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Association between nasopharyngeal colonization with multiple pneumococcal serotypes and total pneumococcal colonization density in young Peruvian children

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ABSTRACT

Objectives: We examined the association of nasopharyngeal (NP) pneumococcal co-colonization (>1 pneumococcal serotype) and pneumococcal density in young Peruvian children enrolled in a prospective cohort study.

Methods: NP swabs collected monthly from children aged <3 years during both asymptomatic and acute respiratory illness (ARI) periods underwent culture-enriched microarray for pneumococcal detection and serotyping and lytA polymerase chain reaction for density assessment. We examined the serotypes commonly associated with co-colonization and the distribution of densities by co-colonization, age, current ARI, and other covariates. The association of co-colonization and pneumococcal density was assessed using a multivariable mixed-effects linear regression model, accounting for repeated measures and relevant covariates.

Results: A total of 27 children contributed 575 monthly NP samples. Pneumococcus was detected in 302 of 575 (53%) samples, and co-colonization was detected in 61 of these 302 (20%). The total densities were higher during ARI than non-ARI periods and lowest among the youngest children, increasing with age. In the multivariable analysis, there was no significant association between pneumococcal density and co-colonization (coefficient estimate 0.22, 95% confidence interval 0.11-0.55; reference: single-serotype detections). Serotypes 23B and 19F were detected significantly more frequently as single isolates.

Conclusion: Pneumococcal co-colonization was common and not associated with increased pneumococcal density. Differential propensity for co-colonization was observed among individual serotypes.

Background

Nasopharyngeal (NP) pneumococcal colonization is a critical initial step in the development of respiratory and invasive pneumococcal disease. However, colonization of the nasopharynx with pneumococcus is very common in young children and is most of-

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ten asymptomatic [1]. The factors that are associated with the transition from asymptomatic NP colonization to pneumococcal disease are not well understood.

The density of NP pneumococcal colonization is thought to be an important factor in the development of pneumococcal disease and may also play a role in transmission [2]. We and other investigators have reported higher NP pneumococcal densities in patients with acute respiratory illness (ARI) or invasive disease than healthy controls, especially when certain respiratory viruses are detected [2–6]. In addition to its role in the pathogenesis of pneumococcal disease, NP pneumococcal colonization may also play a role in pneumococcal immunity and protection from the acquisition of new pneumococcal strains, with epidemiological studies suggesting that both serotype-specific and serotype-independent immunity may be generated by pneumococcal carriage [7,8].

Pneumococcal conjugate vaccines have substantially impacted pneumococcal colonization patterns by decreasing the acquisition and colonization with serotypes included in the vaccines, resulting not only in the direct protection of immunized children but also the indirect protection of unvaccinated individuals by reducing the transmission of the included vaccine serotypes [9–14]. However, traditional culture detection techniques have been limited in their sensitivity to detect the carriage of serotypes present at low levels or the presence of multiple co-colonizing pneumococcal serotypes. With the recent development of enhanced molecular diagnostic techniques, the detection of simultaneous carriage of multiple pneumococcal serotypes and the recognition that certain serotypes may be more difficult to detect in the NP due to lower abundance has been appreciated [13,15,16]. However, the impact of pneumococcal co-colonization with multiple serotypes on nasopharyngeal pneumococcal densities is unclear, with one study identifying an association between pneumococcal co-colonization and increased total pneumococcal densities [15], whereas another study revealed no significant association [16]. Few studies to date have applied methods that are highly sensitive for the detection of multiple serotype carriage of pneumococcus [13,15–18], whereas even fewer have performed these assessments in longitudinal studies of individual children beyond the first year of life. The objective of this study was to examine the association of co-colonization with multiple pneumococcal serotypes and the overall pneumococcal colonization density in young children who were followed up longitudinally in a prospective household-based cohort study in the rural Peruvian Andes.

Methods

Study design and setting

Respiratory Infections in Andean Peruvian Children (RESPIRA-Peru) is a prospective cohort study conducted in the Province of San Marcos, Department of Cajamarca, located in the northern highlands of Peru. The population is primarily rural, with low income and limited access to health care services, as previously described [5,19]. Between May 2009 and September 2011, children aged <3 years residing in the study area were enrolled and prospectively assessed for ARI symptoms during weekly household visits. An ARI episode was defined as the presence of either cough or fever. We also collected data on other respiratory symptoms and signs, including rhinorrhea, ear pain, malaise, tachypnea, nasal flaring, stridor, wheezing, and accessory muscle retractions. We considered a child to be asymptomatic if the child had no ARI symptoms or rhinorrhea alone. The 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in the study region in late 2009 in a two + one schedule (two primary doses at age 3 and 5 months and a booster dose at 12 months). The study was approved by the institutional review boards of Vanderbilt University Medical Center and the Instituto de Investigacion Nutricional in Peru.

