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MAJOR ARTICLE

Changes Over Time in Nasopharyngeal Colonization in Children Under 2 Years of Age at the Time of Diagnosis of Acute Otitis Media (1999–2014)

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Background. In children with acute otitis media (AOM), a decrease in nasopharyngeal (NP) colonization with vaccine serotypes of *Streptococcus pneumoniae* has been noted since the introduction of pneumococcal conjugate vaccines (PCVs). The purpose of this study is to describe corresponding changes in colonization with *Haemophilus influenzae*.

Methods. In 4 separate studies, we obtained NP cultures from children aged 6–23 months presenting with AOM. Cohort 1 was recruited before routine use of PCV7 (1999–2000); 93% of children in cohort 2 (2003–2005) and 100% in cohort 3 (2006–2009) received ≥2 doses of PCV7. All children in cohort 4 (2012–2014) received ≥2 doses of PCV13. Isolates of *H. influenzae* were tested for ß-lactamase production; ß-lactamase negative isolates from cohorts 3 and 4 underwent susceptibility testing.

Results. A total of 899 children were evaluated. NP colonization with *H. influenzae* was found in 26% of children in cohort 1 (n = 175), 41% in cohort 2 (n = 87), 33% in cohort 3 (n = 282), and 29% in cohort 4 (n = 355). Colonization with *H. influenzae* increased initially from cohort 1 to cohort 2 (P = .01), then decreased across cohorts 2, 3, and 4 (P = .03, test for trend). The prevalence rates of ß-lactamase production were 27%, 42%, 33%, and 30% in each of the 4 cohorts, respectively (P = .50).

Conclusions. Although an initial increase in *H. influenzae* colonization was observed, suggesting an impact of PCVs, the most recent prevalence rates of NP colonization with *H. influenzae* and ß-lactamase production were like those observed before universal administration of PCV7. This knowledge is critical to guide appropriate treatment recommendations for children with AOM.

Keywords. AOM; *Haemophilus influenzae*; *Streptococcus pneumoniae*; nasopharyngeal colonization.

Streptococcus pneumoniae and *Haemophilus influenzae* are important pathogens in young children with acute otitis media (AOM). Researchers have suggested that the interaction of viral upper respiratory infections with the bacteria that colonize the nasopharyngeal (NP) may facilitate the transition from bacterial commensal to pathogen in episodes of AOM [1]. Colonization with *S. pneumoniae* and *H. influenzae* is dynamic. Both the introduction of pneumococcal conjugate vaccines and antibiotic selective pressure have resulted in changes in the distribution of these pathogens over time. A 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in the United States in 2000. Widespread adoption of this vaccine led to a dramatic decrease in the incidence of invasive pneumococcal disease in children [2, 3] and to a modest reduction in the incidence of AOM [4–6]. Shortly after the introduction of PCV7, several reports described an initial decrease in colonization with *S. pneumoniae* and an increase in colonization with *H. influenzae* in both healthy children and children with AOM [7, 8]. Between 2008 and 2010, it appeared that prevalence of colonization with *H. influenzae* remained constant [9, 10]. An expanded 13-valent pneumococcal vaccine (PCV13) was introduced in the United States in 2010 [11]; subsequent evidence assessing the impact of PCV13 on NP colonization with *H. influenzae* has been limited [12].

In the present report, we describe changes observed over time regarding NP colonization with *H. influenzae* in 4 cohorts of young children diagnosed with AOM whom we studied during 4 separate periods, respectively: prior to routine vaccination with PCV7, early after the introduction of PCV7, late after PCV7 became routine, and after introduction of PCV13. We also assessed ampicillin resistance in these isolates due to either β-lactamase production (for all isolates) or for ampicillin resistance in isolates that were β-lactamase negative (for those obtained after 2006). Continued surveillance of colonization and antibiotic resistance patterns is critical to guide appropriate treatment recommendations for young children with AOM.

