isolates were significantly lower compared with NTHi.

Antibiotic susceptibility results at healthy and AOM visits against 13 different drugs are shown in Table 2. Ampicillin, trimethoprim/sulfamethoxazole, erythromycin and clarithromycin nonsusceptibility were commonly found (> 25%). Cefuroxime, cefpodoxime, ceftriaxone and amoxicillin-clavulanate nonsusceptibility were detected in small numbers of isolates (<5%). Cefaclor nonsusceptibility was significantly higher in NP isolates at onset of AOM compared with healthy visit isolates ($P = 0.04$). Prior work has shown that NP and AOM isolates are concordant, consistent with pathogenesis.[16,17] We secured more NP samples than MEF samples from the children at onset of AOM, and the additional NP samples led to differing percentages of isolates that we report as antibiotic susceptible. For susceptibility to cefaclor, comparing NP and MEF at onset of AOM, a significant difference was observed (Table 2). However, to confirm that NP and MEF isolates were concordant with regard to antibiotic susceptibility, we compared 17 NP and MEF paired isolates that were cefaclor nonsusceptible and found they were concordant for that antibiotic, and the other 12 antibiotics tested. Antibiotic susceptibility results of *H. influenzae* were further analyzed based on whether the child had prior exposure of antibiotics (30 days before the visit when *H. influenzae* was isolated). No significant difference was detected.

Based on β-lactamase production indicated as β-lactamase positive and β-lactamase negative and AR and AS, isolates were further categorized in 4 groups. 51.0% and 26.5% were β-lactamase negative and AS and β-lactamase positive and AR. 4.1% and 5.1% of isolates were defined as BLNAR and Low-BLNAR, respectively. One isolate (1%) was categorized as BLPACR based on β-lactamase production and amoxicillin-clavulanic acid-resistance and showed multidrug nonsusceptibility against trimethoprim/sulfamethoxazole, cefuroxime, cefpodoxime and ceftriaxone.

DISCUSSION

Understanding the current etiology as well as antibiotic susceptibility is very important to prescribe the most appropriate course of treatment in any clinical setting. In this study, *H. influenzae* isolates were collected from children at health and AOM visits from the NP as well as MEF at onset of AOM. Capsular types and antibiotic susceptibility were determined. Several epidemiologic studies have described *H. influenzae* being the major cause of AOM.[24,25] Since 2015, we have observed that *H. influenzae* has become the predominant otopathogen detected in MEF.[5] The results from this 1-year study support *H. influenzae* as the major cause of AOM (43% of all culture-positive isolates plus 21% of culture negative, polymerase chain reaction–positive MEF). Among all the *H. influenzae* isolates from health or AOM visits, >92% were characterized as NTHi which is consistent with previous reports (85.7–100%).[26–28]

TABLE 2. Antibiotic Susceptibility Among *Haemophilus influenzae* Isolates

	% Non susceptible (I + R)			
	NTHi			
Antibiotics	Healthy (n = 35)	AOM (NP) (n = 28)	AOM (MEF) (n = 28)	Encapsulated (n = 8)
Ampicillin	42.9	39.3	39.3	0.0*
TMP/SMX	34.3	35.7	35.7	37.5
Cefaclor	2.9†	21.4†	7.1	12.5
Cefuroxime	0.0	3.6	7.1	0.0
Cefprozil	5.7	17.9	7.1	25.0
Cefdinir	2.9	10.7	7.1	0.0
Cefixime	2.9	3.6	10.7	0.0
Cefpodoxime	2.9	3.6	3.6	0.0
Ceftriaxone	2.9	3.6	7.1	0.0
Erythromycin	80.0	67.9	58.6	62.5
Azithromycin	5.7	7.1	3.4	0.0
Clarithromycin	28.6	21.4	27.6	37.5
Amoxicillin-clavulanate	0.0	7.1	3.4	0.0

*Ampicillin nonsusceptibility in total NTHi and encapsulated *H. influenzae* $P = 0.02$.
†$P = 0.04$.

In our population of 101 *H. influenzae* isolates, 8 were encapsulated: 7 *H. influenzae* type *f* and 1 *H. influenzae* type *e*. Since the introduction of the Hib vaccine in 1985, the incidence of invasive Hib disease dramatically decreased. Invasive disease has become mainly caused by NTHi (2018, 43/87 49.4%) among less than 5 years old children.[29] *H. influenzae* capsule type *f* has been reported to be the most prevalent type found in invasive diseases among children below 5 years old, followed by type *e*.[3] Emergence of Hia has also been reported, especially in American Indian and Alaska Natives.[3,30] Although some studies detected Hia from MEF at onset of AOM[31,32] as well as during healthy colonization in children,[33] we did not detect any Hia cases. This result might reflect that limited circulation of Hia in our cohort. We found that encapsulated *H. influenzae* isolates were more frequently β-lactamase negative and susceptible to ampicillin, a result similar to reports regarding invasive diseases occurring in France in 2017[34] and Italy in 2012–2016.[35]

The antibiotic recommended by American Academy of Pediatrics[4] as the first-line treatment of AOM is amoxicillin. Amoxicillin is also the first-line antibiotic for children with AOM in most European countries.[36] In our cohort study, 39.7% of all *H. influenzae* isolates and 43% of AOM-causing isolates were β-lactamase producing. We found recent antibiotic prescription was a risk factor of detection of β-lactamase–positive strains but not to specific antibiotics.

