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Density, Serotype Diversity, and Fitness of *Streptococcus pneumoniae* in Upper Respiratory Tract Cocolonization With Nontypeable *Haemophilus influenzae*

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Background. Coinfections by *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are frequently implicated in complex otitis media. Whereas upper respiratory tract carriage precedes disease for both pathogens, interactions between species in cocolonized hosts are poorly understood. We compared colonization densities and the diversity and fitness of pneumococcal serotypes in single-species and mixed-species colonization.

Methods. We analyzed nasopharyngeal pneumococcal carriage and nasopharyngeal and oropharyngeal NTHi carriage in 13 541 samples collected over 6909 study visits from 769 children 2–30 months old in a 7-valent pneumococcal conjugate vaccine dosing trial. We measured density associations between the species and compared pneumococcal serotype diversity during and in the absence of NTHi colonization. We used logistic regression to quantify associations between NTHi colonization and previously published pneumococcal serotype factors related to fitness.

Results. Densities of the 2 species were positively associated when they co-occur in the nasopharynx. NTHi colonization was associated with reduced pneumococcal serotype diversity among children 2–18 months old and was more prevalent among children carrying pneumococcal serotypes with greater capsular thickness, neutrophil resistance, and metabolic efficiency.

Conclusions. Pneumococcal-NTHi cocolonization is associated with an elevated density of both species and with reduced diversity and increased fitness of pneumococcal serotypes. NTHi colonization may create a selective environment favoring pneumococci with immune-evasive phenotypes.

Keywords. *Streptococcus pneumoniae*; nontypeable *Haemophilus influenzae*; carriage; nasopharynx; interspecies interaction; coinfection.

Otitis media (OM) is the leading cause of pediatric healthcare visits and antimicrobial prescriptions in developed countries [1] and poses significant health burden in developing countries due to long-term complications and sequelae [2]. Collectively, *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) cause most OM cases [3]. Coinfections with these 2 pathogens are associated with more-severe disease than either pathogen typically causes alone; in comparison to single-species infections, mixed pneumococci-NTHi OM is more likely to present bilaterally, incur treatment failure, and recur or become chronic [4, 5]. However, limited understanding of the biological mediators of pneumococci-NTHi interaction currently hampers the clinical management and prevention of coinfections.

S. pneumoniae and NTHi share a niche in the upper respiratory tract, and asymptomatic carriage is the source of both infection and onward transmission. Among healthy children, the 2 species appear together in carriage more often than would be expected by chance [6], and cocolonization is associated with higher-density bacterial carriage for both species [7]. These associations cannot be explained by conventional risk factors for carriage, such as age, season, antibiotic receipt, and daycare attendance [8], suggesting that bacterial characteristics or host-immune factors mediate interspecies interaction [9]. Facilitative interactions between species occur in biofilms during OM [10] and may also explain the tendency of different species to occur together in upper respiratory tract carriage. Alternatively, animal models have demonstrated immune-mediated interactions, whereby robust neutrophil responses triggered against *H. influenzae* also clear cocolonizing pneumococci [11–13]. Although competitive in nature, such immune-mediated interactions may contribute to associations by concurrently clearing the 2 species during cocolonization.

The polysaccharide capsule is the defining characteristic of pneumococcal serotypes and a primary immune-evasion factor for the bacterium [14]. Capsule-associated attributes predict the capacity of pneumococcal serotypes to colonize the nasopharynx and the prevalence of pneumococcal serotypes in carriage

[15–17]. Mixed-species pneumococci-NTHi OM episodes have a distinct pneumococcal serotype profile, typically involving less invasive serotypes that are more prevalent in carriage than serotypes causing OM when NTHi is absent [5, 18]. To better understand biological mediators of interactions between these species, we compared bacterial density and the diversity and fitness of pneumococcal serotypes found in single-species and mixed-species colonization among children.

METHODS

Carriage Data

We used data from a preimplementation randomized controlled trial investigating 7-valent pneumococcal conjugate vaccine (PCV7) dosing schedules in southern Israel. The study enrolled healthy children aged 2–30 months from the local Jewish (n = 400) and Bedouin (n = 369) populations. The Bedouin population is transitioning from nomadic lifestyles to permanent settlements and has a lower socioeconomic status, larger family sizes, and higher pneumococcal and NTHi carriage and disease rates than the Jewish population despite access to the same medical care [5, 8, 19, 20].

The study design was described previously [8, 21] and is detailed in Supplementary Table 1. Healthy Bedouin (n = 351) and Jewish (n = 382) children were enrolled at age 2 months and randomly assigned to 2-dose (at 12 and 18 months), 3-dose (at 2, 4, and 6 months or 4, 6, and 12 months), or 4-dose (at 2, 4, 6, and 12 months) PCV7 schedules. Additionally, 18 Bedouin and 18 Jewish children were enrolled at age 18 months and given 1 PCV7 dose. All other routine pediatric vaccinations, including *H. influenzae* type b vaccine, were administered following standard schedules. Swabs were taken from the nasopharynx and oropharynx at ages 2, 4, 6, 7, 12, 13, 18, 19, 24, and 30 months. Of 7474 scheduled visits, 6909 (92.4%) were completed, and 13 541 nasopharyngeal (NP) and oropharyngeal (OP) swabs were analyzed (90.6%).

