Density Interactions between *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* in the Nasopharynx of Young Peruvian Children

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Abstract

Streptococcus pneumoniae, Haemophilus influenzae and *Staphylococcus aureus* are commonly carried in the nasopharynx (NP) of young children, and have been speculated to interact with each other. Although earlier studies used cultures alone to assess these interactions, the addition of real-time quantitative polymerase chain reaction (qPCR) provides further insight into these interactions. We compared results of culture and qPCR for the detection of these three bacteria in 446 NP samples collected from 360 healthy young children in a prospective cohort study in the Peruvian Andes. Patterns of concurrent bacterial colonization were studied using repeated measures logistic regression models with generalized estimating equations. Spearman correlation coefficients were employed to assess correlations among bacterial densities. At a bacterial density $<10^5$ colony forming units (CFU)/ml measured by qPCR, culture detected significantly less carriers (P<0.0001) for all three pathogens, than at a bacterial density $>10^5$ CFU/ml. In addition, there was a positive association between *S. pneumoniae* and *H. influenzae* colonization measured by both culture (OR 3.11 – 3.17, p < 0.001) and qPCR (OR 1.95 – 1.97, p < 0.01). The densities of *S. pneumoniae* and *H. influenzae*, measured by qPCR, were positively correlated (correlation coefficient 0.32, p < 0.001). A negative association was found between the presence of *S. pneumoniae* and *S. aureus* in carriage with both culture (OR 0.45, p = 0.024) and qPCR (OR 0.61, p < 0.05). The impact of density on detection by culture and the observed density-related interactions support use of qPCR in additional studies to examine vaccine effects on diverse bacterial species.

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INTRODUCTION

Several common respiratory bacterial pathogens, including *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus* asymptomatically reside in the human nasopharynx. However, they can occasionally invade adjacent sites or the blood stream and cause disease such as otitis media, pneumonia, bacteremia and meningitis. The pneumococcus is commonly associated with these illnesses, (1) while *H. influenzae* is a common cause of acute otitis media (2) and *S. aureus* is a re-emerging cause of clinically important infections)(3), ranging from mild skin infections and sinusitis to severe diseases such as pneumonia, bacteremia, and endocarditis.

These different bacterial species may interact with each other by competing for resources and by producing chemicals or by inducing host immune responses that influence the growth of other bacteria in the nasopharynx.(4) Several observational studies using culture for bacterial detection have shown that *S. aureus* prevalence is negatively associated with *S. pneumoniae* prevalence,(5, 6) especially with vaccine-type pneumococci,(7-9) while some other studies found no association between them.(10, 11) Earlier studies have shown NP colonization by *S. pneumoniae* and *H. influenzae* to be positively associated.(5, 10-12)

The reported prevalence of nasopharyngeal (NP) colonization with these bacteria varies widely, depending on study population, age, exposure to antibiotics, socioeconomic status and variations in sampling and detection methods, and exposure to vaccines.(13) Previous studies using conventional culture have shown NP colonization of children ranged from 13 – 85% and 6 – 80% for *S. pneumoniae* and *H. influenzae*, respectively,(2, 8) and 10 – 35% for *S. aureus* .(7, 14, 15) Previous interaction studies used culture alone to detect and quantify bacteria. However, cultures have several drawbacks, such as low sensitivity compared with molecular methods, and it is time-consuming and laborious to perform quantification analysis. Real-time quantitative polymerase chain reaction (qPCR) is potentially a more sensitive and rapid alternative to culture.(13) Thus, the addition of qPCR to interaction assessments could provide valuable new insights.

Understanding factors that influence NP colonization by these bacteria is essential since colonization is the initial step for the development of disease. In addition, NP colonization of these pathogens in children is an important source of horizontal transmission to other individuals in the community.(16) Whether bacteria colonize or not is determined by a complex combination of factors including host characteristics that influence the exposure or susceptibility to specific bacterial species and direct interactions between different bacteria. Host factors that have been suggested to influence the colonization prevalence of *S. pneumoniae, H. influenzae,* or *S. aureus* include age, gender, ethnicity, immunity, crowding, number of siblings, daycare attendance, season, antibiotic therapy, acute respiratory infections, vaccine exposure, and environmental exposure to tobacco smoke.(2, 8, 12, 17, 18)

The goals of our study were to (1) compare culture and qPCR for detection of *S. pneumoniae, H. influenzae,* or *S. aureus* in NP samples; (2) describe the prevalence of NP colonization by *S. pneumoniae, H. influenzae,* and *S. aureus* in young children in rural communities of the Peruvian Andes; and (3) investigate the interaction between *S. pneumoniae, H. influenzae,* or *S. aureus* in two ways: first, evaluate whether colonization

status (presence/absence) of one bacterium influences the colonization status of the other two bacteria, and second, evaluate whether the densities of the three bacteria are correlated.

