Title: Pneumococcal phenotype and interaction with nontypeable *Haemophilus influenzae* as determinants of otitis media progression

Running title: Otitis media progression by *S. pneumoniae* and NTHi

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ABSTRACT

Background: Mixed-species otitis media caused by *Streptococcus pneumoniae* (Spn) and nontypeable *Haemophilus influenzae* (NTHi) is associated with complex manifestations that are less common in pneumococcal infections without NTHi. We analyzed the microbiological composition of middle ear fluid (MEF) cultures and nasopharyngeal samples obtained before PCV7/13 rollout to identify bacterial factors influencing single- and mixed-species disease progression.

Methods: Data included OM episodes submitted for MEF cultures during a 10-year prospective study in southern Israel and nasopharyngeal samples from unvaccinated asymptomatic children in the same population. We compared pneumococcal serotype diversity across carriage and disease isolates with and without NTHi co-isolation. We also measured associations between pneumococcal phenotype and rate of progression from colonization to OM in the presence and absence of NTHi.

Results: Whereas pneumococcal serotype diversity in single-species OM is lower than in single-species colonization, serotype diversity does not differ significantly between colonization and OM in mixed-species episodes. Moreover, the pneumococcal serotype distribution of mixed-species OM corresponds more closely to that of mixed-species carriage than to the distribution of pneumococcal serotypes colonizing without NTHi. Pneumococcal phenotypes predicting prolonged carriage duration—such as efficient metabolic properties and strong negative surface charge—are associated with higher rate of progression to single-species OM. These factors are weaker predictors of the progression of mixed-species episodes.

Conclusions: Immune-evasive pneumococcal serotypes able to persist in the nasopharynx have elevated rates of progression to OM. However, these serotype differences in progression rate are attenuated in the presence of NTHi.

INTRODUCTION

Otitis media (OM) has historically been the leading cause of healthcare visits, antimicrobial prescribing, and surgical procedures among young children in high-income countries (1). *Streptococcus pneumoniae* (Spn) and nontypeable *Haemophilus influenzae* (NTHi) are the pathogens most commonly isolated from middle-ear fluid (MEF) in OM. Commensal carriage of these and other bacterial species in the upper-respiratory tract is the reservoir for middle-ear infection. Clinical severity ranges from acute or even asymptomatic presentations to complex OM (e.g., recurrent, nonresponsive, spontaneously-draining, or chronic OM and OM with effusion).

Formation of polymicrobial biofilms involving NTHi and Spn has recently been recognized as a risk factor for complex OM manifestations (2, 3). In comparison to pneumococcal OM episodes where NTHi is not present, mixed-species episodes involving Spn and NTHi more often occur secondary to previous OM, are associated with recurrence and chronicity, and involve pneumococcal serotypes typically believed to be less virulent than those causing episodes without NTHi involvement, notably including pneumococcal serotypes not targeted by conjugate vaccines (PCV7/10/13) (2, 4).

Because nearly all children carry Spn and NTHi in the first years of life, factors influencing progression from colonization to OM are ideal targets for treating or preventing disease. However, determinants of pathogenesis for Spn and NTHi are not well understood. Epidemiological studies in diverse settings have shown the species co-colonize the upper respiratory tract of children more frequently than would be expected by chance (5–9). Co-colonization episodes feature elevated bacterial densities in both species, and entail pneumococcal serotypes that occur disproportionately in mixed-species OM: in comparison to serotypes colonizing the nasopharynx without NTHi, those found in co-colonization tend to have immune-evasive phenotypes favoring prolonged carriage duration (2, 4, 10–12). NTHi enhances pneumococcal biofilm formation and persistence in the middle ear in chinchilla models of OM (13), and the species aid one another in colonizing rats in nasal challenge experiments (14). Nonetheless, the facilitative or competitive nature of species interactions remains unclear (15–17), along with explanations for the distinct pneumococcal serotype distributions of single-species and mixed-species infections (2).

We analyzed isolates from MEF and asymptomatic nasopharyngeal colonization to better understand bacterial determinants of OM progression. Using epidemiological surveillance data from southern Israel prior to PCV7/13 introduction, we compared pneumococcal serotype distributions of single-species and mixed-species carriage and OM, and measured pathogen-specific rates of OM progression from asymptomatic colonization. We found that pneumococcal serotype diversity is higher in mixed-species OM than single-species OM, despite reduced diversity among serotypes co-colonizing with NTHi in comparison to serotypes in single-species colonization. Immune-evasive pneumococcal phenotypes—which predict NTHi co-colonization (4)—have a weaker impact on progression of mixed-species infections in comparison to single-species infections. Our findings demonstrate that NTHi co-occurrence is associated with attenuated differences across pneumococcal serotypes in progression to OM.

RESULTS

Study enrollment

Data came from several studies undertaken prior to PCV7 introduction in southern Israel; we included samples obtained before July, 2008 in analyses (**Table 1**). Nasopharyngeal carriage of Spn and NTHi was monitored among PCV7/13-unvaccinated children ages 2 to 18 months old enrolled in a randomized trial of PCV7 dosing strategies between 2005 and 2008 (5, 18). In total, children submitted 1588 samples, from which Spn was detected in 743 (46.8%) swabs; NTHi was detected in 524 (33.0%) swabs, and the two species were co-isolated in 376 (23.7%). A ten-year prospective study of the incidence of severe OM cases necessitating MEF culture (as indicated by complex manifestations; detailed in **Materials and Methods**) supplied 11,811 cases (2), with 4165 (35.3%) positive for Spn, 4813 (40.8%) positive for NTHi, and 1589 (13.5%) positive for the two species. Other previously-conducted laboratory and epidemiological studies supplied phenotype information about pneumococcal serotypes (12, 19–21). Further descriptive details including age-specific OM incidence and carriage prevalence are included as supporting information (**Figure S1**).