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METHODS

The present report involves data derived from 4 separate clinical trials conducted by our group in children 6 to 23 months of age diagnosed with AOM. Cohort 1 consisted of children enrolled in a study of the effectiveness of influenza vaccine in preventing AOM before the introduction of PCV7 (1999–2000) [13]. Cohort 2 comprised children who were part of a surveillance study of NP colonization in children with AOM early after the introduction of PCV7 (2003–2005) [14]. Cohort 3 included children who were enrolled in a clinical study of antimicrobials vs placebo for AOM, late after use of PCV7 became routine (2006–2009) [15]. Cohort 4 included children who were enrolled in a randomized clinical trial comparing the efficacy of 5 vs 10 days of antimicrobial treatment for AOM after the introduction of PCV13 (2012–2014) [16]. All data were collected from children 6 to 23 months of age who had an NP swab obtained. In some instances, a subgroup of the children enrolled in the original study were selected for analysis in order to make the groups comparable. Specifically, we only included the results of an NP culture that was obtained at the time of diagnosis of a new episode of AOM, before treatment with antimicrobials. For participants who may have had multiple NP swabs obtained during the course of the specific study, we only included results from the first culture performed at the time of diagnosis of the first episode of AOM during study participation.

All children in cohorts 3 and 4 were required to have received at least 2 doses of the available pneumococcal vaccine as a condition for inclusion. In our area, vaccination coverage with PCVs has been >80% since 2004; the current rate is 87.4% [17]. *H. influenzae* type B vaccine was widely available in the United States for infants since the early 1990s and is not believed to impact colonization rates of nontypeable *H. influenzae* [18]. For each study, we obtained written, informed consent from the children's parent(s) before undertaking any research procedures, and all 4 studies were approved by the University of Pittsburgh or Western Institutional Review Boards. We recruited children from the Primary Care Center and the Emergency Department of the Children's Hospital of Pittsburgh of UPMC, its affiliated Pediatric PittNet Practice-Based Research Network, and a pediatric practice in rural Kentucky (S.L.B.). A medical record review was conducted at the time of study enrollment to ensure that each participant met all the inclusion and exclusion criteria and verified vaccine status. We excluded children with Down syndrome, any craniofacial abnormality, known immunodeficiency, or indwelling tympanostomy tube(s). Children who were toxic in appearance or hospitalized at the time the NP swab was obtained were excluded.

Procedures for examining children, diagnosing AOM, obtaining and processing NP specimens, and statistical analysis have been described, in detail, previously [14, 19]. In brief, examinations were conducted by validated otoscopists using pneumatic otoscopy. The diagnosis of AOM was based on the presence of middle-ear effusion accompanied by one or more of the following: acute ear pain, marked redness of the tympanic membrane, and substantial bulging of the tympanic membrane. The criteria for diagnosing AOM were identical across all cohorts. NP specimens were obtained using a dacron tipped swab in the standard fashion in order to sample the NP. Each swab was placed in Amies transport media without charcoal and inoculated onto chocolate agar and 5% sheep blood agar. Cultures were incubated with 5% CO_2 for 48 hours at 37°C and processed using standard techniques in the same laboratory (M.G.) [14, 19]. The presence of *H. influenzae* or *S. pneumoniae* by bacterial cultures performed on the NP swabs was considered to indicate colonization with these organisms.

All isolates of *H. influenzae* were screened, and the typeable isolates were serotyped based on agglutination. β-lactamase production was assessed with a cefinase disk screen. All isolates found to be β-lactamase negative in cohort 3 were tested for susceptibility to ampicillin by the E-test method, and in cohort 4 isolates were tested for susceptibility by broth microdilution methods according to the most current Clinical Laboratory Standards Institute (CLSI) guidelines [20]. Isolates of *H. influenzae* that were ß-lactamase negative were considered to be ampicillin nonsusceptible if the minimum inhibitory concentration (MIC) was >1 μg/mL. Due to limitations in funding, no further assessment was performed to identify possible gene mutations.

We defined recurrent AOM as the occurrence of ≥3 episodes in 6 months or ≥4 episodes in 12 months and day care attendance as the exposure to ≥3 children ≥10 hours per week.