MATERIALS AND METHODS

Study population and data collection

The population for this study was derived from a prospective cohort study of ~500 children 0-35 months of age in the District of San Marcos, Cajamarca, Peru. The study aimed to investigate whether indoor air pollution and acute respiratory infections (ARI) influenced nasopharyngeal colonization with *S. pneumoniae* in healthy rural children living between 2000 and 4000 meters above sea level. Children 0-35 months of age were enrolled and baseline demographic and socioeconomic information collected; the presence of ARI or pneumonia symptoms was collected at baseline and at weekly visits to the homes by trained field workers. Routine nasopharyngeal swabs for bacterial colonization were collected monthly on all children using Rayon swabs immediately placed in 1 ml of transport medium (skim-milk tryptone glucose glycerol, STGG), processed at a local laboratory according to WHO standards and stored at −70°C. Enrollment into this study started in May 2009. Here we present data for 446 consecutive NP samples collected among children 360 without ARI symptoms between August and September 2009.

Bacteriologic cultures

To increase the sensitivity of cultures, 200 μl of STGG sample were enriched in THY broth (Todd-Hewitt broth supplemented with 0.5% of yeast extract) containing 1% of rabbit serum and incubated for 6 h at 37°C with 5% CO_2.(13) To identify *S. pneumoniae* strains, the enrichment broth was inoculated onto blood agar plates (BHI agar containing 5% sheep blood) and incubated overnight at 37°C in a 5% CO_2 atmosphere.(13) Presumptive pneumococcus-like colonies were confirmed by the optochin susceptibility test and bile solubility. Equivocal results were confirmed by PCR using primers that target the *cps*A gene(19) and DNA extracted from the *S. pneumoniae* isolates using the Chelex method (see below).(20)

For detection of *S. aureus*, the enriched THY broth was inoculated onto mannitol salt agar and incubated at 37°C in 5% CO_2 for 24 hours; colonies morphologically suggestive of *S. aureus* were confirmed by performing PCR on Chelex-extracted DNA using published primers targeting the *nuc* gene.(21)

For detection of all serotypes and non-typable *H. influenzae*, 200 μl of STGG sample was enriched in brain heart infusion broth with 5% Fildes enrichment (BD Diagnostics, NJ, USA) for 6 h at 37°C in 5% CO_2; the enriched broth was then plated onto chocolate agar with bacitracin and incubated overnight. The presence of *H. influenzae* was confirmed by performing PCR using the primers targeting the *hpd* gene which detects all *H. influenzae* strains(22) on DNA extracted from suspected colonies using the extraction method previously described by LaClaire *et al.*(23)

DNA extraction from cultured isolates using the Chelex method for PCR

A loopful of bacteria from the culture plate was placed into a 1.5 ml micro-centrifuge tube and mixed with 200 μl of 5% Chelex-100 resin (Bio-Rad) and 2 μl of Proteinase K (20mg/ml, QIAgen).(20) After incubation at 56°C for an hour then at 95°C for 10 minutes, the sample was mixed and then centrifuged at 13,000 rpm for 5 minutes to completely separate the layers. The DNA-containing supernatant was used as template in PCR reactions.

DNA extraction for qPCR from the original nasopharyngeal specimen and reference strains

Two-hundred µl of STGG sample were added with 100 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer containing 0.04 g/ml lysozyme and 75 U/ml of mutanolysin and then incubated for 1 h at 37°C in a water bath. Using the QIAamp DNA Mini protocol, DNA was eluted in 100 µl of elution buffer and kept at −70°C. Genomic DNA from the reference strains of *S. pneumoniae* (ATCC 33400 or TIGR4),(24) *S. aureus* (ATCC 25923) and *H. influenzae* type b (CDC reference strain M5216) was also extracted from overnight cultures using the QIAamp kit. DNA concentrations were measured by Nanodrop method (Nanodrop Technologies, Wilmington, DE) and serial dilutions in DNase-, RNase-free water were made to obtain the qPCR standards.