Pneumococcal serotype distribution in single-species and mixed-species colonization and otitis media

If serotype factors play a role in progression of pneumococcal colonization to OM, the diversity of serotypes isolated from MEF would be expected to differ from the diversity of serotypes carried in the nasopharynx (4). To investigate this hypothesis, we calculated the Simpson's Diversity Index (*D*) for pneumococcal serotypes isolated from MEF and from carriage, with and without co-occurring NTHi, and tested for differences in serotype diversity (**Figure 1**); *D* measures the probability for any two randomly-chosen isolates to belong to different serotypes (see **Materials and Methods**).

Pneumococcal serotype diversity was higher in nasopharyngeal isolates than in MEF isolates both in the presence and in the absence of NTHi ($p<10^{-4}$). However, in mixed infections, the difference was no longer significant ($p=0.11$). Whereas serotype diversity was lower in the presence of NTHi during colonization, an opposite relationship was seen in MEF isolates, where diversity was significantly lower in single-species than in mixed-species infections ($p=0.022$).

We next sought to determine whether similar pneumococcal serotype diversity in mixed-species colonization and OM owed to the isolation of serotypes from nasopharyngeal and MEF samples at similar frequencies. We used Kullback-Leibler divergence to measure the relatedness of serotype distributions from single-species OM, single-species carriage, and mixed-species carriage to the serotype distribution of mixed-species OM (**Figure 2**). We identified lower divergence between serotype distributions of mixed-species carriage and OM than between serotype distributions of single-species carriage and mixed-species OM ($p<0.01$). Divergence between the pneumococcal serotype distributions of mixed-species OM and single-species OM was lower, however, suggesting a role of pneumococcal serotype in both single-species and mixed-species OM progression.

Determinants of progression from colonization to OM

We next compared pathogen-specific rates of progression to OM episodes necessitating MEF in order to identify bacterial factors associated with virulence. We derived the odds ratio of disease as an expression for the relative rate of progression from asymptomatic colonization to OM (see **Materials and Methods**).

Progression rates for pneumococcal serotypes spanned over two orders of magnitude (**Figure 3**). The highest progression rates were detected among PCV13 serotypes, including 1, 3, 5, and 7F; for the latter two serotypes, which were detected in 24 and 101 single-species OM cases respectively, no instances of single-species carriage were detected. Although we also detected no single-species carriage of 7B and 24F, these serotypes accounted for only 6 and 4 single-species OM episodes, respectively. Though not detected in mixed-species colonization, serotypes 8, 33F, and 15B/C were isolated from 4, 28, and 48 instances of mixed-species OM, respectively. Unencapsulated pneumococci had the lowest measured progression rates for single-species OM, while no instances of single-species OM involved serotypes 22F or 19B. We did not identify strong statistical evidence (defined by a 95% confidence interval entirely above one) that any serotype had a higher progression rate than NTHi.

Serotype differences in progression for mixed-species episodes were attenuated in comparison to differences in progression for single-species episodes. We identified lower progression rates in mixed-species episodes involving serotypes that tended otherwise to be virulent when colonizing in the absence of NTHi, including 6A, 6B, 14, 18C, 17F, 19A, 19F, and 31. Only nontypeable pneumococci and serotypes 20 and 31 showed increased progression rates in association with NTHi.

To understand this variation in progression to OM, we used regression models to calculate associations between phenotypes and progression rates for single-species and mixed-species episodes. Phenotypic attributes associated with fitness in colonization (greater ability to evade neutrophils, thicker encapsulation, and greater metabolic efficiency (12); stronger anionic charge (19); and higher case-fatality ratio in IPD (23)) predicted elevated rates of serotype-specific OM progression (**Table 2**), both in the presence and absence of NTHi. In addition, we identified that serotypes at high risk of causing IPD when carried (21) generally had lower rates of progression to OM. However, associations between pneumococcal phenotypes and progression rates were weaker for mixed-species episodes in comparison to single-species episodes. These analyses also revealed a 77.5% (46.3%, 114.5%) higher progression rate during the months with the highest degree of respiratory virus transmission (December to March), compounding elevated carriage prevalence during these months as a driver of seasonal OM incidence (5).

DISCUSSION

We sought to better understand the progression of Spn and NTHi to complex OM manifestations using prospectively-gathered epidemiological surveillance data from Israel. In contrast to the finding that single-species pneumococcal OM episodes have lower serotype diversity than single-species pneumococcal carriage episodes, we did not identify differential diversity in carriage and OM when pneumococcal serotypes co-occur with NTHi. Identifying variation in the

rates at which pneumococcal serotypes progress from colonization to OM in young children, we found that immune-evasive phenotypes predict higher rates of progression, and that this effect of pneumococcal phenotype is weaker in mixed-species episodes.

We also found that serotypes with high rates of progression to OM are distinct from those with high risk of causing IPD when carried (21, 24, 25). This finding is consistent with previous studies (26, 27), reaffirming a differential role of capsular factors in pathogenesis of mucosal diseases. The association of immune-evasive pneumococcal phenotype with higher rates of OM progression suggests pathogenesis may be influenced by the ability of robust serotypes to maintain higher bacterial density in the nasopharynx, or by the stronger inflammatory responses necessary to clear these serotypes from the nasopharynx and middle ear (28, 29). Although our analysis does not characterize the contribution of viral infections to disease progression (30, 31), their impact is suggested in our analysis by elevated progression rates during the winter months.

Several epidemiologic findings from our study corroborate the findings of laboratory studies addressing the impacts of microbial interaction on OM pathogenesis. Because mixed-species OM episodes frequently involve biofilm formation, the weaker associations we identify between pneumococcal capsular phenotype and mixed-species disease progression are consistent with previous evidence of down-regulated pneumococcal capsule expression in biofilms (32). In addition, most serotypes did not show increased progression rates in the presence of NTHi in our analysis, similar to previous findings that mixed-species biofilms form “stable” infections with diminished risk of progressing to systemic disease in comparison to single-species infections (13). The previously-reported tendency for children experiencing mixed-species OM to suffer severe symptoms (2) may thus partially reflect susceptibility to severe manifestations among children at risk for mixed-species OM progression (33–35).