Statistical Analyses

We used a logistic regression model to compare the cohorts regarding the distribution of *H. influenzae*. We conducted tests for trend using the Cochran-Armitage test for trend in binomial proportions. We performed all analyses using 2-sided tests with the significance level set at an α of .05. Demographic data were compared using the chi-square test. For all analyses, we used SAS software (version 9.3; SAS/STAT 9.3 User's Guide, SAS Institute Inc., Cary, NC).

RESULTS

Demographics

A total of 899 children were enrolled in the 4 cohorts. Age, sex, race, and other demographic and clinical characteristics are described in Table 1. There were differences between the cohorts for race, maternal education, health insurance status, exposure to household cigarette smoke and incidence of recurrent AOM. There were no significant differences in demographic and clinical characteristics between children in cohorts 3 and 4. All children in cohort 1 had no PCV doses; 93% of the children in cohort 2 and all children in cohort 3 had at least 2 doses of PCV7, while all children in cohort 4 had at least 2 doses of PCV13 (90% received at least 3 doses).

Table 1. Demographic and Clinical Characteristics of Young Children Aged 6–23 Months Enrolled in the Studies

	Cohort 1 1999–2000 (n = 175) No. (%)	Cohort 2 2003–2005 (n = 87) No. (%)	Cohort 3 2006–2009 (n = 282) No. (%)	Cohort 4 2012–2014 (n = 355) No. (%)	Total (n = 899) No. (%)	*P*
Pneumococcal vaccination status	≥2 doses	≥2 doses of PCV7	≥2 doses of PCV7	≥2 doses of PCV13		
	0 (0)	81/87 (93)	282 (100)	355 (100)		
Age at entry, mo						
6–11	73 (42)	42 (48)	156 (55)	178 (50)	449 (50)	
12–17	58 (33)	30 (35)	76 (27)	105 (30)	269 (30)	.15
18–23	44 (25)	15 (17)	50 (18)	72 (20)	181 (20)	
Sex						
Male	95 (54)	50 (57)	149 (53)	185 (52)	479 (53)	.82
Female	80 (46)	37 (43)	133 (47)	170 (48)	420 (47)	
Race						
Caucasian	100 (57)	28 (32)	124 (44)	166 (47)	418 (47)	.001 white vs nonwhite
African American	59 (34)	53 (61)	119 (42)	149 (42)	380 (42)	
Other	16 (9)	6 (7)	39 (14)	40 (11)	101 (11)	
Maternal education						
Less than high school	13 (7)	19 (22)	40 (14)	42 (12)	114 (13)	
High school graduate	112 (64)	54 (62)	179 (64)	220 (62)	565 (63)	.02
College graduate	50 (29)	14 (16)	62 (22)	92 (26)	218 (24)	
Unknown	0	0	1	1	2	
Health insurance status						
Private	94 (54)	17 (20)	81 (29)	109 (31)	301 (34)	
Public	71 (40)	70 (80)	196 (69)	241 (68)	578 (64)	<.001
None	10 (6)	0 (0)	5 (2)	5 (1)	20 (2)	
Exposure to household cigarette smoke						
Yes	57 (33)	31 (36)	84 (30)	84 (24)	256 (28)	.05
No	118 (67)	56 (64)	198 (70)	271 (76)	643 (72)	
Other children in household						
Yes	116 (66)	59 (68)	185 (66)	241 (68)	601 (67)	.93
No	59 (34)	28 (32)	97 (34)	114 (32)	298 (33)	
Recurrent AOM[a]						
Yes	52 (30)	11 (13)	49 (17)	68 (19)	180 (20)	.002
No	123 (70)	76 (87)	233 (83)	287 (81)	719 (80)	
Day care[b]						
Yes	58 (33)	26 (30)	137 (49)	198 (56)	419 (47)	<.001
No	117 (67)	61 (70)	145 (51)	157 (44)	480 (53)	

[a]Defined as at least 3 AOM episodes in the preceding 6 months or 4 episodes in the preceding year.