Bacteriology procedures have been described elsewhere [8, 21]. Pneumococcal carriage was determined by a culture-positive NP or OP swab. Because OP swabs were tested for pneumococci only if the NP sample was negative for pneumococci, we limited our analyses to NP pneumococcal carriage; OP swabs were positive in only 2% of visits with negative NP swabs. NP and OP swabs were analyzed for *H. influenzae*. Pneumococcal serotypes were determined by the Quellung reaction, and *H. influenzae* isolates were serotyped by polyvalent antisera to groups a, b, c, d, e, and f. Nonagglutinating isolates were considered NTHi. Bacterial density was measured semiquantitatively as the quadrants of an agar plate with colony growth following 4 streaks.

Pneumococcal Serotype Attributes

We used previously collected measures of pneumococcal capsular attributes to determine how serotype characteristics influence pneumococci-NTHi associations in carriage [17]. Resistance of pneumococcal serotypes to neutrophil-mediated killing was measured as the proportion of cells surviving an in vitro complement-independent surface killing assay, the degree of encapsulation was measured by the zone of exclusion of fluorescent dextran molecules, and metabolic efficiency was quantified by the inverse of the number of carbons per capsular polysaccharide repeat unit (Table 1).

Because polymicrobial OM is associated with less pathogenic pneumococcal serotypes [5], we also examined the relationship between NTHi cocolonization and measures of the virulence of serotypes. These included invasiveness, measured as the probability for a carriage episode to lead to invasive pneumococcal disease (IPD) [22], and serotype-specific risk of death in IPD cases [23]. We summarize pair-wise correlations among serotype measurements in Supplementary Table 3.

Statistical Analysis

Bacterial Density and Cocolonization

To examine whether pneumococcal and NTHi carriage densities were correlated during cocolonization, we calculated the Spearman correlation coefficient (ρ) for semiquantitative

Table 1. Data Available for Pneumococcal Serotype Attributes

Serotype Attribute, Variable	Measurement	Source	Serotypes With Available Data, No.[a]	Carriage Episode Coverage, No. (%)[a,b,c] (n = 3701)
Colonization fitness				
Resistance to neutrophil-mediated killing	Survival in a complement-independent in vitro surface killing assay	[17]	14	1998 (54)
Capsular size	Zone of exclusion of dextran molecules	[17]	15	2107 (57)
Capsular metabolic efficiency	Carbons per polysaccharide repeat unit (inverse)	[17]	54	3047 (82)
Virulence				
Invasiveness	IPD incidence per carriage incidence	[22]	36	3033 (82)
IPD case-fatality ratio	30-d mortality during IPD	[23]	37	3122 (84)

Abbreviation: IPD, invasive pneumococcal disease.

[a] Serotypes for which each item is available are listed in Supplementary Table 6.

[b] The number of carriage isolates for each serotype is listed in Supplementary Table 3 with nontypeable *Haemophilus influenzae* cocolonization frequency.

[c] Indeterminate and nontypeable pneumococcal isolates (180 of 3656 nasopharyngeal isolates [4.9%]) were excluded from the analysis.

measures of density between the species. We expected that host-mediated interactions may lead to differences in cocolonization patterns across ages, such as differences due to immune maturation or onset of naturally acquired immunity, and therefore stratified analyses by age (2–18 months and 19–30 months). We also stratified by anatomical site of NTHi colonization (NP, OP, or both NP and OP) to determine how NP and OP NTHi density each related to NP pneumococcal density, thereby testing whether interactions are mediated locally within the host. We defined NTHi-negative children as the comparator group for analyses. We computed 95% confidence intervals (CIs) by using a cluster bootstrap of individuals, thus accounting for repeated sampling of each child in measures of uncertainty [24].

We also used logistic regression models to assess whether pneumococcal and NTHi colonization density predicted colonization by the other bacterium, measuring odds ratios (ORs) and adjusted ORs (aORs) for the presence of each bacterium associated with the density of the other species. Models were fitted using generalized estimating equations to address repeated sampling of individual children. We included both NP and OP NTHi carriage density as covariates in regression models predicting pneumococcal colonization, to distinguish independent, anatomical site-specific associations. Again, we stratified analyses by age group and constructed separate models treating NP and OP NTHi carriage as outcome measures; in these analyses, we adjusted for NTHi density on the other swab to better distinguish the impact of NP pneumococcal density. Multivariate models also included age (log-transformed within the age stratum included in each model), Jewish or Bedouin ethnicity, season, antibiotic use in the preceding month, number of child contacts in the home or daycare center (log-transformed), and receipt of 1, 2, or ≥3 PCV7 doses, which we previously found to predict serotype carriage but not species-level carriage of *S. pneumoniae* or *H. influenzae* [8]. We accounted for seasonality by including harmonic covariates with periods of 4, 6, and 12 months and accounted for differences in age and seasonal patterns in the Jewish and Bedouin populations, using interactions between these variables and ethnic group.