Real-time quantitative PCR (qPCR)

The total density of *S. pneumoniae*, measured in CFU/ml, was determined using pre-optimized concentrations of the forward primer (5′-ACGCAATCTAGCAGATGAAGCA-3′; 200 nM), reverse primer (5′-TCGTGCG TTTTAATTCCAGCT-3′; 200 nM), and probe (5′-FAM-TGCCGAAAACGCTTGATACAG GGAG-3-BHQ1; 200 nM) targeting the *lytA* gene as described previously.(24) To quantify the molecular bacterial load (CFU/ml) purified genomic DNA from *S. pneumoniae* reference strain TIGR4 (from our laboratory collection) was serially diluted to prepare standards representing 4, 4×10^1, 4×10^2, 4×10^3, 4×10^4, 4×10^5 or 4×10^6 CFU as previously described.(25) These standards were run along with our samples in a CFX96 real time PCR-detection system (Bio-Rad, Hercules CA) and final *S. pneumoniae* CFU/ml were calculated using the software Bio-Rad CFX manager.

The total density of *S. aureus*, measured in CFU/ml, was determined using the forward primer (5′-GTTGCTTAGTGTTAACTTTAGTTGTA-3′; 800 nM), reverse primer (5′-AATGTCGCAGGTTCTTTATGTAATTT-3′; 800 nM), and probe (5-FAM-AAGTCTAAGTAGCTCAGCAAATGCA-3-BHQ1; 400 nM) targeting the *nuc* gene.(21) To create standard curves, purified genomic DNA of the whole genome sequenced *S. aureus* reference strain N315 was used, assuming a genome size of 2.8 Mb. (26)

The total density of *H. influenzae*, measured in CFU/ml, was determined using recently published primers and probe: forward primer (5′-GGTTAAATATGCCGATGGTGTTG-3′; 100 nM), reverse primer (5′-TGCATCTTTACGCACGGTGTA-3′; 300 nM), and probe (5′-HEX-TTGTGTACACTCCGT "T-BHQ1" GGTAAAAGAACTTGCAC-3′; 100 nM) targeting the *hpd* gene.(22) To create standard curves, the purified genomic DNA of *H. influenzae* reference strain M5029 (a gift from Leonard Mayer, CDC, Atlanta GA, USA), was used, assuming a genome size of 1.8 Mb.

Real-time quantitative PCR was performed using the Bio-Rad CFX96™ Real-Time PCR Detection System (Hercules, CA, USA) in a reaction volume of 25 µl containing the EXPRESS qPCR Supermix Universal (Invitrogen by Life Technology, CA, USA), 2.5 µl of sample DNA, forward and reverse primers and fluorogenic probe with concentrations described above. For *S. pneumoniae*, the qPCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min; samples with cycle threshold (Ct) values 35 were considered positives. For *H. influenzae*, the cycling conditions included 50°C for 2 min, 95°C for 10 min, and 45 cycles of 15 s at 95°C followed by 1 minute at 60°C; and samples with Ct values 35 were considered positives. For *S. aureus*, the conditions were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Positive samples were samples with Ct values 38.

Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS institute, Inc, Cary, NC, USA). To assess whether colonization by one bacterial species was associated with colonization by the other two bacterial species, repeated measures logistic regression models with generalized estimating equations (GEE) were used because some children contributed more than one swab to the analyses. We modeled colonization by *S. pneumoniae, H. influenzae* and *S. aureus* separately, and each model included variables indicating the presence/absence of the other two bacterial species as the main exposures of interest. Covariates to be controlled for potential confounding included age in months, gender and antibiotic usage within the 7 days prior to sample collection. Separate analyses used bacterial culture and qPCR to define the colonization status of each bacterium.

To assess the degree of correlation between densities of each pair of bacteria in all samples, we used Spearman correlation coefficients. This test was preferred because bacterial densities determined by qPCR were not normally distributed because a large proportion was qPCR negative, and were assigned a density of 0. We then focused on those samples that were positive for two bacteria and re-calculated Spearman correlation coefficients. Positive samples were also categorized into high density and low density using median as a cutoff. Chi-square tests were performed to examine whether there was an association among bacterial densities (classified as high or low) of the three bacteria.