Respiratory sample collection and testing

NP swabs were routinely collected monthly from each child, whether or not ARI symptoms were present, and were obtained according to World Health Organization recommendations for identifying pneumococcal colonization [20]. Briefly, deep NP samples were collected using a nonflocked rayon polyester swab and immediately placed in a tube with 1 ml of skim milk-tryptone-glucose-glycerine transport media and transported to the local laboratory within 8 hours of collection for storage at −70°C. NP specimens were tested at Emory University or Vanderbilt University using methods standardized between sites, including bacterial culture for pneumococcal identification and quantitative polymerase chain reaction (qPCR) of the panpneumococcal *lytA* gene for pneumococcal detection, and the total pneumococcal density was measured by *lytA* polymerase chain reaction (PCR) without a previous culture amplification [4,5]. Aliquots of samples in which pneumococci were detected by culture and/or *lytA* PCR subsequently underwent molecular serotyping by microarray after culture amplification [15,21]. A sweep of all pneumococcal colonies from dilution series on selective culture plates were collected for DNA extraction. DNA was fragmented and fluorescently labeled, and microarray studies were conducted using Senti-SP v1.6 (BUGS Bioscience), which reports the identity and relative abundance of all serotypes present within a sample. The individual serotype-specific density was determined by multiplying the total pneumococcal density (determined by *lytA* qPCR before culture amplification) by the individual serotype's relative abundance (proportion as determined by microarray after culture amplification).

Study population

We selected a subset of all children enrolled in the RESPIRA-Peru study who met the following criteria: monthly NP samples collected for a period of at least 12 months, provided at least 12 NP samples, no gap in NP sample collection for >1 year, and at least one pneumococcal detection while under observation. All samples from these children were tested for pneumococcus by culture and/or PCR, and the samples with pneumococci detected by culture and/or RT-PCR underwent molecular serotyping, as described previously.

Statistical analysis

We assessed the sociodemographic characteristics of the selected and overall study population, including age, gender, number of household children, and 7-valent pneumococcal conjugate vaccination receipt. We also assessed the total pneumococcal density according to single or co-colonization status, age group (<6 months, 6-11 months, 12-23 months, >24 months), ARI status, and PCV7 vaccination status (previous receipt of at least one pneumococcal conjugate vaccine [PCV] dose at the time of sample collection). We also determined the frequency and serotypes associated with pneumococcal detection in the study children and the proportion of samples in which multiple serotypes were detected (co-colonization). For individual serotypes, we reported the proportion of single-serotype and co-colonization detections with 95% confidence intervals (CIs) [22]. In samples with multiple serotypes detected, we explored the relative abundance of individual serotypes included in the PCV7 vaccine (vaccine type [VT], nonvaccine type [NVT], nontypeable [NT]). Serotypes that comprised ≥70% of the relative abundance of the sample derived from microarray results were considered 'dominant,' those that comprised <70% of the

Table 1
Demographic and baseline characteristics of participants comprising analytic subset and overall RESPIRA-Peru cohort.

	Children with only single-colonization detections (n = 8)	Children with at least one co-colonization detection (n = 19)	All included children (n = 27)	Overall cohort (n = 892)
Age at enrollment (months); median (IQR)	8.6 (6.6, 12.9)	10.4 (6.7, 15.8)	9.5 (6.7, 14.5)	4.6 (0.5, 17.1)
Male sex, %	75	58	63	52
Household size; median (IQR)	5 (4, 6)	5 (4, 6)	5 (4, 6)	5 (4, 6)
Household children <3 years; median (IQR)	1 (1, 1)	2 (1, 2)	1 (1, 2)	1 (1, 2)
Household room number; median (IQR)	2 (2, 4)	2 (1, 3)	2 (1, 3)	2 (1, 3)
Maternal highest education, %				
No formal	12.5	5.3	7.4	7.6
Incomplete primary/secondary school	50.0	68.4	63.0	51.6
Completed primary school	12.5	10.5	11.1	28.9
Completed secondary school	25.0	10.5	14.8	8.6
Completed technical school	0	5.3	3.7	<1.0
Completed or some higher education	0	0	0	2.4
PCV7 status at enrollment, %				
At least one dose	0	12.5	3.7	5.2
Unknown	0	0	0	6.1
PCV7 status at end of follow-up, %				
0 doses	62.5	52.6	55.6	36.4
1 dose	0	26.3	18.5	10.0
2 doses	12.5	10.5	11.1	20.2
3 doses	25.0	10.5	14.8	27.4
Unknown	0	0	0	6.1