Our findings also aid interpretation of pneumococcal conjugate vaccine impact against OM. We found that pneumococcal serotypes included in PCV13 generally had higher rates of progression from colonization to OM in comparison to non-vaccine serotypes. This finding may explain why pneumococcal serotype replacement in carriage has not offset overall reductions in OM incidence following PCV7/13 rollout in Israel (22, 36) and other settings (37, 38) to the extent reported in IPD incidence (39). Because tissue damage sustained during early-life OM episodes historically associated with PCV13 serotypes contributes to risk for secondary infections (34), PCV13-mediated protection against OM caused by these virulent agents may also contribute indirectly to reduced incidence of OM caused by other pathogens (22, 40).

Our analysis has certain limitations. An ideal prospective study of OM progression would monitor nasopharyngeal carriage and OM incidence with MEF culture within a cohort of children to assess pathogen-specific OM incidence rates during colonization. Because the sample size needed to characterize serotype differences in progression would be prohibitively large in such a study, however, microbiologically-detailed prospective studies of carriage and disease provide a good alternative for characterizing pathogen-specific virulence (25–27, 40). The validity of evaluating progression as OM incidence per carrier is suggested by meta-analytic findings of high concordance (>80%) in Spn and NTHi detection from nasopharyngeal and MEF cultures in severe OM cases (41), such as those necessitating MEF culture in our study.

Nonetheless, molecular diagnostic tools may offer enhanced sensitivity in comparison to culture as performed here (42–44).

It is important to note that our sample was enriched with complex OM cases due to the indications for obtaining MEF culture in the original prospective study. Thus, comparisons in our analysis reflect differences in progression to severe OM, and may not represent the broader spectrum of illness including acute OM episodes. While our analysis identified a higher progression rate for NTHi than certain pneumococcal serotypes, this may not owe entirely to differences in virulence between the species. In comparison to Spn, NTHi tends to be carried at older ages (**Figure S1**), and has high colonization prevalence among otitis-prone children (35, 45). High rates of progression for this pathogen may thus reflect its predominance in secondary infections following tissue damage associated with early-life OM (34), in particular among otitis-prone children (35).

Our study provides data about species interactions between Spn and NTHi in children prior to the establishment of widespread immunity against virulent pneumococcal serotypes through routine PCV7/13 immunization. Our finding that the association between pneumococcal phenotype and OM progression rate is altered in the presence of NTHi helps to account for the distinct serotype distributions of single-species and mixed-species infections (2).

METHODS

Setting

Previously-published studies provided data on incidence of severe OM necessitating MEF culture (22) and prevalence of bacterial carriage (5, 18) among Bedouin and Jewish children in the Negev region of southern Israel. The Bedouin population is transitioning from nomadic lifestyles to permanent settlements, and has larger family sizes, higher levels of overcrowding, and lower socioeconomic status in comparison to the nearby Jewish population (46). Bedouin children tend to experience higher rates of bacterial carriage and disease than Jewish children, despite receiving care from the same facilities (5).

Datasets

OM incidence and carriage prevalence

Incidence of OM episodes necessitating MEF culture is monitored routinely at the Soroka University Medical Center (SUMC) through an ongoing prospective, population-based epidemiological surveillance program. Over 95% of children in the Negev region receive care at SUMC. Indications for MEF culture are based on clinical severity and include, but are not limited to, previous OM or tube insertion at any time, high-grade fever or toxic appearance, and spontaneous drainage, as detailed previously (22); these criteria have not changed during the study period.

Prevalence of nasopharyngeal Spn and NTHi carriage was monitored in a pre-implementation trial of PCV7 dosing schedules, wherein receipt of a first vaccine dose was randomized to

between 2 and 18 months of age (18); data regarding pneumococcal and NTHi co-colonization have been published previously (4, 5). We included data from all visits by unvaccinated children up to age 18 months.

Bacteriological procedures

Samples of MEF were obtained from tympanocentesis or spontaneous drainage and placed in MW173 Amies transport medium (Transwab; Medical Wire and Equipment, Potley, UK) before being plated, within 16 hours, on trypticase agar (5% sheep blood and 5 µg/mL gentamicin) and chocolate agar media, followed by 48 hours' incubation at 35ºC in 5% CO_2. Laboratory procedures for identification of Spn and NTHi were consistent in the studies of carriage and MEF, and have been described previously (18). Pneumococcal serotypes were determined by the Quellung reaction (antisera from Statens Serum Institut, Copenhagen, Denmark). Studies received ethics approval from SUMC, and secondary analyses were exempted by the institutional review board at Harvard TH Chan School of Public Health.

Pneumococcal serotype measurements

We used previously-obtained measurements of pneumococcal phenotypes in our analyses of factors associated with progression rate. These included the negative surface charge of the capsule (a determinant of susceptibility to phagocytosis) (19); the metabolic efficiency of capsule production, measured by the inverse of the number of carbons per repeat unit of the polysaccharide (12); the ability of serotypes to survive neutrophil-mediated killing in an *in vitro* surface assay (12); the width of the capsule (12); the likelihood for serotypes to cause death during invasive pneumococcal disease (IPD) (20); and the likelihood for serotypes to progress from carriage to IPD (21).

Statistical analysis

Serotype distribution in carriage and otitis media

We measured serotype diversity (D) as

$$D = 1 - \sum_i p_i^2$$

where p_i values indicated the proportion of isolates belonging to serotype i. We used bootstrap resampling to measure confidence intervals around estimates and to test for differences in diversity in carriage and OM, applying a cluster bootstrap of children to account for repeated sampling in the carriage studies (4).

We used Kullback-Leibler divergence to measure similarity of the pneumococcal serotype distribution in mixed-species OM to the serotype distributions of the following clinical entities: pneumococcal OM without NTHi, pneumococcal carriage without NTHi, and mixed-species carriage of Spn and NTHi. We sampled from Dirichlet-multinomial posterior distributions of serotype frequencies, applying a flat (Jeffreys) prior to account for uncertainty in sparse

observations (47). Measures closer to zero indicated greater similarity to the pneumococcal serotype distribution of mixed-species OM. Consistent with our analyses of Simpson diversity, we generated credible intervals and conducted hypothesis testing via the bootstrap for disease isolates, and via the cluster bootstrap for carriage isolates.