Ethical Approvals

This study was approved by the Ethical Review Boards (ERB) of the Instituto de Investigación Nutricional, Vanderbilt University and Emory University. An ERB-approved written informed consent form was obtained from one parent (usually the mother) of participating subjects at enrollment. The study was also approved by the local health authorities, and by community leaders.

RESULTS

Study population

A total of 446 consecutive nasopharyngeal samples from 360 children aged 0 – 35 months (mean 18.5 months, median 18.3 months), 53% male were included in this analysis. Among the enrolled children, 309 children had vaccination information available, and the percentages of children who received at least one dose of pneumococcal conjugate vaccines and *H. influenzae* type b vaccines before the swabs were collected were 9.1% and 67.3%, respectively. Of the 446 swabs, only 10 (2.2%) were collected from children who had received antibiotics within the 7 days prior to sample collection.

Comparison of culture and qPCR

All 446 samples were tested by both culture and qPCR for *S. pneumoniae* and *S. aureus*. For *H. influenzae*, 446 samples were tested using qPCR while only 351 samples were cultured because of inadequate remaining sample volume. A comparison of bacterial culture with qPCR showed that qPCR detected more pneumococcal carriage (in 77.4% of children compared to 60.1% of children by culture); and similarly, qPCR detected 37.3% carriage of *H. influenzae* compared to 23.6% by culture. For *Staphylococcus aureus* carriage qPCR detected carriage in 40.8% of children while only 11.9% were detected by culture (Table 1). For all three bacteria, all culture-positive swabs were also positive by qPCR and all qPCR-negative swabs were also negative by culture. As the bacterial density measured by qPCR increased, the detection by culture increased (Table 2). At a carriage density of $<10^5$ colony forming units (CFU)/ml as measured by qPCR, culture was significantly less sensitive than PCR at detecting pneumococcal carriage. Carriage was detected by culture in 196 of 273

(71.8%) of children carrying at low density, compared to detection by culture in 72/72 (100%) at a density $>10^5$ CFU/ml density (P<0.00001). Lesser sensitivity of culture at a density measured by PCR of $<10^5$ CFU/ml compared to a CFU $>10^5$ was also true for *Haemophilus influenzae* (detection of 1/19 (5.2%) versus 82/112 (73.2%); P< 0.0001) and staphylococcal carriage (detection of 13/132 (9.8%) versus 40/50 (80%); P<0.0001) (Table 2). The proportion of 351 samples indicating colonization by single or multiple species, by culture and qPCR, are shown in Table 3. Results of cultures showed that none, 1, or 2 bacteria species were concurrently present in 28.2%, 49.3% and 22.5% of NP swabs respectively, while qPCR detected none, 1, or 2 bacteria species in 8.0%, 40.2% and 51.8% of NP swabs, respectively.

Prevalence and incidence of colonization by *S. pneumoniae, H. influenzae*, and *S. aureus* as determined by qPCR

Among the first swabs collected from the 360 children, the prevalence of colonization by *S. pneumoniae, H. influenzae,* and *S. aureus* was 76.9%, 36.4% and 40.3%. For 86 children who contributed two swabs taken one month apart, 47.0% of 17 children who were originally not colonized by *S. pneumoniae* were colonized after one month, and 13.0% of 69 children who were originally colonized by *S. pneumoniae* cleared the bacteria. The percentages of acquisition and clearance after one month were 28.8% and 32.4% for *H. influenzae*, and 39.5% and 54.2% for *S. aureus*, respectively. All specimens were collected for this analysis between Aug and Sep 2009 to exclude any potential seasonality effect. Over the limited age range of the study (0 – 36 months) we did not detect a significant negative impact of increasing age on the density of these three pathogens. Overall density of pneumococcal carriage was not significantly different among the 9.1% of children previously in receipt of at least a single dose of PCV, nor was Haemophilus density influenced by previous receipt of Hib vaccine.