IQR, interquartile range; PCV7, 7-valent pneumococcal conjugate vaccine

relative abundance were considered 'nondominant' [23]. We also displayed the frequency of co-colonization with individual pneumococcal serotypes using a hierarchical edge bundle visualization method [24]. The Pearson chi-square test was performed for comparisons of categorical data.

To assess the association of colonization pattern (colonization with a single serotype or co-colonization with ≥2 serotypes) and pneumococcal density, we used a multivariable mixed-effects linear regression model, with subject identifier as the random effect to account for repeated measures from individual children. Because pneumococcal densities are highly skewed, to stabilize the distribution of values, we applied $\log_{10}$-transformation to colonization density values [4]. The covariates included age at sample collection, sex, calendar month, ARI symptom status, and PCV vaccination status.

All analyses were conducted using R version 4.1.3 along with ggplot2, lmerTest, and lme4 packages.

Results

A total of 892 children were enrolled in the RESPIRA-Peru cohort study; 27 of these children met the selection criteria for this analysis, which included consecutive monthly samples for at least 12 months and detection of pneumococcus in at least one sample. The median age at enrollment for these 27 children was 9.5 months (interquartile range [IQR] 6.7-14.5 months); 63% were male (Table 1). A total of 12 of 27 (44%) had received at least one dose of PCV7 at enrollment. The median duration of study follow-up for these 27 children was 25.0 months (IQR 21.5-26.2) and median time between sample collections was 1.1 months (IQR 0.9-2.3).

These 27 children contributed a total of 575 NP samples. Pneumococci were detected by culture and/or *lytA* PCR in 361 of the 575 (63%) samples. Of these samples, 340 of 361 (94%) contained pneumococcus and/or closely related Streptococcal species bearing *cps* gene homologues by microarray, and 302 of 361 (84%) contained pneumococcus for the analysis of pneumococcal serotype and density characterization. Most samples (258 of 302 [85.4%]) were collected during asymptomatic periods, whereas a few (44 of 302 [14.6%]) were collected during ARI periods, during which antibiotic use was uncommon, preceding only approximately 2% of the NP samples collected in the study [25]. A total of 40 pneumococcal serotypes or NT strains were detected (Supplementary Figure 1), including all seven PCV7 vaccine serotypes (VT), 31 nonvaccine serotypes (NVT), and two NT strains. The serotypes initially designated '19A-like' and '23B-like' by microarray exhibited a similarity in the *cps* gene content consistent with the known variants of serotypes 19F and 23B, respectively, and thus are subsequently defined as 19F and 23B [26,27].

Co-colonization with more than one pneumococcal serotype or NT strain

Co-colonization with more than one pneumococcal serotype or NT strain was present in 61 of the 302 (20%) samples contributed by 19 of 27 children (Table 1); 9 of 19 (47%) of these children had received at least one dose of PCV7. Among these 61 samples, two pneumococcal strains were detected in 46 (75%) samples, three in 12 (20%), four in two (3%), and six in one (<2%). Co-colonization occurred in 55 of 258 (21.3%) samples from asymptomatic children and 6 of 44 (13.6%) samples from children during ARI ($P = 0.24$). Co-colonization was observed in 34 of the 161 (21%) samples that were preceded by at least one dose of PCV7 (25 samples with two serotypes, six samples with three serotypes, two samples with four serotypes, and one sample with six serotypes) and 27 of 141 (19%) samples that were collected before any receipt of PCV7 (21 samples with two serotypes, six samples with three serotypes; $P = 0.80$).

Pneumococcal colonization densities and the association with co-colonization

The distribution of unadjusted log-transformed pneumococcal densities stratified by age group, co-colonization status, vaccination status, and VT/NVT are displayed in Figure 1. The aggregate unadjusted pneumococcal densities were higher during the ARI periods than the asymptomatic periods and lowest in those children aged <6 months, increasing and stabilizing at older ages. Other unadjusted analyses showed few differences in density by PCV7 vaccination status and VT detection.

