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When Co-Colonizing the Nasopharynx *Haemophilus influenzae* Predominates over *Streptococcus pneumoniae* Except Serotype 19A Strains to Cause Acute Otitis Media

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Abstract

Of 368 acute otitis media (AOM) cases among 7-valent pneumococcal conjugate (PCV-7) vaccinated children, 43.5% were colonized by multiple otopathogens in the nasopharynx but only 7.1% experienced polymicrobial AOM. When co-colonization occurred, *Haemophilus influenzae* predominated over all *Streptococcus pneumonia* strains except 19A strains to cause AOM. *Haemophilus influenzae* and *Streptococcus pneumonia* both predominated over *Moraxella* catarrhalis to cause AOM.

Keywords

Streptococcus pneumoniae (Spn); Haemophilus influenzae (Hflu); Moraxella catarrhalis (Mcat); polymicrobial colonisation; acute otitis media

Introduction

The prevalence of *Streptococcus pneumoniae (Spn)* as a bacterial pathogen decreased following the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7); however, the relative increase in proportion of non-PCV7 serotypes following PCV7 introduction soon resulted in non-PCV7 serotypes, mainly serotype 19A, becoming a major otopathogen (1). A serotype 19A strain of *Spn* was isolated from children in Rochester NY that was resistant to all 18 antibiotics approved for the acute otitis media (AOM) treatment indication in children (2).

Spn, Haemophilus influenzae (Hflu), and *Moraxella* catarrhalis (Mcat) are the most common bacterial pathogens associated with AOM in the US. The initial step of AOM pathogenesis is nasopharyngeal (NP) colonization by otopathogenic bacteria. Although otopathogenic bacteria often coexist in the NP, knowledge is limited regarding which otopathogens are more likely to predominate in causing AOM when multiple otopathogens colonize the NP. We hypothesized that polymicrobial otopathogen NP colonization generally results in a predominant species progressing from the NP via the eustachian tube to the middle ear. In this study we sought to determine which otopathogens successfully emerged from

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polymicrobial NP colonization and caused AOM in PCV7-vaccined children. Based on our previous work describing the success of *Spn* expressing serotype 19A as a frequent otopathogen in the PCV7 vaccine era, we especially sought to determine the outcome of polymicrobial NP colonization by 19A strains compared with non-19A strains.

Material and Methods

Study design

This study is a secondary analysis of data collected from June 2006 to end of 2010 from children enrolled in a 5-year prospective study supported by the National Institute on Deafness and Communication Disorders. Healthy children without previous episodes of AOM were enrolled at 6 months of age from a middle class, suburban sociodemographic pediatric practice in Rochester, NY. When AOM was suspected, a tympanocentesis was performed to confirm the diagnosis as previously described (1). At the time of an AOM diagnosis, middle ear fluid (MEF), nasal swab (NS) and oropharyngeal (OP) samples were obtained for bacterial pathogen cultures. Hereafter NP results include those obtained from both NS and OP samples. All of the children received age-appropriate standard vaccinations including the PCV-7 (Prevnar, Wyeth Pharmaceuticals, Collegeville, PA). The demographic characteristics of the children were similar to those described previously (1). This study was approved by the Institutional Review Board of University of Rochester and Rochester General Hospital, and written informed consent was obtained from parents of all children prior to enrollment in the study.

Sample collection and microbiology

Sample collection, the tympanocentesis procedure and microbiology methods were as previously described (1). All *Spn* isolates were serotyped by Quellung reaction.

Statistics—Statistical significance was determined by repeated measures logistic regression with generalized estimating equations and Fisher's exact test. All p values were two tailed and a p value of < 0.05 was considered significant.

Results

We analyzed NP and MEF samples obtained during 368 AOM visits (including 198 first AOM and 170 recurrent AOM) from 231 children aged 6-30 months. There were no significant differences in culture positive rates of multiple otopathogens according to subject age, gender, or between first and recurrent AOM (data not shown). Overall, the culture positive rate of multiple otopathogens in the NP was 43.5% (160/368), which was significantly higher than the frequency of polymicrobial AOM (7.1%, 26/368, p<0.0001). The NP positive rate of the combination *Spn-Mcat* (16.0%, 59/368) was significantly higher than *Hflu-Mcat* (7.1%, 26/368, p=0.002), and *Spn-Hflu-Mcat* (8.7%, 32/368, p=0.003), but not higher than *Spn-Hflu* (11.7%, 43/368, p=0.1). The MEF culture positive rate of combinations *Spn-Mcat* (4.3%, 16/368) was significantly higher than *Hflu-Mcat* (0.3%, 1/368, p=0.0002), and than *Spn-Hflu-Mcat* (0%, 0/368, p<0.0001), but not higher than *Spn-Hflu* (2.4%, 9/368, p=0.2). Each combination had higher positive rates in NP than in MEF (all p values <0.0001).