Serotype Diversity During Cocolonization

We assessed how NTHi cocolonization related to pneumococcal serotype diversity by comparing Simpson diversity indices for pneumococcal serotypes among children carrying only pneumococcus or carrying pneumococcus together with NTHi [25]. This index quantifies diversity in terms of the number of serotypes and the evenness of their representation, measuring the probability that 2 randomly selected pneumococcal isolates belong to different serotypes. We defined NTHi-negative child-visits as the comparator group and again stratified analyses by the anatomical site of NTHi colonization and age (2–18 or 19–30 months). We accounted for repeated sampling of

children in CIs and hypothesis tests via a cluster bootstrap of individuals.

Pneumococcal Capsular Determinants of Cocolonization

To understand pneumococcal factors underlying density and diversity associations, we next assessed how NTHi cocolonization prevalence varied across pneumococcal serotypes according to their biological attributes. Considering the subset of visits where children carried pneumococci in the nasopharynx, we constructed logistic regression models with generalized estimating equations to determine ORs and aORs of NTHi colonization associated with measures of pneumococcal serotype fitness and virulence (Table 1). This approach prevents confounding by fitness factors that also predict variation in pneumococcal serotype prevalence [26]. Models controlled for the same covariates included in the density analyses and were again stratified by children's age (2–18 or 19–30 months) and NTHi colonization site, with NTHi-negative child-visits providing the comparator group. Owing to multicollinearity among measurements of pneumococcal serotype attributes (Supplementary Table 3), we measured each phenotypic association individually in separate models. We tested interaction terms between capsular attributes and pneumococcal density to assess density-mediated associations.

RESULTS

Carriage Profile in the Study Population

Carriage patterns within the study population have been described previously in detail [8, 21]. Pneumococcal prevalence in the nasopharynx was 70% and 39% at visits by Bedouin and Jewish children, respectively (Table 2). Whereas prevalence was constant in Bedouin children ages 2–18 and 19–30 months (70%), prevalence increased from 35% to 48% among Jewish children at these ages. Over 90% of all *H. influenzae* isolates were nontypeable, and NTHi was carried in both sites at 96% and 86% of visits where it was detected on the NP or OP swab, respectively. Similar to pneumococcal carriage, the prevalence of NTHi carriage increased from 26% to 49% in the nasopharynx and from 31% to 57% in the oropharynx among Jewish children between ages 2–18 months and 19–30 months. Likewise, NTHi carriage was relatively stable across ages among Bedouin children, with 50% and 58% overall prevalence in the nasopharynx and oropharynx, respectively. At visits where children carried NTHi, the likelihood of detecting the bacterium concurrently in the nasopharynx and oropharynx was higher among those aged 19–30 months (85%), compared with those aged 2–18 months (73%; Supplementary Figure 1).

Species Associations in Nasopharyngeal and Oropharyngeal Carriage

Pneumococcal carriage prevalence was higher when children also carried NTHi. Children experiencing concurrent NP plus OP NTHi carriage were 1.75 times (95% CI, 1.65–1.85) more likely to carry pneumococci than children who did not carry

Table 2. Nasopharyngeal and Oropharyngeal *Streptococcus pneumoniae* (*Sp*) and *Haemophilus influenzae* (*Hi*) Colonization Among 369 Bedouin Children and 400 Jewish Children Aged 2–18 Months or 19–30 Months

	Bedouin Children		Jewish Children	
Variable	2–18 mo	19–30 mo	2–18 mo	19–30 mo
Visits completed, no.	2242	1066	2523	1078
NP swabs analyzed among completed visits, no.	2230	992	2514	1037
OP swabs analyzed among completed visits, no.	2232	989	2514	1033
Sp-positive swabs/swabs analyzed[a]	1594/2230 (71)	698/988 (71)	904/2513 (36)	505/1033 (49)
Sp-positive NP swabs/NP swabs analyzed	1577/2230 (71)	692/993 (70)	888/2514 (35)	499/1037 (48)
Sp-positive OP swabs/*Sp*-negative NP swabs	21/653 (3)	8/301 (3)	18/1626 (1)	8/538 (1)
Hi-positive NP swabs/NP swabs analyzed	1233/2231 (55)	571/992 (58)	672/2514 (27)	526/1037 (51)
Hi type b–positive NP swabs/*Hi*-positive NP swabs	10/1233 (1)	5/569 (1)	8/672 (1)	0/524 (0)
NTHi-positive NP swabs/*Hi*-positive NP swabs	1145/1233 (93)	528/569 (93)	648/672 (96)	511/524 (98)
NTHi-positive OP swabs/NTHi-positive NP swabs	1088/1145 (95)	517/526 (98)	612/648 (94)	494/508 (97)
Sp-positive NP swabs/NTHi-positive NP swabs	894/1144 (78)	422/528 (80)	370/648 (57)	319/511 (62)
Hi-positive OP swabs/OP swabs analyzed	1363/2232 (61)	649/989 (66)	825/2514 (33)	608/1033 (59)
Hi type b–positive OP swabs/*Hi*-positive OP swabs	11/1363 (1)	5/649 (1)	12/825 (1)	1/608 (<1)
NTHi-positive OP swabs/*Hi* positive OP swabs	1260/1363 (92)	605/649 (93)	790/825 (96)	587/608 (97)
NTHi-positive NP swabs/NTHi-positive OP swabs	1088/1260 (86)	517/605 (85)	612/790 (77)	494/587 (84)
Sp-positive NP swabs/NTHi-positive OP swabs	963/1260 (76)	465/605 (77)	424/790 (54)	353/587 (60)

Data are proportion (%), unless otherwise noted.