Figure 1. Distribution of $\log_{10}(x + 1)$-transformed absolute total (a-e) or individual serotype-specific (f) pneumococcal densities by (a) colonization status (co-colonization vs single colonization), (b) co-colonization status (two serotypes vs three+ serotypes), (c) age group, (d) ARI status, (e) PCV7 vaccination status, and (f) PCV7 vaccination status and PCV7 vaccine type among 27 participants from the RESPIRA-Peru study. ARI, acute respiratory illness; PCV7, 7-valent pneumococcal conjugate vaccine.

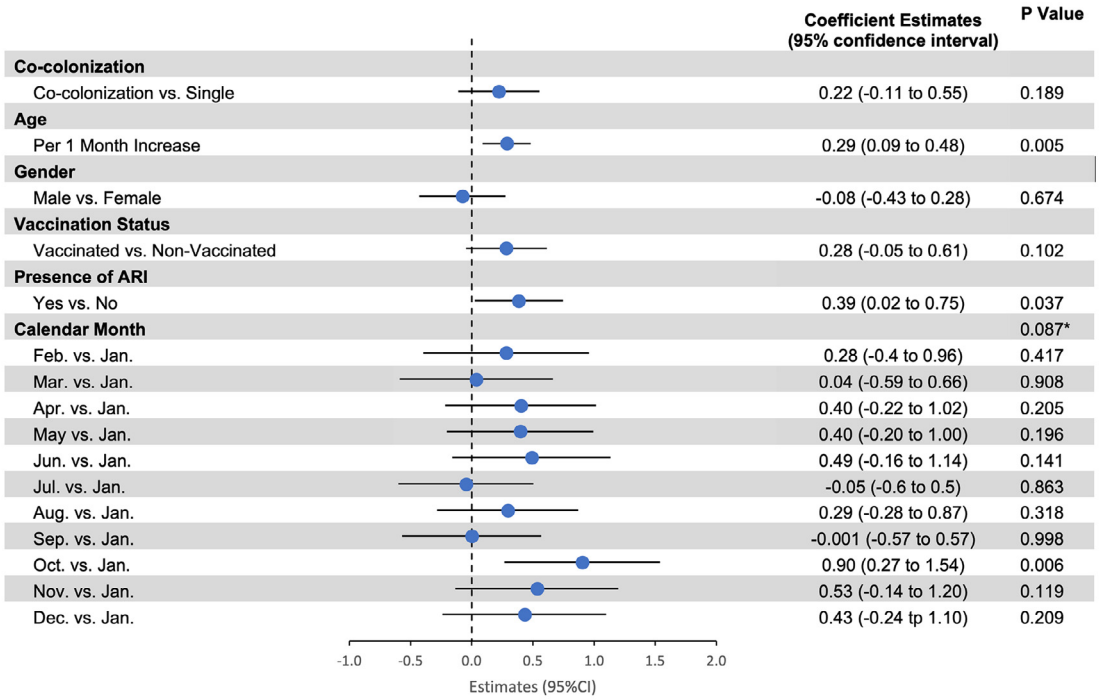

Figure 2. Multivariable analysis of co-colonization status and pneumococcal colonization density, adjusted for age at sample collection (continuous), gender, calendar month, 7-valent pneumococcal conjugate vaccination status (any dose), and ARI status (binary: presence of ARI) among a subset of 27 children in RESPIRA-Peru cohort. References: single colonization, younger age, female gender, January, no PCV7 dose, asymptomatic status. ARI, acute respiratory illness; CI, confidence interval; PCV7, 7-valent pneumococcal conjugate vaccine.

In the multivariable analysis, no significant association was observed between the absolute pneumococcal density and the presence of co-colonization (Figure 2). Increasing age (coefficient 0.29 [95% CI 0.09-0.48], $P = 0.005$) and current ARI (0.39 [95% CI 0.02-0.75], $P = 0.037$) were associated with higher pneumococcal densities than younger age and asymptomatic status, respectively.