[b]Defined as ≥10 hours per week with ≥3 other children.

Comparison of the Cohorts

NP colonization with *H. influenzae* increased from 26% in cohort 1 (prior to PCV7) to 41% in cohort 2 (2003–2005; *P* = .01) (Table 2), then decreased across cohorts 2, 3, and 4 from 41% to 33% and 29%, respectively (*P* = .03, test for trend). The odds ratio for NP colonization with *H. influenzae* for the comparison of cohort 1 to cohort 2 is 2.04, with a 95% confidence interval of 1.18–3.52. The prevalence rates of β-lactamase production were 27%, 42%, 33%, and 30% in each of the 4 cohorts, respectively (*P* = .51). Prevalence rates of ampicillin nonsusceptibility in ß-lactamase-negative isolates were only analyzed for cohorts 3 and 4. In cohort 3, only 1 of 63 isolates had an MIC >1 μg/mL. In cohort 4, 3 (4%) of 72 isolates of *H. influenzae* had an MIC of 1 μg/mL, and 4 (6%) of 72 isolates had an MIC >1 μg/mL. The majority of the *H. influenzae* isolates in all cohorts were nontypeable. Colonization with *S. pneumoniae* did increase slightly but was not statistically different between cohorts 1 and 2 (*P* = .10). The test for trend across cohorts 2, 3, and 4 was not significant (*P* = .10).

Figures 1 and 2 show the proportion of children in each cohort who were colonized with *S. pneumoniae*, *H. influenzae,* both, or neither. The proportion of children colonized with both pathogens simultaneously did not differ in each of the 4 cohorts (*P* = .74). Logistic regression was used to adjust for cohort differences in race, maternal education, health insurance status, exposure to household cigarette smoke, incidence of recurrent AOM, and day care, respectively. After adjusting, results were

Table 2. Bacterial Culture Results From Nasopharyngeal Specimens Obtained at the Time of Diagnosis of Acute Otitis Media From Children Aged 6–23 Months

Study Topic	Cohort 1 Effectiveness of Influenza Vaccine in Preventing AOM	Cohort 2 Pneumococcal NP Colonization Before and After Introduction of PCV7	Cohort 3 Antibiotic vs Placebo for AOM	Cohort 4 5 d vs 10 d of Antibiotic for AOM
Period	1999–2000	2003–2005	2006–2009	2012–2014
No. of children from parent study with NP culture obtained at diagnosis of AOM	175	87	282	355[a]
NP colonization with *H. influenzae*	45/175 (26%)	36/87 (41%)	94/282 (33%)	103/355 (29%)
H. influenzae ß-lactamase negative	33/45 (73%)	21/36 (58%)	63/94 (67%)	72/103 (70%)
Ampicillin MIC ≤ 1 µg/mL	NA	NA	62	68
Ampicillin MIC > 1 µg/mL	NA	NA	1	4

Abbreviations: AOM, acute otitis media; MIC, minimum inhibitory concentration; NA, not available, no ampicillin testing was done in the first 2 cohorts; NP, nasopharyngeal; PCV13, 13-valent pneumococcal conjugate vaccine; PCV7, 7-valent pneumococcal conjugate vaccine.

[a]Enrolled prior to March 30, 2014.

similar to the unadjusted results. While the 4 cohorts differed in day care attendance, there was no significant interaction between day care and each cohort ($P = .60$). In addition, there was no significant difference between cohorts, adjusting for day care ($P = .45$).

DISCUSSION

The findings reported here describe our serial observations of colonization with *H. influenzae* in young children diagnosed with AOM beginning prior to the introduction of PCV7 and ending after the introduction and widespread use of PCV13. Following introduction of PCV7, we initially observed an increase in NP colonization with *H. influenzae* (41% between the years 2003–2005). However, ongoing surveillance of colonization prevalence of *H. influenzae* in cohorts 3 and 4 (33% and 29%, respectively) showed a regression trend toward the NP colonization prevalence observed before introduction of PCV7 (26% in 1999–2000). Remarkably, the prevalence of colonization with either *S. pneumoniae* or *H. influenzae* stayed the same over this time period despite changes in pneumococcal vaccine history in the children (Figure 2).