Abbreviations: NP, nasopharyngeal; NTHi, nontypeable *H. influenzae*; OP, oropharyngeal.

[a] The number of carriage isolates for each serotype is listed in Supplementary Table 3 with NTHi cocolonization frequency.

NTHi. In addition, NP-only and OP-only carriage of NTHi were associated with 1.32-fold (95% CI, 1.09–1.57) and 1.28-fold (95% CI, 1.16–1.41) higher prevalence of pneumococcal carriage, respectively, compared with pneumococcal prevalence among children who did not carry NTHi. Concurrent NP plus OP NTHi carriage thus contributed a 36% (95% CI, 25%–49%) increase in pneumococcal carriage prevalence than was associated with OP-only carriage.

Pneumococcal colonization was associated with increased likelihood of detecting NTHi together in both the nasopharynx and oropharynx. The prevalence of concurrent NP plus OP NTHi carriage was 2.13-fold (95% CI, 1.97–2.31) higher among pneumococcal carriers than noncarriers. However, children who carried pneumococci were not more likely than noncarriers to experience NP-only or OP-only NTHi colonization (relative risk, 0.99 [95% CI, .68–1.48] and 0.93 [95% CI, .79–1.10], respectively). Among children carrying NTHi in the oropharynx, those carrying pneumococci were 1.16 times (95% CI, 1.12–1.21) more likely to carry NTHi in the nasopharynx simultaneously. Similarly, among children carrying NTHi in the nasopharynx, those carrying pneumococci were 1.04 times (95% CI, 1.01–1.06) more likely to carry NTHi concurrently in the oropharynx.

Nasopharyngeal Interactions Mediate Between-Species Presence and Density Associations

Pneumococcal and NTHi densities were positively correlated among children carrying the 2 species together. The Spearman rank-correlation coefficient between species densities in the nasopharynx took values of 0.38 (95% CI, .34–.42) and 0.52 (95% CI, .47–.57) for children aged 2–18 months and 19–30 months, respectively. However, the positive relationship between NTHi density in the oropharynx and pneumococcal density in the nasopharynx depended upon whether NTHi was concurrently present at both sites. Among children carrying NTHi in both the nasopharynx and oropharynx, we noted a weak positive correlation between OP NTHi density and NP pneumococcal density in the younger ($\rho = 0.24$; 95% CI, .18–.29) and older ($\rho = 0.35$; 95% CI, .28–.41) age groups. Correlation values were lower among children who carried NTHi only in the oropharynx, among whom we did not detect associations in species densities at ages 2–18 months ($\rho = 0.05$; 95% CI, −.10–.19; $P_{Interaction} = .007$) or 19–30 months ($\rho = 0.02$; 95% CI, −0.19–0.23; $P_{Interaction} = 0.002$).

We observed strong positive correlations between NP and OP NTHi density among children who carried the bacterium at both sites ($\rho = 0.66$ [95% CI, .63–.68] for ages 2–18 months and $\rho = 0.60$ [95% CI, .56–0.64] for older children).

In multivariate analyses, we found that positive associations between the species were mediated by density and anatomical site of NTHi colonization in a similar manner (Figure 1 and Supplementary Table 4). Adjusted odds of pneumococcal carriage in the nasopharynx increased incrementally with NTHi density in the NP (Figure 1). Whereas pneumococcal carriage in the nasopharynx was also positively associated with NTHi carriage in the oropharynx, this relationship was not mediated by pneumococcal density. Similarly, the adjusted odds of

carrying NTHi in the nasopharynx increased with NP pneumococcal density, while the adjusted odds of carrying NTHi in the oropharynx did not differ according to pneumococcal density. We determined that age did not affect interspecies density associations by testing an interaction term between age (log-transformed or greater/less than 19 months) and density of the other species in models including all visits by children.

NTHi Cocolonization Is Associated With Reduced Pneumococcal Serotype Diversity

Sixty-eight distinct pneumococcal serotypes were detected in NP swabs (Supplementary Table 2). At ages 2–18 months, pneumococcal serotype diversity was lower among children who carried NTHi (Figure 2). These patterns persisted in analyses stratified by ethnicity, although effect sizes were statistically significant only within the Jewish subsample (Supplementary Table 5).

Reductions in serotype diversity among children carrying NTHi were not apparent at ages >18 months (Figure 2). Comparing across ages, pneumococcal serotype diversity tended to

be lower at 2–18 months of age, compared with 19–30 months of age, among children with concurrent NP plus OP NTHi carriage ($P = .07$), a trend that was also apparent in association with NP ($P = .05$) and OP ($P = .1$) NTHi carriage. We noted no significant difference in serotype diversity across ages among children who did not carry NTHi ($P = .4$).