Variation in otitis media progression rate

Odds ratios calculated from counts (Y) of pathogen-specific carriage and disease episodes supplied the relative rate of progression from colonization to OM, measured as (cases/year)/carrier. The theoretical basis of this interpretation is as follows: defining the prevalence of colonization by agent(s) i as π_i and the rate of OM incidence as λ_i,

$$OR_{ij} = \frac{Y_i^{OM} Y_j^{Car}}{Y_j^{OM} Y_i^{Car}} = \frac{\left(\frac{Y_i^{OM}}{PYAR(OM)}\right)\left(\frac{Y_j^{Car}}{N(Car)}\right)}{\left(\frac{Y_j^{OM}}{PYAR(OM)}\right)\left(\frac{Y_i^{Car}}{N(Car)}\right)} = \frac{\lambda_i \pi_j}{\lambda_j \pi_i} = \frac{\left(\frac{\lambda_i}{\pi_i}\right)}{\left(\frac{\lambda_j}{\pi_j}\right)}$$

, where $PYAR$(OM) and $N(Car)$ refer to total person-years at risk for OM and children at risk for carriage, respectively, in the two datasets.

Based on the same premise, we used the odds ratio of OM to quantify the association between pneumococcal serotype factors and rate of progression from colonization to OM in the presence and absence of NTHi. Defining e^{α_0} and e^{β_0} as the "baseline" rate of OM incidence and prevalence of colonization, respectively, and $e^{\alpha_1 X}$ and $e^{\beta_1 X}$ as the multiplicative impact of a serotype factor X on incidence and prevalence, respectively,

$$OR_{X=1} = \frac{\lambda_{X=1}\pi_{X=0}}{\lambda_{X=0}\pi_{X=1}} = \frac{(e^{\alpha_0+\alpha_1})(e^{\beta_0})}{(e^{\alpha_0})(e^{\beta_0+\beta_1})} = \frac{e^{\alpha_1}}{e^{\beta_1}}$$

, which again represents the relative progression rate as the fold increase in incidence for X=1 (ref. X=0), normalized by any effect of X on carriage prevalence.

We used logistic regression to calculate odds ratios and adjusted odds ratios, controlling for the seasonal peak in virus transmission (months December to March) and Jewish or Bedouin ethnicity. Because differing numbers of children were retained in the unvaccinated group of the carriage study across ages, it was not possible to adjust for age in the odds ratio formulation above; however, the narrow age range considered (≤18 months) limits bias. We fitted models via generalized estimating equations with an exchangeable correlation structure to account for repeated sampling of children in the carriage studies.

REFERENCES

1. Rovers MM. 2008. The burden of otitis media. Vaccine 26:G2-4.
2. Dagan R, Leibovitz E, Greenberg D, Bakaletz L, Givon-Lavi N. 2013. Mixed pneumococcal-nontypeable *Haemophilus influenzae* otitis media is a distinct clinical

entity with unique epidemiologic characteristics and pneumococcal serotype distribution. J Infect Dis 208:1152–1160.
3. Casey JR, Adlowitz DG PM. 2010. New patterns in otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. Pediatr Infect Dis J 29:304–9.
4. Lewnard JA, Huppert A, Givon-Lavi N, Pettigrew MM, Regev-Yochay G, Dagan R, Weinberger DM. 2016. Density, serotype diversity, and fitness of *Streptococcus pneumoniae* in upper respiratory co-colonization with nontypeable *Haemophilus influenzae*. J Infect Dis 214:1411–1420.
5. Lewnard JA, Givon-Lavi N, Huppert A, Pettigrew MM, Regev-Yochay G, Dagan R, Weinberger DM. 2016. Epidemiological markers for interactions among *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* in upper respiratory tract carriage. J Infect Dis 213:1596–1605.
6. Shak JR, Vidal JE, Klugman KP. 2013. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol 21:129–135.
7. Jacoby P, Watson K, Bowman J, Taylor A, Riley T V., Smith DW, Lehmann D. 2007. Modelling the co-occurrence of *Streptococcus pneumoniae* with other bacterial and viral pathogens in the upper respiratory tract. Vaccine 25:2458–2464.
8. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. 2010. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infect Dis 10:304.
9. Madhi SA, Adrian P, Kuwanda L, Cutland C, Albrich WC, Klugman KP. 2007. Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae*—and associated interactions with *Staphylococcus aureus* and *Haemophilus influenzae* colonization—in HIV-Infected and HIV-uninfected children. J Infect Dis 196:1662–1666.
10. Chien Y-W, Vidal JE, Grijalva CG, Bozio C, Edwards KM, Williams J V., Griffin MR, Verastegui H, Hartinger SM, Gil AI, Lanata CF, Klugman KP. 2012. Density interactions between *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in the nasopharynx of young Peruvian children. Pediatr Infect Dis J 32:72–77.
11. Lipsitch M, Abdullahi O, D'Amour A, Xie W, Weinberger DM, Tchetgen Tchetgen E, Scott JAG. 2012. Estimating rates of carriage acquisition and clearance and competitive ability for pneumococcal serotypes in Kenya with a Markov transition model. Epidemiology 23:510–519.
12. Weinberger DM, Trzciński K, Lu Y-J, Bogaert D, Brandes A, Galagan J, Anderson PW, Malley R, Lipsitch M. 2009. Pneumococcal capsular polysaccharide structure predicts serotype prevalence. PLoS Pathog 5:e1000476.
13. Weimer KEDED, Armbruster CEE, Juneau RA a, Hong W, Pang B, Swords WEE. 2010. Coinfection with *Haemophilus influenzae* promotes pneumococcal biofilm formation during experimental otitis media and impedes the progression of pneumococcal disease. J Infect Dis 202:1068–1075.
14. Margolis E, Yates A, Levin BR. 2010. The ecology of nasal colonization of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*: the role of competition and interactions with host's immune response. BMC Microbiol 10:59.
15. Lysenko ES, Lijek RS, Brown SP, Weiser JN. 2010. Within-host competition drives