Assessing the associations between the colonization status of *S. pneumoniae, H. influenzae*, and *S. aureus Bacterial cultures*

Repeated measures logistic regression models of colonization (presence or absence) by *S. pneumoniae* and *H. influenzae* determined by cultures are shown in Table 4. The model of colonization by *S. pneumoniae* indicated that colonization by *H. influenzae* was positively associated with *S. pneumoniae* (OR 3.11, 95% CI 1.73 – 5.59, p < 0.001), while the presence of *S. aureus* was negatively associated with colonization by *S. pneumoniae* (OR 0.45, 95% CI 0.22 – 0.90, p = 0.024), controlling for age, gender, and antibiotic usage. A positive association between *S. pneumoniae* and *H. influenzae* was also observed in the model of colonization by *H. influenzae* (OR 3.17, 95% CI 1.76 – 5.72, p < 0.001).

Quantitative PCR

Repeated measures logistic regression models of colonization by *S. pneumoniae*, *H. influenzae*, and *S. aureus* determined by qPCR indicated a positive association between *S. pneumoniae* and *H. influenzae* (OR 1.97, 95% CI 1.18 – 3.29, p = 0.009) and a significant negative association with *S. aureus* (OR 0.61, 95% CI 0. 39 – 0.97, p = 0.031). The model of *H. influenzae* colonization also showed a positive association between *H. influenzae* and *S. pneumoniae* (OR 1.95, 95% CI 1.19 – 3.21, p = 0.009). The model of *S. aureus* colonization also showed that *S. pneumoniae* was negatively associated with *S. aureus* (OR 0.61, 95% CI 0.40 – 0.96, p = 0.039).

Assessing the correlation of bacterial densities of *S. pneumoniae, H. influenzae* and *S. aureus*

The Spearman correlation coefficients for the density between *S. pneumoniae* and *H. influenzae*, was 0.32 (p < 0.001). When only positive swabs (density > 0) were considered, the Spearman correlation coefficient was 0.38 (p < 0.001). When the density of the positive swabs was categorized into high and low for each bacterium, swabs with high density of *S. pneumoniae* were associated with high density of *H. influenzae* (OR 3.67, p<0.001). No association was found between S. *pneumoniae* and *S. aureus* or between *H. influenzae* and *S. aureus* densities.

DISCUSSION

In this study, we describe the colonization of young children in the rural Andes using molecular methods to complement information from traditional cultures. Our data indicate that molecular approaches increase the yield of detection of bacterial colonization by 17% to 29% for the three bacteria studied, suggesting that these molecular assays may become the assays of choice to detect bacterial colonization in the nasopharynx.

When comparing the results of culture and qPCR for detection of *S. pneumoniae, H. influenzae* and *S. aureus* for nasopharyngeal samples stored in the WHO standard medium for detection of pneumococci in the nasopharynx (STGG),(27) culture had low sensitivity when bacterial density was low, and its performance may be suboptimal for detecting low density carriage. The much higher detection rate using qPCR than using culture suggests that previous studies using culture alone underestimated the prevalence of bacterial colonization in the nasopharynx. However, qPCR could detect both viable and nonviable bacterial cells, which could lead to an overestimation of bacterial colonization prevalence. The use of molecular techniques with high sensitivity to detect pneumococcal colonization is important for evaluation and formulation of pneumococcal vaccines.(13)

To our knowledge, this is the first study to use qPCR to examine the prevalence of *H. influenzae* and *S. aureus* colonization in healthy Andean children. The clinical and epidemiologic relevance of using qPCR for detection of *H. influenzae* and *S. aureus* in nasopharyngeal samples is still to be determined. Several studies suggest that qPCR may provide a rapid quantification that can be used to distinguish between infection and colonization because bacterial density may be higher during infection than during colonization.(28-31) We found that age in months, gender and antibiotic usage were not associated with colonization by any of the three bacterial species we examined using either cultures or qPCR results. Our data suggest that if age related reductions in bacterial density occur, then they occur after three years of age which was the limit of age of children in this study. However, there were strong relationships among these three species. The negative association between *S. pneumoniae* and *S. aureus* colonization observed in this study is consistent with previous reports in Europe, Asia, America and Africa.(5-9) One possible mechanistic explanation for this bacterial interference phenomenon is that hydrogen peroxide produced by *S. pneumoniae*(32) may "protect" the host against *S. aureus* colonization. A recent randomized controlled trial showed that children who received heptavalent pneumococcal conjugative vaccine in The Netherlands were twice as likely to be colonized with *S. aureus* around twelve months of age compared to unvaccinated controls, but this effect might be transient. Whether this transient increase in *S. aureus* colonization influences the health of vaccinated children is currently unknown.(33)