Capsular Markers of Pneumococcal Fitness Predict NTHi Cocolonization

Among children 2–18 months old carrying pneumococci, the odds of cocolonization with NTHi in the nasopharynx and oropharynx were higher when children carried serotypes that have previously been identified as fitter, as measured by capsule thickness (aOR, 1.46; 95% CI, 1.00–2.12), survival in a neutrophil-mediated killing assay (aOR, 1.58; 95% CI, 1.02–2.44), and metabolic efficiency of capsule production (aOR, 1.43; 95% CI, 1.03–1.97; Table 3). Associations between NTHi colonization and pneumococcal phenotypes were not dependent upon pneumococcal density, and measures of pneumococcal virulence
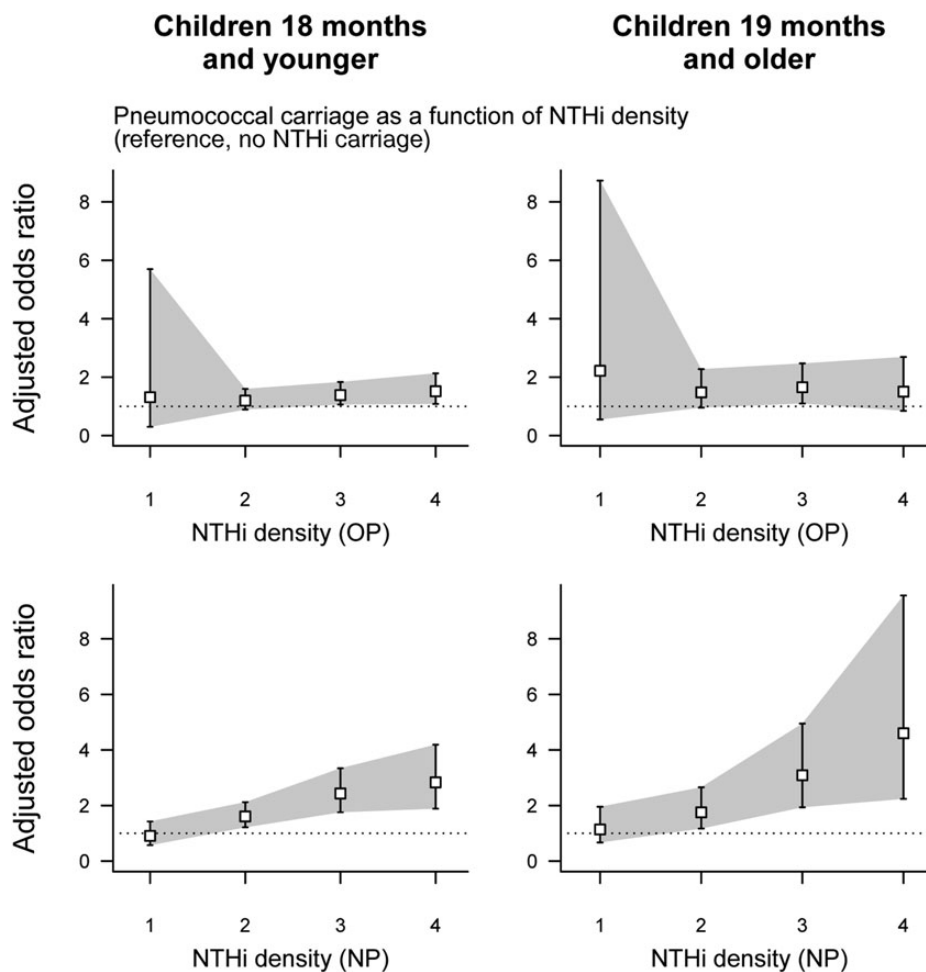

Figure 1. Cross-species density associations with *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) carriage by anatomical site of colonization. NTHi carriage in the nasopharynx has a density-mediated positive association with pneumococcal carriage in the nasopharynx, whereas the positive association between NTHi carriage in the oropharynx and pneumococcal carriage in the nasopharynx is not density mediated (top two rows).
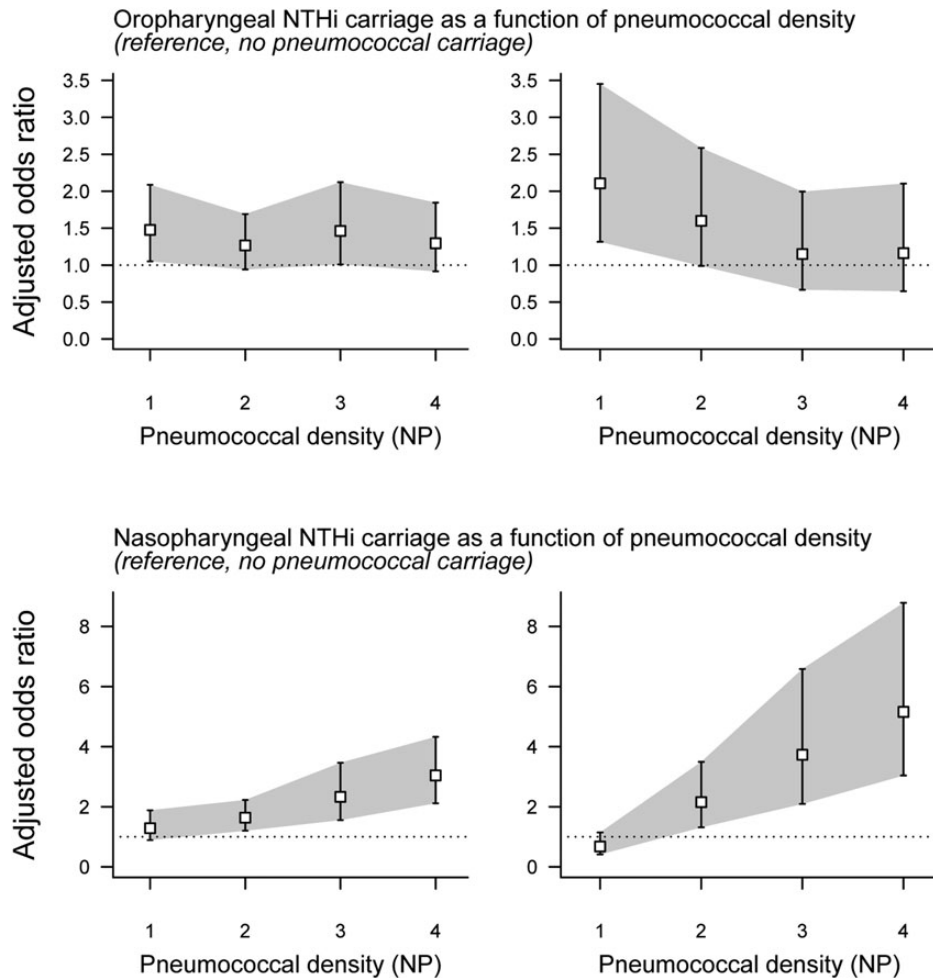
Oropharyngeal NTHi carriage as a function of pneumococcal density
(reference, no pneumococcal carriage)