selection for the capsule virulence determinant of *Streptococcus pneumoniae*. Curr Biol 20:1222–1226.
16. Lysenko ES, Ratner AJ, Nelson AL, Weiser JN. 2005. The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. PLoS Pathog 1:0003–0011.
17. Pericone CD, Overweg K, Hermans PW, Weiser JN. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect Immun 68:3990–3997.
18. Dagan R, Givon-Lavi N, Porat N, Greenberg D. 2012. The effect of an alternative reduced-dose infant schedule and a second year catch-up schedule with 7-valent pneumococcal conjugate vaccine on pneumococcal carriage: a randomized controlled trial. Vaccine 30:5132–5140.
19. Li Y, Weinberger DM, Thompson CM, Trzciński K, Lipsitch M. 2013. Surface charge of *Streptococcus pneumoniae* predicts serotype distribution. Infect Immun 81:4519–4524.
20. Harboe ZB, Thomsen RW, Riis A, Valentiner-Branth P, Christensen JJ, Lambertsen L, Krogfelt KA, Konradsen HB, Benfield TL. 2009. Pneumococcal serotypes and mortality following invasive pneumococcal disease: a population-based cohort study. PLoS Med 6:e1000081.
21. Sleeman KL, Griffiths D, Shackley F, Diggle L, Gupta S, Maiden MC, Moxon ER, Crook DW, Peto TE a. 2006. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. J Infect Dis 194:682–8.
22. Ben-Shimol S, Givon-Lavi N, Leibovitz E, Raiz S, Greenberg D, Dagan R. 2016. Impact of widespread introduction of pneumococcal conjugate vaccines on pneumococcal and non-pneumococcal otitis media. Clin Infect Dis 63:611–618.
23. Weinberger DM, Harboe ZB, Sanders E a M, Ndiritu M, Klugman KP, Rückinger S, Dagan R, Adegbola R, Cutts F, Johnson HL, O'Brien KL, Scott JA, Lipsitch M. 2010. Association of serotype with risk of death due to pneumococcal pneumonia: a meta-analysis. Clin Infect Dis 51:692–699.
24. Brueggemann AB, Peto TEA, Crook DW, Butler JC, Kristinsson KG, Spratt BG. 2004. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J Infect Dis.
25. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. 2003. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 187:1424–32.
26. Shouval DS, Greenberg D, Givon-Lavi N, Porat N, Dagan R. 2006. Site-specific disease potential of individual *Streptococcus pneumoniae* serotypes in pediatric invasive disease, acute otitis media and acute conjunctivitis. Pediatr Infect Dis J 25:602–7.
27. Hanage WP, Auranen K, Syrjänen R, Herva E, Mäkelä PH, Kilpi T, Spratt BG. 2004. Ability of pneumococcal serotypes and clones to cause acute otitis media: implications for the prevention of otitis media by conjugate vaccines. Infect Immun 72:76–81.
28. Bakaletz LO. 2010. Immunopathogenesis of polymicrobial otitis media. J Leukoc Biol 87:213–22.
29. Juhn SK, Jung M-K, Hoffman MD, Drew BR, Preciado DA, Sausen NJ, Jung TTK, Kim BH, Park S-Y, Lin J, Ondrey FG, Mains DR, Huang T. 2008. The role of inflammatory mediators in the pathogenesis of otitis media and sequelae. Clin Exp Otorhinolaryngol 1:117–38.

30. Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T. 2011. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. J Clin Microbiol 49:3750–3755.
31. Chonmaitree T, Revai K, Grady JJ, Clos A, Patel JA, Nair S, Fan J, Henrickson KJ. 2008. Viral upper respiratory tract infection and otitis media complication in young children. Clin Infect Dis 46:815–823.
32. Chao Y, Marks LR, Pettigrew MM, Hakansson AP. 2015. *Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease. Front Cell Infect Microbiol 4:194.
33. Faden H, Brodsky L, Waz MJ, Stanievich J, Bernstein JM, Ogra PL. 1991. Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. Ann Otol Rhinol Laryngol 100:612–615.
34. Dagan R, Pelton S, Bakaletz L, Cohen R. 2016. Prevention of early episodes of otitis media by pneumococcal vaccines might reduce progression to complex disease. Lancet Infect Dis 16:480–492.
35. Kaur R, Morris M, Pichichero ME. 2017. Epidemiology of acute otitis media in the post-pneumococcal conjugate vaccine era. Pediatrics 140:e20170181.
36. Ben-Shimol S, Givon-Lavi N, Leibovitz E, Raiz S, Greenberg D, Dagan R. 2014. Near-elimination of otitis media caused by 13-valent pneumococcal conjugate vaccine (PCV) serotypes in southern Israel shortly after sequential introduction of 7-valent/13-valent PCV. Clin Infect Dis 59:1724–1732.
37. De Wals P, Carbon M, Sévin E, Deceuninck G, Ouakki M. 2009. Reduced physician claims for otitis media after implementation of pneumococcal conjugate vaccine program in the province of Quebec, Canada. Pediatr Infect Dis J 28:e271-5.
38. Marom T, Tan A, Wilkinson GS, Pierson KS, Freeman JL, Chonmaitree T. 2014. Trends in otitis media-related health care use in the United States, 2001-2011. JAMA Pediatr 168:68–75.
39. Weinberger DM, Malley R, Lipsitch M. 2011. Serotype replacement in disease after pneumococcal vaccination. Lancet.
40. Lewnard JA, Givon-Lavi N, Weinberger DM, Lipsitch M, Dagan R. 2017. Pan-serotype reduction in progression of Streptococcus pneumoniae to otitis media after rollout of pneumococcal conjugate vaccines. Clin Infect Dis: cix673.
41. van Dongen TM, van der Heijden GJ, van Zon A, Bogaert D, Sanders EM, Schilder AGM. 2013. Evaluation of concordance between the microorganisms detected in the nasopharynx and middle ear of children with otitis media. Pediatr Infect Dis J 32:549–52.
42. Xu Q, Kaur R, Casey JR, Adlowitz DG, Pichichero ME, Zeng M. 2011. Identification of *Streptococcus pneumoniae* and *Haemophilus in*fluenzae in culture-negative middle ear fluids from children with acute otitis media by combination of multiplex PCR and multi-locus sequencing typing. Int J Pediatr Otorhinolaryngol 75:239–244.
43. Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, Nosten F, Goldblatt D. 2011. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. J Clin Microbiol 49:1784–1789.
44. Cilveti R, Olmo M, Pérez-Jove J, Picazo J-J, Arimany J-L, Mora E, Pérez-Porcuna TM, Aguilar I, Alonso A, Molina F, del Amo M, Mendez C. 2017. Epidemiology of otitis media with spontaneous perforation of the tympanic membrane in young children and