To investigate which species was more likely to cause AOM when multiple otopathogens were in the NP, we analyzed the culture outcomes of each otopathogen in MEF when multiple otopathogens were present in the NP. *Spn* isolates with the same serotype in NP and MEF were considered identical. Since there is no serotyping for *Hlfu,* we used multilocus sequencing typing (MLST) data from the children as previously reported (3). The

Hflu isolates with same MLST in NP and MEF were considered identical. Among the 43 AOM cases when both *Spn* and *Hflu* were simultaneously detected in the NP, *Hflu* AOM cases (65.1%) were significantly higher than *Spn* AOM cases (25.6%, p=0.0005 Figure 1A). Among 59 AOM cases when both *Spn* and *Mcat* were simultaneously detected in the NP, *Spn* AOM (64.4%) were significantly higher than *Mcat* AOM cases (39.0%) (p=0.01, Figure 1B). Among 26 AOM cases when both *Hflu* and *Mcat* were simultaneously detected in the NP, *Hflu* AOM cases (69.2%) were significantly higher than Mcat cases (23.1%) (p=0.002, Fig.1C). Among the 32 AOM cases that were culture positive for all three otopathogens in the NP, 50.0% were culture positive in MEF for *Hflu*, 28.1% for *Spn*, and 3.1% for *Mcat* (Fig.1D).

To assess whether strains of *Spn* expressing particular serotypes were more likely to cause AOM, we analyzed the proportion of each individual serotype that was present in MEF when detected in the NP. Overall, we found that 19A was the most frequent serotype, followed by serotype 15 in both the NP and MEF. Among 188 visits that were culture-positive in the NP for *Spn*, 36.2% expressed the 19A serotype, and 16.5% expressed serotype 15. Among 99 *Spn* AOM episodes, 43.4% were caused by 19A strains and 16.2% were caused by serotype 15 strains. The subtypes of serotype 15 were not determined. When present in NP, 19A strains (63.2%, 43/68) were more likely than non-19A strains than (46.3%, 57/123) to be present in MEF to cause AOM (p=0.03).

At the time of AOM, when *Spn co*-colonized with *Hflu* in the NP, the culture-positive rate of *Hflu* predominated over non-19A strains (64.7%, 33/51 vs. 19.6%, 10/51, p<0.0001), but not 19A strains (42.9%, 9/21 vs. 42.9%, 9/21, p=1.0);The culture-positive rate of 19A strains (42.9%, 9/21) in MEF was significant higher than that of non-19A strains (19.6%, 10/51, p=0.04, Figure 1E). When *Spn* co-colonized with *Mcat* in the NP, both 19A (61.8%, 21/34) and non-19A strains of *Spn* (45.6%, 26/57) predominated over *Mcat* (26.5%, 9/34, p=0.007, 26.32%, 15/57, p=0.05, respectively), but there was no difference in the culture-positive rate in MEF between 19A (61.8%, 21/34) and non-19A strains (45.6%, 26/57, p=0.2, Figure 1F).

Discussion

This study investigated the predominant otopathogens causing AOM when multiple otopathogens co-colonize the NP. We found that: (1) culture-positive rates of multiple otopathogens in NP were significantly higher than those in MEF. The combination of *Spn*-*Mcat* was the most frequent multiple otopathogen combination in both the NP and MEF; (2) when multiple otopathogens co-colonized the NP, *Hflu* predominated over *Mcat* and non-19A but not 19A *Spn* strains. *Hflu* and *Spn* predominated over *Mcat* to cause AOM; (3) *Spn* expressing the 19A capsular serotype of *Spn* colonized the NP more frequently than any other capsular types, and 19A strains were more likely than non-19A strains to cause AOM when they colonized the NP.

There are previous studies involving AOM describing rates of multiple bacterial pathogens in MEF (4, 5), however the results are inconsistent probably due to differences in population dynamics, PCV-7 vaccine use, frequency of viral upper respiratory tract infections, antibiotics use, otopathogen culture or detection methods, study designs, diagnostic method s (tympanostomy or otorrhea) and others. The rate in this study (7.1% of AOM cases) is consistent with other prior studies in the US (6).

Current knowledge is limited regarding which otopathogens have a greater capacity to cause AOM when multiple bacterial otopathogens co-colonize the NP. Similar to our results, Syrijanen et al (7) found that if both *Spn* and *Hflu* were in the NP, *Hflu* was more likely

cultured from MEF. However, we also found that the predominance of *Hflu* over Spn was dependent on the capsular serotype. While *Hflu* predominated over *Spn* expressing non-19A serotypes, this wan not the case for 19A *Spn* stains. The implications of these findings may be that elimination of *Spn* strains expressing the 19A serotype by vaccination with PCV-13 results in an increased predominance of *Hflu* as an otopathogen. Since we found that both *Hflu* and *Spn* appear to predominate over *Mcat* when co-colonization occurs it seems less likely that *Mcat* will emerge as a more important otopathogen in the near future.