A report involving *H. influenzae* isolates from the NP at the time of AOM children collected from 2012 to 2014 described 30% β-lactamase–producing strains.[37] In 2019, Wald and DeMuri[38] reported that 31 (44%) of 71 NP isolates obtained from 228 children with presumptive sinusitis were β-lactamase positive.

We found an increase in β-lactamase–producing strains associated with recent antibiotic exposure, similar to the work by Eliasson et al.[39] Two prior studies of *H. influenzae* did not find an increase in β-lactamase–producing strains associated with recent antibiotic exposure (2–3 months before the isolation of *H. influenzae*).[40,41] This result differs from those observed with *S. pneumoniae*, where a more consistent association has been observed between recent antibiotic exposure and increased isolation of antibiotic nonsusceptible strains.[42]

In the case of penicillin allergies, cefdinir, cefuroxime, cefpodoxime and ceftriaxone are recommended.[4] The overall rate of nonsusceptibility to these 4 antibiotics was low. However, there were notable differences in nonsusceptibility rates to cephalosporins among NP isolates when comparing healthy and AOM visits. Among NP isolates collected at health, rates of nonsusceptibility to cefaclor, cefuroxime, cefprozil, cefdinir and amoxicillin-clavulanate were consistently lower than among NP isolates collected at onset of AOM. Antibiotic nonsusceptibility rates for cephalosporins, amoxicillin and amoxicillin-clavulanate among NP isolates collected at onset of AOM matched better with MEF isolates than NP isolates collected at times of health. The further analysis on paired samples (NP and MEF at the time of AOM) confirmed concordance in antibiotic susceptibility. The results suggest that antibiotic susceptibility testing of NP isolates collected at onset of AOM better correlates with results of MEF cultures when MEF isolates are not available.

We detected 9.2% (BLNAR + low-BLNAR) in our cohort. In an early multicenter studies in the United States (1994–1995, 2000–2001), the prevalence rate of BLNAR *H. influenzae* strains was low (2.5% BLNAR + low-BLNAR and 0.9%/0.1% BLNAR/low-BLNAR, respectively).[43,44] 6.8% of invasive *H. influenzae* cases from 2013 to 2016 reported from European countries were caused by BLNAR strains[45] and 6.9% in France from 2017.[34] Perhaps, BLNAR strains have started circulating more commonly in the United States. Furthermore, we also detected 1 case of BLPACR that was from an AOM treatment failure case. This isolate showed multidrug resistance as defined by nonsusceptibility to 3 or more antibiotic categories.[46,47] Continuous epidemiologic monitoring and antibiotic susceptibility of circulating strain of *H. influenzae* is required.

We have limitations to note. This study involved collection of *H. influenzae* isolates during 1 year and derived from a predominantly middle-class, suburban population of children in upstate NY. Therefore, our results may not be representative of other types of populations in the United States or those in other countries. During the second half of this study period, a NY State "stay at home" executive order because of ongoing coronavirus disease 2019 circulation was implemented that may have influenced the results. Another limitation is we adapted a phenotypic definition for BLNAR and BLPACR by following CLSI guidelines. Guidelines and interpretation criteria for ampicillin susceptibility set by different committees differ (CLSI: 10 μg disc content, >2 μg/mL minimum inhibitory concentration breakpoint, whereas European Committee on Antimicrobial Susceptibility Testing: 2 μg disc content, >1 μg/mL minimum inhibitory concentration breakpoint).[15,48] Some groups have reported higher sensitivity to detect BLNAR (AR) with a reduced dose of disc[22,49] that would favor European Committee on Antimicrobial Susceptibility Testing susceptibility methods.

In conclusion, this study provides novel data on the prevalence of *H. influenzae* at the time of health and AOM in children, the proportion of capsulated strains and antibiotic susceptibility among strains collected during colonization and onset of AOM in children during 2019–2020. While the results provide overall and up-to-date trends of antibiotic susceptibility of *H. influenzae* in children, the situation is dynamic, supporting the need for continuous monitoring of *H. influenzae* as a pathogen.

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