Similar to several other epidemiologic studies,(5, 10-12) we found a positive association between colonization of *S. pneumoniae* and *H. influenzae*, and in addition, we found that their densities in the nasopharynx were positively correlated. This is consistent with a recent

study showing that the presence of *H. influenzae* increases pneumococcal biofilm formation in vivo and in vitro.(34) However, in vitro experiments suggest that hydrogen peroxide and neuraminidase produced by *S. pneumoniae* can inhibit the growth of *H. influenzae*.(35, 36) Studies in mice suggest that the immune response primarily elicited by *H. influenzae* reduces the density of some pneumococcal strains,(4, 37) while the presence of *S. pneumoniae* facilitates the colonization by a new *H. influenzae* population.(4) Another epidemiologic study showed that these two bacteria were negatively associated in children with ARI, but the association shifted from negative to positive in the presence of *M. catarrhalis*.(38) Therefore, the mechanism by which *S. pneumoniae* and *H. influenzae* influence each other in the nasopharynx is complex and may be affected by which one initially colonized the nasopharynx, the host immune response, the presence of ARI symptoms, and other bacterial species present in the nasopharynx.

The observed association between bacteria in our study as well as in other epidemiologic studies may be a result of direct bacterial interaction or due to unmeasured host-specific confounders that simultaneously influence colonization status of different bacteria. Jacoby *et al.* recently used a hierarchical multivariate logistic model to analyze longitudinal data and to simultaneously model colonization status of different pathogens, trying to differentiate the host-level interaction and microbe-level interaction.(10) Interestingly, they found a positive association between *S. pneumoniae* and *H. influenzae* at host-level only among aboriginal children, not among non-aboriginal children, and they found neither host-level nor microbe-level associations between *S. pneumoniae* and *S. aureus*, which is contrary to many previous studies. We could not use their approach to analyze our data because only a small subset of children contributed more than one swab. Although we have controlled some host-level potential confounders, we cannot rule out that other factors may have influenced the observed bacterial associations.

Our study has several other limitations. Other pathogens potentially involved in interactions with the three bacteria we examined, such as *M. catarrhalis* (38) and coagulase-negative staphylococci,(18) were not studied. In addition, information on other potential confounders, such as number of siblings, family size and daycare attendance was only available on 65.7% of this sample. However, the association between the three bacterial species did not seem to be confounded by these factors in analyses confined to the subset of children from whom complete information was available (not shown). Bacterial density data on this population living at high altitude may differ from those living at lower altitudes but there are no data to date on the association of altitude with nasopharyngeal bacterial density. There are as yet limited data on serotype specific measures of nasopharyngeal density in children and these may add to the complexity of the density relationships in the nasopharynx.

The relationship between bacterial density in the nasopharynx and disease is attracting increasing attention. Increased nasopharyngeal density of respiratory bacteria has been linked to acute otitis media(39) in children as well as to pneumonia (30), but the relatively low specificity of the association has not allowed a critical level of density to be used diagnostically to date in children. Serotype specific density may be valuable as a diagnostic in the future. Nasopharyngeal pneumococcal density has however recently been shown to useful in the diagnosis of pneumococcal pneumonia in adults with a four log difference in nasopharyngeal density demonstrated between asymptomatic carriage and disease(40). The detection of low density carriage by molecular methods may thus be more important for understanding persistence of pneumococcal serotypes and transmission of the pneumococcus and other respiratory bacteria, than in making an association with disease in an individual patient.

Understanding how bacteria interact with each other in the nasopharynx, whether synergistically or competitively, is essential for designing preventive measures. This is especially true in this era of vaccines and antimicrobials which target specific bacteria and may unexpectedly influence the bacterial flora. Our study confirmed the previously observed negative association between *S. pneumoniae* and *S. aureus* and positive association between *S. pneumoniae* and *H. influenzae* using culture and qPCR to test nasopharyngeal samples collected from young children in rural communities of the Peruvian Andes. Our study demonstrates for the first time the impact of bacterial density on the detection of these bacteria by culture and the relationship between the density of pneumococcal and Haemophilus colonization. As colonization density may be an essential precursor to disease caused by these bacteria and may also be important for transmission, our study suggests that future colonization studies of these pathogens should also measure bacterial density.