Nasopharyngeal NTHi carriage as a function of pneumococcal density
(reference, no pneumococcal carriage)

Figure 1 *continued.* Similarly, pneumococcal density in the nasopharynx mediates the positive association with NTHi carriage in the nasopharynx (bottom row). Multivariate models account for NP and OP NTHi and/or pneumococcal density, age, season, ethnicity, antibiotic receipt within prior month, child contacts at home or daycare, and PCV7 doses received. Adjusted and unadjusted effect sizes are presented in Supplementary Table 4. Abbreviations: NP, nasopharyngeal; OP, oropharyngeal.
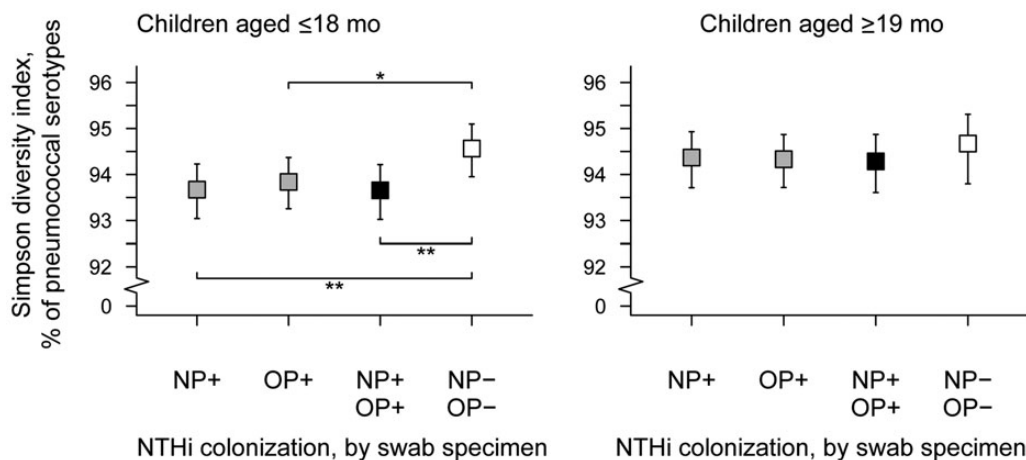
Figure 2. Lower diversity of pneumococcal serotypes found cocolonizing with nontypeable *Haemophilus influenzae* (NTHi) among children aged ≤18 months. We present measures of diversity together with indicators of statistical significance based on cluster-bootstrap resampling. These findings are replicated in stratified analyses within the Jewish and Bedouin populations (Supplementary Table 5). *$P < .05$ and **$P < .01$. Abbreviations: NP, nasopharyngeal; OP, oropharyngeal; −, absent; +, present.

(invasiveness and case-fatality ratios) were not associated with carrying NTHi among children 2–18 months old.

In contrast to what was observed among children aged 2–18 months, pneumococcal capsular thickness, immune evasiveness, and metabolic efficiency were not associated with NTHi cocolonization among older children. However, NTHi had higher adjusted odds of being found in the nasopharynx and oropharynx, and at both sites among older children carrying less invasive pneumococcal serotypes (Table 3).