Serotype-specific co-colonization and density assessments

The 10 most frequently detected serotypes are displayed in Figure 3, with the frequency of single- and co-colonization detections and associated 95% CI. Serotypes 23B and 19F were detected significantly more often as single isolates. No other significant differences in propensity for single versus co-colonization were identified among the other serotypes. Serotypes 4, 21, 20B, 35A, 19B, and 38 were infrequently detected alone or in co-colonization, making it challenging to reliably assess their propensity for co-colonization. Serotypes 22A (7 detections), 23A (5 detections), 12F (1 detection), and 33B (1 detection) were never detected in single-colonization events. The frequency and combinations of individual serotype co-detections among 302 samples are displayed in Supplementary Figure 2. The supplementary table outlines the frequency of individual serotypes as dominant or nondominant serotypes in co-colonization events. PCV7 serotypes were the dominant serotypes in 16 of the 57 (28%) co-colonization detections, whereas NVT were dominant in 26 of the 75 (35%) co-colonization detections ($P = 0.54$). Although the number of NVT co-colonization detections were small (6C [n = 4] and 19B [n = 2]), they were typically nondominant serotypes in the co-colonization detections.

The absolute colonization densities for individual serotype detections are plotted in Figure 4, grouped by VT, NVT, and NT and sorted in order of descending total median densities between groups, stratified according to co-colonization or single-serotype carriage.

Discussion

Among children aged <3 years enrolled in a prospective cohort study in rural Peru and followed up with monthly NP sampling for at least 12 months, co-colonization with more than one pneumococcal serotype or NT strain was detected in 20% of samples by culture-enriched microarray. However, there was no association between co-colonization and pneumococcal density. Higher densities were identified during ARI periods relative to asymptomatic periods and in older children than younger children. Serotypes 19F, 6B, and 11A were most commonly detected among all samples, and serotypes 6B and 11A were also detected most frequently in co-colonization detections. We observed serotype-specific differences in the frequency of co-colonization, with 23B and 19F more likely to be detected in single-colonization than co-colonization events.

Higher pneumococcal colonization densities have been associated with symptomatic ARI and pneumonia, and a previous study by our group suggested a role for increased pneumococcal densities during asymptomatic periods that was associated with an increased risk of subsequent ARI [28]. Our finding that there was no detectable association between pneumococcal density and co-colonization is similar to findings from another study in which no association was identified between the nasopharyngeal pneumococcal density and number of pneumococcal serotypes in a similarly sized sample of preschool-aged Portuguese children in 2011 [16]. However, a study of 50 older Dutch children from June 2012, when PCV10 was in use in the national immunization program, reported a correlation between the number of colonizing serotypes and pneumococcal abundance, although this study assessed saliva rather than NP samples and detected the colonizing pneumococcal serotypes with qPCR [29]. In addition, in a longitudinal assessment of Indonesian children followed up from age ~2 months up to 12 months during 2014-2015, multiple serotype carriage was associated with higher pneumococcal density after adjustment for antibiotic use, ARI, age, and repeated sampling [15]. In the Indonesian