association with bacterial nasopharyngeal carriage, recurrences and pneumococcal vaccination in Catalonia, Spain: the prospective HERMES Study. PLoS One 12:e0170316.
45. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D. 1994. Nasopharyngeal colonization with nontypeable *Haemophilus influenzae* and recurrent otitis media. J Infect Dis 170:862–866.
46. Israel Central Bureau of Statistics. 2016. Statistical Abstract of Israel No. 67.
47. Jeffreys H. 1946. An invariant form for the prior probability in estimation problems. Proc R Soc London Ser A Math Phys Sci 186:453–461.

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CONFLICTS OF INTEREST

JAL received research funds from Pfizer to Harvard University for the study (grant CP147216). JAL has also received consulting fees from Pfizer. RD has received grants and research support from Berna/Crucell, Wyeth/Pfizer, Merck, and Protea; has been a scientific consult for Berna/Crucell, GlaxoSmithKline, Novartis, Wyeth/Pfizer, Merck, and Protea; has been a speaker for Berna/Crucell, GlaxoSmithKline, and Wyeth/Pfizer; and is a shareholder of Protea/NASVAX. NG-L and PT report no conflicts.

TABLES

Table 1: Data sources

Variables measured	Measurement details	Coverage	Citation
Pneumococcal OM incidence	All episodes of OM necessitating MEF culture from children <18 months old presenting for care at Soroka University Medical Center between July 1999 and June 2008	11811 episodes, with 9397 Spn or NTHi positive	(22)
Nasopharyngeal pneumococcal carriage prevalence	Unvaccinated Bedouin and Jewish children sampled at scheduled visits, ages 2-18 months, enrolled in a PCV7 dosing study	769 children submitting 1588 swabs, with 891 Spn or NTHi positive	(5)
Resistance to neutrophil-mediated killing[1]	Proportion surviving a complement-independent *in vitro* surface killing assay for each pneumococcal serotype	14 serotypes[2]	(12)
Magnitude of anionic surface charge[1]	(Negative) zeta potential of fixed-density suspension of pneumococcal serotype in phosphate-buffered saline	48 serotypes[2]	(19)
Capsular size[1]	Zone of exclusion of fluorescent dextran molecules around pneumococcal serotype diplococcus	15 serotypes[2]	(12)
IPD case-fatality ratio	Serotype-specific 30-d mortality during IPD	37 serotypes[2]	(20)

Metabolic efficiency	Inverse of number of carbons per capsular polysaccharide repeat unit of pneumococcal serotype	54 serotypes[2]	(12)
Invasiveness	Proportion of carriage events leading to IPD	36 serotypes[2]	(21)

[1]*In vitro* measurements of serotype properties were obtained from isogenic capsular-switch mutants
[2]We list serotypes for which phenotypic data were collected in **Table S1**.

Table 2: Pneumococcal phenotype associations with otitis media progression

Pneumococcal attribute[1]	Relative progression rate, per 1SD increase in covariate (95% CI)[2]	Adjusted relative progression, per 1SD increase in covariate (95% CI)[2,3]	Adjusted relative increase in progression rate, per 1SD increase in covariate, for Spn+NTHi (95% CI)
Metabolic efficiency (12)			
Spn only	**1.55 (1.33, 1.81)**	**1.54 (1.32, 1.80)**	ref.
Spn+NTHi	**1.22 (1.08, 1.39)**	**1.22 (1.07, 1.39)**	**0.79 (0.65, 0.97)**
Surface anionic charge (19)			
Spn only	**1.63 (1.40, 1.89)**	**1.62 (1.39, 1.88)**	ref.
Spn+NTHi	**1.31 (1.15, 1.49)**	**1.30 (1.14, 1.48)**	**0.80 (0.66, 0.97)**
Resistance to neutrophil-mediated killing (12)			
Spn only	1.12 (0.99, 1.27)	**1.13 (1.01, 1.28)**	ref.
Spn+NTHi	1.05 (0.90, 1.24)	1.07 (0.91, 1.26)	0.94 (0.77, 1.16)
Capsule width (12)			
Spn only	**1.33 (1.18, 1.51)**	**1.34 (1.19, 1.52)**	ref.
Spn+NTHi	**1.32 (1.13, 1.55)**	**1.34 (1.14, 1.57)**	1.00 (0.82, 1.22)
Case-fatality ratio in IPD (20)			
Spn only	**1.53 (1.32, 1.77)**	**1.56 (1.34, 1.81)**	ref.
Spn+NTHi	**1.22 (1.07, 1.38)**	**1.23 (1.08, 1.41)**	**0.79 (0.65, 0.96)**
Invasiveness (IPD per carriage episode) (21)			
Spn only	**0.70 (0.63, 0.78)**	**0.70 (0.62, 0.78)**	ref.
Spn+NTHi	**0.81 (0.71, 0.91)**	**0.81 (0.72, 0.91)**	1.16 (0.99, 1.35)

[1]Pneumococcal attributes are defined in Table 1.
[2]Progression rates are compared via the odds ratio, as calculated by logistic regression with an exchangeable correlation structure to account for repeated carriage observations from individual children. Due to collinearity among serotype measurements, individual models are fitted for each association. Our derivation of the odds ratio as the relative progression rate is in the Methods.
[3]Models control for Jewish/Bedouin ethnicity and respiratory virus season (December to March).