Acknowledgments

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Table 1

Comparison of bacterial culture and qPCR for detection of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* in nasopharyngeal swabs.

No. (%)												
	S. pneumoniae			*H. influenzae*			*S. aureus*					
	Culture (−)	Culture (+)	Total	Culture (−)	Culture (+)	Total	Culture (−)	Culture (+)	Total			
qPCR(−)	101 (22.6%)	0 (0.0%)	101 (22.6%)	220 (62.7%)	0 (0.0%)	220 (62.7%)	264 (59.2%)	0 (0.0%)	264 (59.2%)			
qPCR(+)	77 (17.3%)	268 (60.1%)	345 (77.4%)	48 (13.7%)	83 (23.6%)	131 (37.3%)	129 (28.9%)	53 (11.9%)	182 (40.8%)			
Total	178 (39.9%)	268 (60.1%)	446 (100.0%)	268 (76.4%)	83 (23.6%)	351 (100.0%)	393 (88.1%)	53 (11.9%)	446 (100.0%)			

Table 2

Bacterial detection by culture, stratified by bacterial density (qPCR)

	No. culture positive / No. in density category by qPCR (%)		
Bacterial density by qPCR (CFU/ml)	*S. pneumoniae*	*H. influenzae*	*S. aureus*
0 >0 - 10^4 >10^4 - 10^5 >10^5 -10^6 >10^6	0/101 (0.0%) 65/131(49.6%) 131/142 (92.3%) 66/66 (100%) 6/6 (100%)	0/220 (0.0%) 0/2 (0.0%) 1/17 (5.9%) 13/33(39.4%) 69/79 (87.3%)	0/264 (0.0%) 1/43 (2.3%) 12/89 (13.5%) 18/25 (72.0%) 22/25 (88.0%)
Total	268/446 (60.1%)	83/351 (23.6%)	53/446 (11.9%)

Table 3

identification of *Streptococcus pneumoniae, Staphylococcus aureus*, and *Haemophilus influenzae* in 351 nasopharyngeal swabs tested for all three bacteria using culture and qPCR

Colonizing bacteria	Culture results (n=351)	qPCR results (n=351)
none	99 (28.2%)	28 (8.0%)
S. pneumoniae alone	135 (38.5%)	97 (27.6%)
S. aureus alone	22 (6.3%)	33 (9.4%)
H. influenzae alone	16 (4.6%)	11 (3.1%)
One species total	173 (49.3%)	141(40.2%)
S. pneumoniae + S. aureus	17 (4.8%)	99 (28.2%)
S. pneumoniae + H. influenzae	61 (17.4%)	72 (20.5%)
S. aureus + H. influenzae	1 (0.3%)	11 (3.1%)
Two species total	79 (22.5%)	182 (51.8%)
S. pneumoniae + S. aureus + H. influenzae	0 (0%)	0 (0%)
Any *S. pneumoniae*	213 (60.7%)	268 (85%)
Any *H. influenzae*	78 (24.8%)	94 (29.8%)
Any *S. aureus*	40 (12.7%)	143 (45.4%)

Table 4

Association between colonization with *Streptococcus pneumoniae* or *Haemophilus influenzae* (based on culture data) and selected variables

	OR (95% CI)[1] for colonization with selected bacteria:	
	S. pneumoniae (n=351)	*H. influenzae* (n=351)
S. pneumoniae Absent (ref) Present	-	1 **3.17 (1.76 – 5.72)**
H. influenzae Absent (ref) Present	1 **3.11 (1.73 – 5.59)**	–
S. aureus Absent (ref) Present	1 **0.45 (0.22 – 0.90)**	1 0.71 (0.29 – 1.74)
Age (1-mo increase)	1.00 (0.98 – 1.03)	1.02 (0.99 – 1.05)
Sex Female (ref) Male	1 1.42 (0.90 – 2.25)	1 0.61 (0.36 – 1.04)
Antibiotics No (ref) Yes	1 0.97 (0.22 – 4.29)	1 2.50 (0.53 – 11.9)

[1]OR, odds ratio; CI, confidence interval. Significant ORs and 95% CI are shown in boldface. Model for colonization of each bacterium included variables representing presence/absence of the other two bacteria and potential confounders: age, sex and antibiotic usage within the past 7 days (Antibiotics).