Interactions among bacterial optothogens may influence species persistence in the NP. Negative associations have been observed between otopathogens in previous report(8). Host innate immune responses during concurrent colonization by otopathogens may play an important role in the outcome of interspecies interactions at mucosal surfaces (9). PCV-7 vaccination changes the environment in the NP by eliminating vaccine-strains. That in turn changes the balance between strains, reflecting their absolute fitness, ultimately shifting their abundance (10). Our previous studies have shown that a proportional reduction in NP colonization and AOM caused by *Spn* following introduction of PCV-7 vaccine was subsequently followed by a gradual and steady resurgence of *Spn* strains (1), led by serotype 19A capsular organisms (2). In the long-term, disease caused by non PCV-7 strains could partially undermine the impact of the vaccine.

Limitations of this study are that we did not employ quantitative detection methods to evaluate the impact of bacterial loads on culture outcomes, nor did we assess viral-bacterial interactions and impact of antibiotic treatment on culture outcomes.

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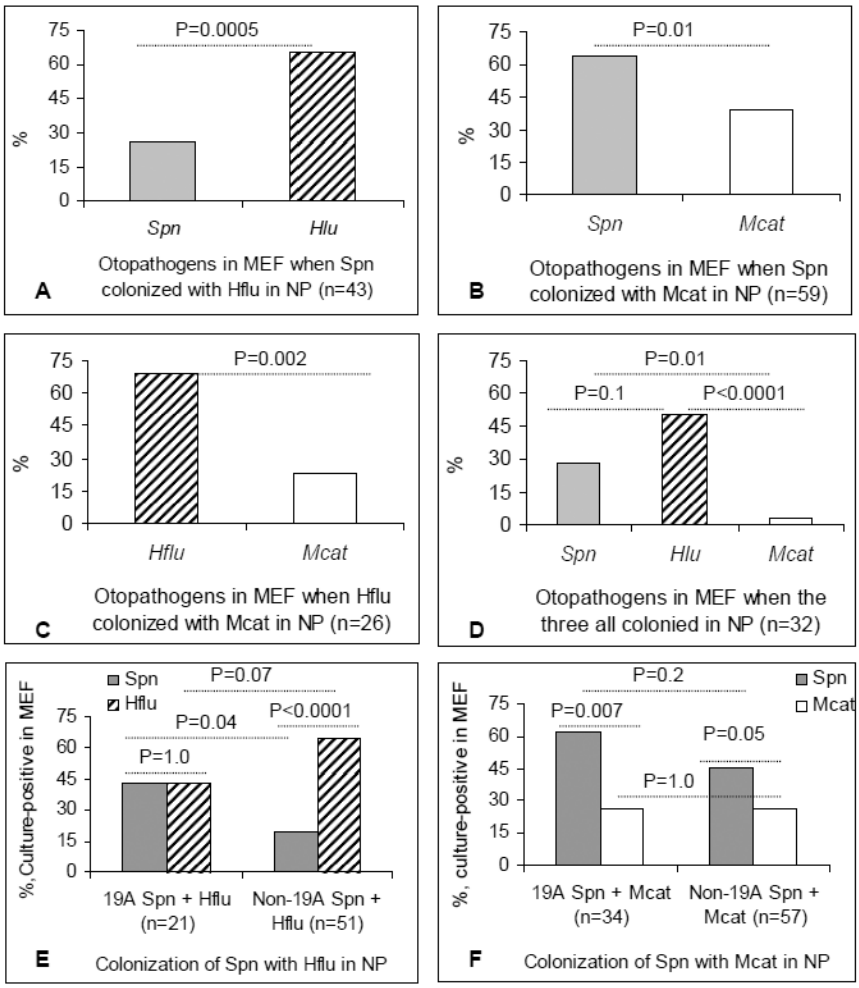

Figure 1. The predominant otopathogen to cause AOM when multiple otopathogens were simultaneously present in the NP

When multiple otopathogens were simultaneously present in the NP at the time of AOM the culture-positive rate in MEF was compared using logistic regression with repeated measures and Fisher's exact test. The percentage of each otopathogen causing AOM when co-colonization occurred involving, A, *Spn* with *Hflu*; B, *Spn* with *Mcat*; C, *Hflu* with *Mcat;* D, all three otopathogens; E, 19A or non-19A strains of *Spn* with *Hflu*; B, 19A or non-19A strains of *Spn* with *Mcat*. NP: Nasopharynx; MEF: middle ear fluid; *Spn*, *S. pneumonia*; *Hflu*: *H. influenzae*; *Mcat*: *M.catarrhalis*.