FIGURES

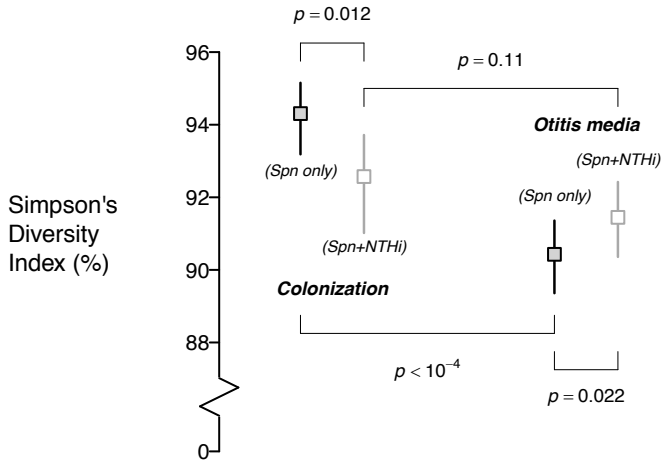

Figure 1: Pneumococcal serotype diversity in carriage and MEF isolates, with and without co-occurring NTHi. Pneumococcal serotype diversity is lower in single-species OM than single-species colonization, suggesting a role of serotype factors in progression. This difference is not apparent in mixed-species colonization and OM.

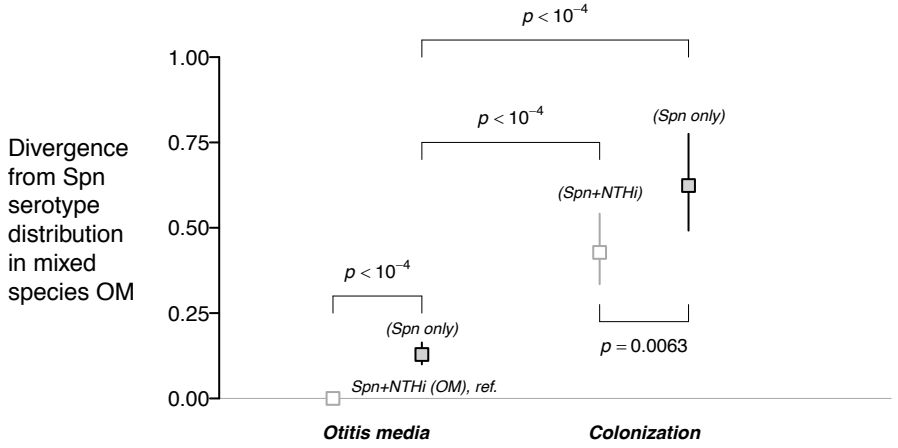

Figure 2: Divergence of pneumococcal serotype distribution in single-species and mixed-species carriage and OM. To determine whether serotype-specific interactions of *S. pneumoniae* with NTHi contribute to the pneumococcal serotype distribution of mixed-species OM episodes, we calculated Kullback-Leibler divergence in pneumococcal serotype distributions from that of mixed-species OM episodes for: single-species (Spn) OM, mixed-species carriage (Spn+NTHi), and single-species carriage. A value of zero indicates an exact match with the pneumococcal serotype distribution of mixed-species OM episodes; increasing values reflect greater divergence.

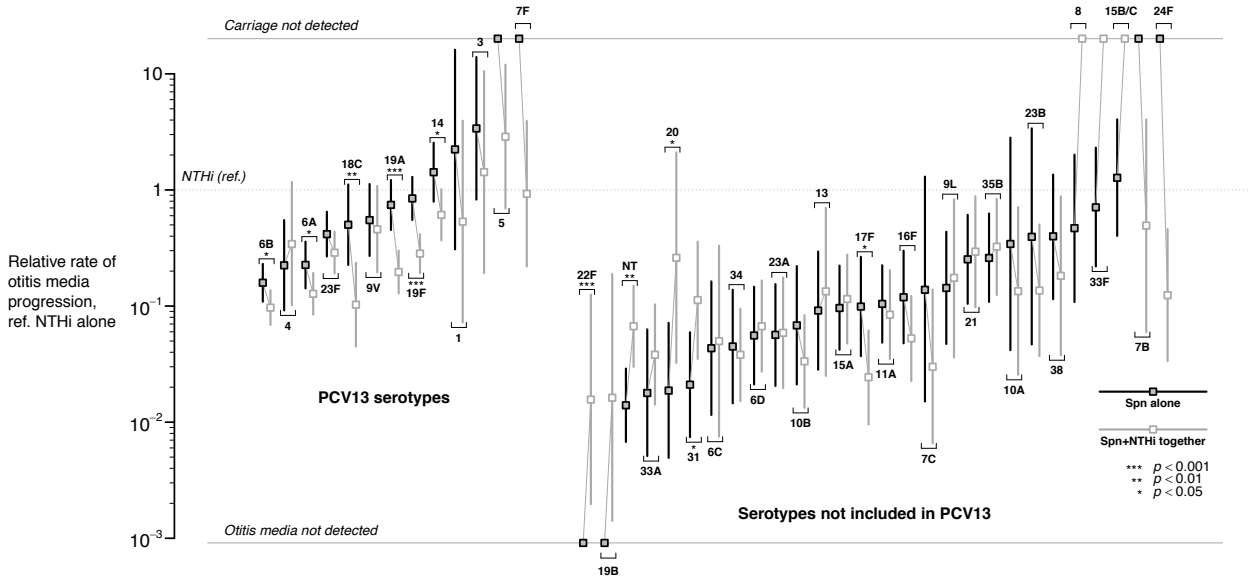

Figure 3: Pneumococcal serotype associations with otitis media progression. The rate ratio of OM progression from colonization (as measured by the adjusted odds ratio; see Methods for derivation) is calculated for each *S. pneumoniae* serotype, in single-species and mixed-species episodes, relative to NTHi. Analyses control for season (Dec-Mar or other) and Jewish/Bedouin ethnicity. Unencapsulated serotypes are labeled "NT". Models are estimated with an exchangeable correlation structure to account for repeated sampling of individual children in the carriage data.