Faden et al. examined the relationship between NP colonization and risk of development of otitis media in young children and described how this fluctuates over time [21]. Acquisition of NP colonization and how it changes with immunization is dynamic. An unexpected benefit of the introduction of *H. influenzae* type B conjugate vaccines was its impact on decreasing NP colonization with this pathogen (not the nontypeable *H. influenzae* that causes AOM) as a result of herd protection [18, 22]. Subsequent introduction of pneumococcal conjugate vaccines raised the expectation that they would also reduce NP

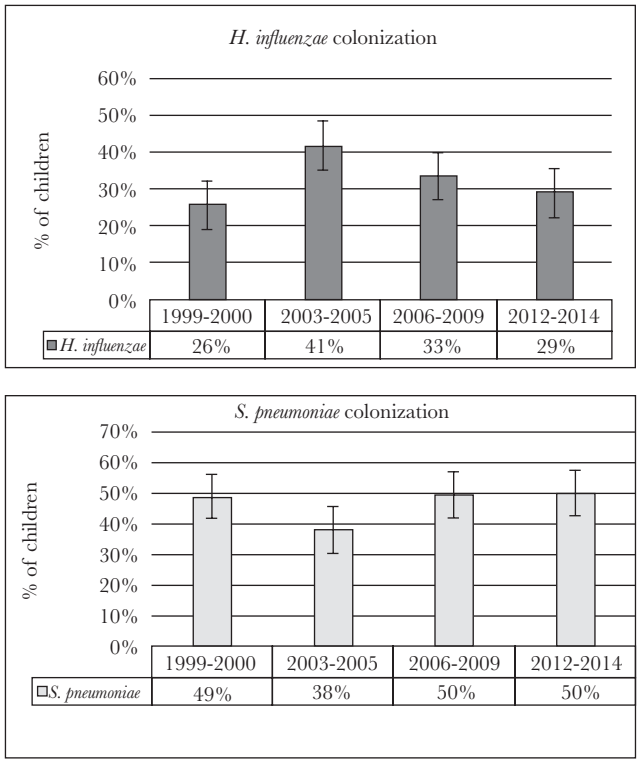

Figure 1. Percentage of children colonized with *H. influenzae* or *S. pneumoniae* by cohort.

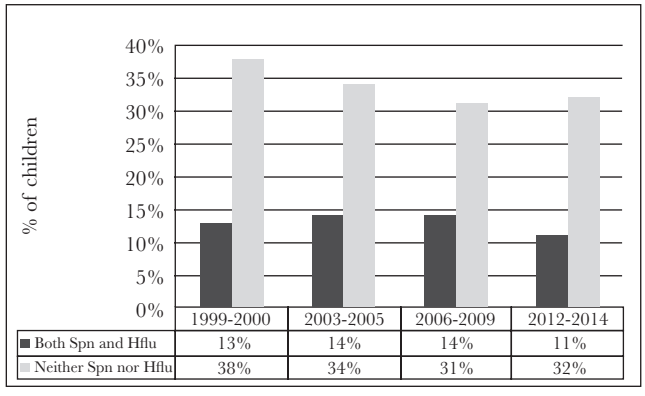

Figure 2. Dual colonization with Spn and Hflu.

colonization with *S. pneumoniae,* and subsequent episodes of AOM and invasive disease. Our data suggest that the initial decline in NP colonization with vaccine serotypes of *S. pneumoniae* associated with introduction of PCV7 created an ecologic opportunity for expansion of colonization with nontypeable *H. influenzae*. Our previous report of these same 4 cohorts describes the changes in *S. pneumoniae* serotypes in each group [19]. These observations have also been noted by other authors [23, 24]. However, our timeline extends beyond 2010, and we hypothesize that subsequent colonization with replacement strains of *S. pneumoniae* resulted in a change in the dynamics, with a return to the baseline prevalence of colonization with *H. influenzae* that we observed. These 2 organisms have the same ecologic niche and seem to balance each other [23, 24]. Some authors have suggested that this dynamic may play a role in biofilm formation, which certainly warrants further investigation [25–27].