DISCUSSION

Mixed-species OM caused by *S. pneumoniae* and NTHi is associated with complex disease symptoms, poor treatment outcomes, and significant healthcare burden [4, 5]. Because upper respiratory tract carriage is the source of infections, we investigated cocolonization patterns between the 2 species to characterize their within-host interactions. Our findings are threefold. Positive associations in pneumococcal and NTHi colonization and density [7] are influenced by the co-occurrence of the species in the nasopharynx. Second, at ages 2–18 months, pneumococci isolated from children carrying NTHi have lower serotype diversity in comparison with pneumococci colonizing in the absence of NTHi. Third, among children carrying pneumococcus, prevalence of NTHi colonization increases with the immune evasiveness and metabolic fitness of

pneumococcal serotypes at ages 2–18 months. Taken together, these observations characterize mixed-species pneumococci-NTHi colonization in young children as a distinct state involving higher-density bacterial populations and a characteristic pneumococcal serotype profile.

Reduced pneumococcal serotype diversity and evidence for fitness advantages among pneumococci cocolonizing with NTHi suggest a selective environment during cocolonization. Similarly, animal models of cocolonization by pneumococcus and *H. influenzae* point to immune-mediated competition between the bacteria as a driver of serotype associations. In mice and rats, *H. influenzae* colonization and pneumococcus–*H. influenzae* cocolonization elicit greater upper respiratory tract neutrophil infiltration than hosts typically mount against pneumococci alone [11–13], possibly augmenting advantages afforded to highly replicative, neutrophil-resistant pneumococcal serotypes as noted in our study [17, 27]. However, the limited selection of pneumococcal serotypes (6B, 4, and 23F) and use of *H. influenzae* serotype d rather than NTHi in animal models limit our ability to compare outcomes [11–13]. Additionally, our study measured pneumococcal resistance to neutrophils in a complement-independent killing assay [17], whereas animal models have examined cross-species opsonophagocytic responses.

Immune responses are a primary mediator of pneumococcal and *H. influenzae* colonization dynamics [11, 12, 28]. Although

Table 3. Pneumococcal Capsular Predictors of Nontypeable *Haemophilus influenzae* (NTHi) Cocolonization Among Children Aged 2–18 Months or ≥19 Months

NTHi Colonization Outcome, Serotype Factor[a,b,c]	Children Aged 2–18 mo		Children Aged ≥19 mo	
	OR (95% CI)	aOR[d] (95% CI)	OR (95% CI)	aOR[d] (95% CI)
Any NP carriage (vs NTHi negative)				
Neutrophil resistance	1.51 (1.02–2.23)	1.51 (.98–2.32)	0.89 (.47–1.67)	0.96 (.49–1.87)
Encapsulation	1.32 (.94–1.86)	1.45 (1.00–2.09)	0.90 (.51–1.60)	0.94 (.51–1.74)
Metabolic efficiency	1.41 (1.05–1.89)	1.42 (1.03–1.96)	0.94 (.60–1.47)	0.99 (.65–1.51)
Invasiveness	1.03 (.96–1.10)	1.02 (.95–1.09)	0.93 (.87–.98)	0.91 (.84–.99)
IPD CFR	0.78 (.54–1.13)	0.74 (.49–1.11)	0.68 (.38–1.23)	0.85 (.45–1.59)
Any OP carriage (vs NTHi negative)				
Neutrophil resistance	1.44 (.98–2.11)	1.42 (.94–2.16)	0.91 (.49–1.68)	0.90 (.47–1.75)
Encapsulation	1.25 (.90–1.75)	1.35 (.94–1.93)	0.97 (.55–1.72)	0.97 (.53–1.78)
Metabolic efficiency	1.34 (1.01–1.78)	1.33 (.97–1.82)	1.01 (.66–1.55)	1.05 (.69–1.59)
Invasiveness	1.03 (.97–1.09)	1.01 (.96–1.07)	0.93 (.88–.98)	0.92 (.86–.98)
IPD CFR	0.81 (.56–1.16)	0.81 (.54–1.20)	0.74 (.41–1.31)	0.87 (.47–1.62)
Concurrent NP and OP carriage (vs NTHi negative)				
Neutrophil resistance	1.57 (1.05–2.33)	1.58 (1.02–2.44)	0.90 (.48–1.70)	0.94 (.48–1.86)
Encapsulation	1.33 (.95–1.88)	1.46 (1.00–2.12)	0.92 (.52–1.63)	0.96 (.52–1.79)
Metabolic efficiency	1.43 (1.07–1.92)	1.43 (1.03–1.97)	1.01 (.66–1.55)	0.97 (.62–1.52)
Invasiveness	1.02 (.96–1.09)	1.01 (.95–1.07)	0.92 (.86–.99)	0.90 (.83–.99)
IPD CFR	0.80 (.55–1.17)	0.77 (.51–1.17)	0.68 (.38–1.22)	0.83 (.44–1.56)

Abbreviations: aOR, adjusted odds ratio; CFR, case-fatality ratio; CI, confidence interval; IPD, invasive pneumococcal disease; NP, nasopharyngeal; OP, oropharyngeal; OR, odds ratio.

[a] Measurements are defined in Table 1.

[b] All serotype measurements are scaled to values observed for pneumococcal serotype 9N.

[c] Separate models are fitted for each predictor.