SUPPORTING INFORMATION

Table S1: Serotype coverage for phenotypic measurements.

Attribute	Serotypes
Resistance to neutrophil-mediated killing	1, 4, 5, 6A/C, 6B, 7F, 9N, 9V, 11A, 14, 18C, 19A, 19F, 23F
Capsular size	1, 4, 5, 6A/C, 6B, 7F, 9N, 9V, 11A, 14, 18C, 19A, 19F, 23F, 35B
Metabolic efficiency	1, 2, 3, 4, 5, 6A/C, 6B, 7A, 7B, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10F, 11A, 11B, 11C, 11F, 12A, 12F, 13, 14, 15A, 15B, 15C, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 22F, 23F, 27, 29, 31, 32A, 32F, 33B, 33F, 34, 35A, 35B, 37, 43
Invasiveness	1, 3, 4, 5, 6A/C, 6B, 7F, 8, 9A, 9N, 9V, 10A, 11A, 12F, 13, 14, 15A, 15B, 15C, 16F, 17F, 18B, 18C, 19A, 19F, 20, 21, 22F, 23A, 23B, 23F, 31, 33F, 35F, 37, 38
Magnitude of anionic surface charge	1, 2, 3, 4, 5, 6A/C, 6B, 7A, 7B, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10F, 11A, 11C, 11F, 12A, 12F, 13, 14, 15A, 15B, 15C, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20, 22F, 23F, 27, 29, 31, 32A, 32F, 33B, 33F, 34, 35A, 35B, 37, 43
IPD case-fatality ratio	1, 2, 3, 4, 5, 6A/C, 6B, 7F, 8, 9A, 9N, 9V, 10A, 11A, 12A, 12F, 13, 14, 15A, 15B, 15C, 16F, 17F, 18C, 19A, 19F, 20, 22F, 23A, 23F, 24F, 31, 33F, 34, 35B, 35F, 38

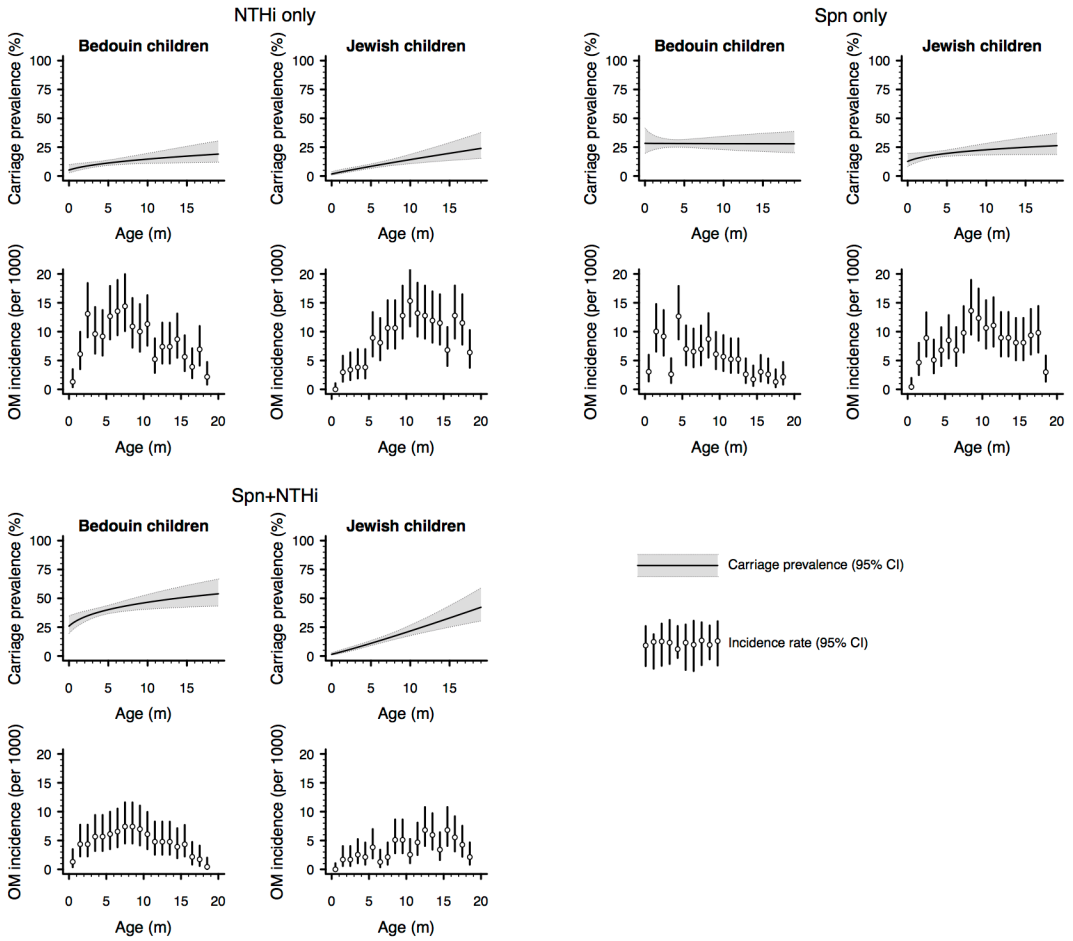

Figure S1: Carriage prevalence and otitis media incidence among Jewish and Bedouin children. We calculated age-specific prevalence of Spn and NTHi carriage by fitting regression models with a log link function to data from scheduled visits at ages 2, 4, 6, 7, 12, and 18 months; we estimated the continuous trend over ages using a log transformation, which we determined was superior to first, second, or third-order polynomial terms via the Bayesian information criterion. We used an exchangeable correlation structure to account for repeated sampling of individual children. Incidence rate analyses were limited to data from 2004 to 2008 for compatibility with the timeframe of the carriage studies, and with previous studies of epidemiological circumstances preceding rollout of PCV7/13 in southern Israel (22, 40).