The most common mechanism by which *H. influenzae* becomes resistant to antibiotics is production of β-lactamase. Prevalence of ampicillin resistance due to ß-lactamase production remained stable over the 4 cohorts, with 27%–42% of all *H. influenzae* isolates being ß-lactamase positive. This observation is similar to previous reports [7, 9, 23, 28]. Recent interest has focused on isolates of *H. influenzae* that are β-lactamase negative and resistant to ampicillin due to mutations in the *fts1* gene that codes for PBP-3. Isolates expressing this phenotype are referred to as β-lactamase negative ampicillin resistant (BLNAR) [29, 30]. While in general BLNAR prevalence rates have remained low worldwide, several countries (Korea, Spain, and France) have reported increased prevalence. Prevalence rates as high as 34.5% have been reported in children with *H. influenzae* type B meningitis in Japan [31–34]. Relatively few studies have reported US prevalence, but it is believed to be low (0.6%) [35, 36]. Efforts to accurately determine current and potential changes in the prevalence of colonization and disease with BLNAR *H. influenzae* are limited by lack of consensus on how to best identify these isolates. Garcia-Cobos et al. carried out a comparison of microdilutional MIC testing with susceptibility testing done using disk diffusion and E-test methodologies for isolates of *H. influenzae* that are β-lactamase negative. These authors recommended testing with the broth microdilution method and suggested breakpoints for BLNAR isolates for ampicillin and amoxicillin (susceptible, MIC ≤ 0.5 μg/mL; intermediate, MICs = 1–2 μg/mL; and resistant, MICs ≥ 4 μg/mL) [29]. Our group has routinely performed MIC testing on β-lactamase-negative isolates of *H. influenzae* since 2006, observing an increase in MICs over time. We identified a single nonsusceptible isolate between 2006 and 2009; however, in cohort 4, we identified 7 (10%) of 72 isolates with an MIC ≥1 μg/mL, with 4 (6%) having an MIC of ≥2 μg/mL by the broth microdilution method. This represents the phenotypic appearance of a possible BLNAR; however, further testing for the resistance mechanisms would be required to further characterize these isolates.

A limitation of our study is that these cohorts differed in some demographic characteristics that could influence NP colonization, such as race, maternal education, health insurance status, exposure to household cigarette smoke, incidence of recurrent AOM, and day care. To address this, we adjusted for these differences, and the adjusted results were similar to the unadjusted results. Regarding NP colonization with *H. influenzae*, there was no significant interaction between day care and cohort (P = .60) and no significant difference between cohorts, adjusting for day care (P = .45). Most importantly, the age of the children enrolled and the stringency in the diagnosis of AOM were similar for all cohorts [13–16, 19]. We report only NP colonization, and extrapolation of these data to the treatment of AOM should be made with caution. Studies have shown that pathogens causing AOM are almost invariably simultaneously recovered from the nasopharynx and that NP pathogens recovered at the time of diagnosis of AOM more likely reflect the epidemiology of otopathogens than NP pathogens recovered from healthy children [9, 12, 37]. An additional limitation of this study is that the methods for identification of *H. influenzae* isolates may not have distinguished *H. influenzae* from isolates of *H. haemolyticus* that were not hemolytic [38, 39]. However, all specimens were identified in the same laboratory (M.G.) using identical methods; therefore, trends should accurately reflect changes over time.

Continued surveillance will be required to monitor the effect of PCV13 on colonization with *S. pneumoniae* and *H. influenzae* and overall changes in antibiotic resistance. The current prevalence rates of ampicillin nonsusceptibility in ß-lactamase-negative isolates of *H. influenzae* appear to be low, but ongoing observation is warranted. This knowledge is critical to guide appropriate treatment recommendations for young children with AOM.

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