[d] Models control for age (log transformed), season (4-, 6-, and 12-month harmonics), Jewish or Bedouin ethnicity (interacted with age and season), antibiotic use in the preceding month, number of child contacts in the home or daycare center (log transformed), and receipt of 1, 2, or ≥3 7-valent pneumococcal conjugate vaccine doses, following previous analyses [8].

positive associations between species frequently signify facilitative interactions, nonspecific phagocytic effectors may drive positive associations by concurrently eliminating cocolonizing species or by preventing both species from recolonizing hosts. Under this scenario, immune parameters including local phagocyte densitie may predict the species' persistence in carriage. Whereas this possibility is consistent with the hypothesis that immune responses drive pneumococcal serotype associations with NTHi cocolonization, different biological mechanisms may account for densities and serotype distributions associated with mixed-species colonization. In our study, serotype diversity and fitness associations, unlike density associations, were age dependent.

Another explanation is that highly fit pneumococcal serotypes have distinct mutualistic interactions with NTHi. However, such interactions would be predicted to increase the density of these serotypes when the species colonize together; we did not identify density-mediated associations with pneumococcal capsular attributes. In addition, reduced carriage of serotypes 6B, 18C, 19F, and 23F among children receiving PCV7 would be expected to cause concomitant reductions in NTHi carriage if mutualistic interactions occurred between NTHi and thickly encapsulated, immune-evasive pneumococcal serotypes. In contrast, PCV7 receipt did not influence NTHi or *H. influenzae* colonization in the present study and in other trials measuring multiple-species carriage end points [8, 29, 30]. Animal models and in vitro studies of pneumococcal and *H. influenzae* interaction moreover do not provide evidence of mechanisms other than immune responses causing serotype-specific species interactions [10–13, 31, 32].

In our study, pneumococcal serotype diversity and phenotype were associated with NTHi cocolonization among children 2–18 months old [4, 5]. Immune maturation over these ages reverses the neonatal CD4+/CD8+ lymphocyte balances [33] amid changes in T-helper type 1 (Th1), Th2, and interferon γ profiles [34, 35]. CD4+ T-cell responses mediate immunity to pneumococcal and NTHi carriage [36–38], and their diminishing prominence among older children may underlie the disappearance of species associations. In contrast, associations mediated by direct bacterial interactions would be expected to be age-independent, although acquired anticapsular immunity against previously carried serotypes may confound associations in older children [26, 39].

Pneumococcal serotypes prevalent in carriage with lower disease potential are disproportionately represented in polymicrobial pneumococci-NTHi OM [5]. We identify that determinants of these serotypes' capacity to colonize the upper respiratory tract also predict serotype-specific NTHi cocolonization prevalence. These concordant observations suggest species interactions during upper respiratory tract carriage drive serotype patterns in coinfections. We also find low pneumococcal invasiveness is associated with NTHi cocolonization among older children (age, >18 months), although biological explanations of this pattern are unclear. Contrary to our observations, *H. influenzae* permits the virulent pneumococcal serotype 4 to outcompete the less virulent serotype 23F in cocolonized mice, contrary to *H. influenzae*–independent competition outcomes [11]. Whereas we examined culture-positive carriage of planktonic bacteria, the 2 species form a biofilm in middle-ear infections [5, 10]. Studies of polymicrobial OM pathogenesis may elucidate the relationship between mixed-species colonization and infection.

Our analysis has several limitations. *Haemophilus haemolyticus* may be misclassified as NTHi in a proportion of OP isolates [40], potentially explaining the weaker associations we noted with NTHi-classified carriage in the oropharynx. While heterogeneous sample quality on concomitantly collected NP and OP swabs may influence density associations, highly correlated NP and OP NTHi densities suggest this was not an important limitation in our study. Although our data came from a PCV7 trial, the study design should not impact the interpretation or reliability of our findings about species interactions because vaccination did not affect *H. influenzae* or NTHi carriage [8]. Because our analyses compared NTHi cocolonization prevalence by pneumococcal serotype, outcomes were not confounded by vaccination and other determinants of pneumococcal serotype carriage. Last, the infrequency of pneumococcal colonization episodes where children experienced NP-only or OP-only NTHi carriage (53 and 175 visits, respectively) prevented us from testing whether diversity and phenotype patterns were specific to either anatomical site via interactions [41].

While we did not assess multiple-serotype carriage, future studies should characterize the effect of NTHi cocolonization on pneumococcal serotype interactions and the impacts of viruses and other microbial species on pneumococci-NTHi interaction [9]. Data on host immune status may further elucidate determinants of cocolonization: whereas our study enrolled only HIV-negative children, the positive association between pneumococcal and *H. influenzae* colonization is greater among HIV-negative than HIV-positive children [42], suggesting immune-mediated species interactions. Inverse carriage associations between *H. influenzae* and *Staphylococcus aureus* and between pneumococcus and *S. aureus* similarly appear only among HIV-negative children [42, 43]. Otitis-prone children experience compromised CD4+ T-cell responses to pneumococcal and *H. influenzae* carriage and OM [38], further suggesting an immunological basis for species interactions in carriage and disease [44, 45].

We identify distinct bacterial density and pneumococcal serotype patterns underlying the frequently reported carriage association between *S. pneumoniae* and NTHi. These outcomes strengthen support for the idea of biologically mediated interactions. Clarifying species interaction mechanisms may provide

insight into dynamics of respiratory tract microbial communities and the pathogenesis, prevention, and optimal management of coinfections.

Supplementary Data

Notes

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