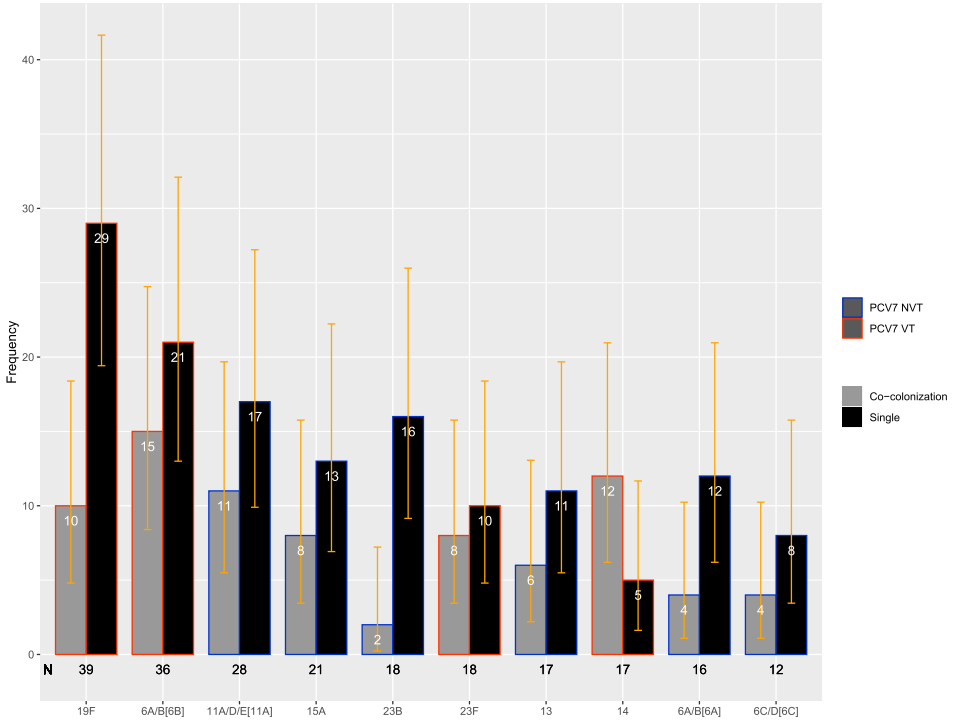

Figure 3. Frequencies of co-colonization and single-colonization detections (with 95% confidence intervals) for each of the 10 most frequently detected pneumococcal serotypes detected among the subset of 27 RESPIRA-Peru participants. NVT, nonvaccine serotypes; PCV7, 7-valent pneumococcal conjugate vaccine; VT, vaccine serotypes.

study when the national PCV immunization program did not exist and infants with a previous PCV receipt were excluded, carriage density decreased slightly with age, with a decrease in the mean pneumococcal density by 0.03 $\log_{10}$ for each month increase in age up to 12 months. This contrasts with our finding where densities increased with age and then stabilized. However, given the highly variable nature of pneumococcal densities, the precision of these estimates may be limited, particularly when the number of observations is not large. In concordance with our previous findings, the Indonesian study also found higher pneumococcal densities among children during periods with ARI independent of viral detection. The variations in the study design, PCV vaccination history, and inclusion of older children in our study relative to the Indonesian study may account for some of these differences. Taken together, the findings from these studies underscore the dynamic nature of pneumococcal carriage and carriage density and the multiple factors that influence these dynamics.

We found that certain pneumococcal serotypes, including PCV7 serotype 19F and NVT 23B were more likely to be detected in single-colonization detections than in co-colonization detections in this partially vaccinated cohort, with only 44% of children having received at least one dose of PCV7 and only 15% completing a three-dose series by the end of study follow-up. Other studies have described the serotype-specific differences in the patterns of single- versus multiple-colonization detections, which may have been influenced by PCV receipt [13,23]. In one study, children who had received the 13-valent PCV13 had lower-than-expected frequencies of co-colonization with nonvaccine serotypes than unvaccinated children, suggesting that nonvaccine serotypes, expanded after PCV implementation, may have enhanced the ability to outcompete the other pneumococcal strains to colonize the nasopharyngeal niche [23]. Decreases in co-colonization may translate into fewer opportunities for the horizontal transfer of genes associated with pathogenicity and antimicrobial resistance, thus representing a potential additional benefit of PCVs.

A recent report from the PCV13 era in the UK (2014-2020) reported that among young children with nasopharyngeal carriage of PCV13 serotypes, a higher proportion of PCV13 serotypes were detected as minor serotypes in co-colonization than nonvaccine serotypes, as opposed to single serotypes or 'major' serotypes in co-colonization events [30]. In that report, VT colonization was also associated with lower colonization density than NVT. We observed no significant difference in the colonization densities among PCV7 VT compared with NVT; however, the majority of children had received no PCV7 vaccine doses and very few had completed a three-dose series. Although PCV7 VT 19F and NVT 23B were more likely to be detected in single-colonization events, both serotypes were equally likely to be present as a nondominant or dominant serotype in co-colonization detections. Overall, the proportion of nondominant serotypes in co-colonization did not differ from PCV7 VT in co-colonization compared with NVT in co-colonization. As mentioned previously, the UK study represented a more mature PCV13 program.

The main limitation of this study was the relatively small number of subjects that contributed samples in these repeated assessments. Although we were able to rule out substantial differences in colonization density, smaller differences may be difficult to detect with our more limited number of observations. The total pneumococcal densities were determined by *lytA* PCR without culture amplification, whereas the relative abundance of individual serotypes in co-colonization were determined by microarray after culture amplification. Our determination of serotype-specific densities assumed that the relative proportions of individual serotypes remained stable after culture amplification, which may not have been the case if some serotypes were more amplified in culture than others. Furthermore, the small number of observations precluded the determination of the role of individual serotypes in co-colonization dynamics. Moreover, our study design, in which nasal samples were only tested for respiratory viruses during ARI periods, did not enable the assessments of the impact of respiratory

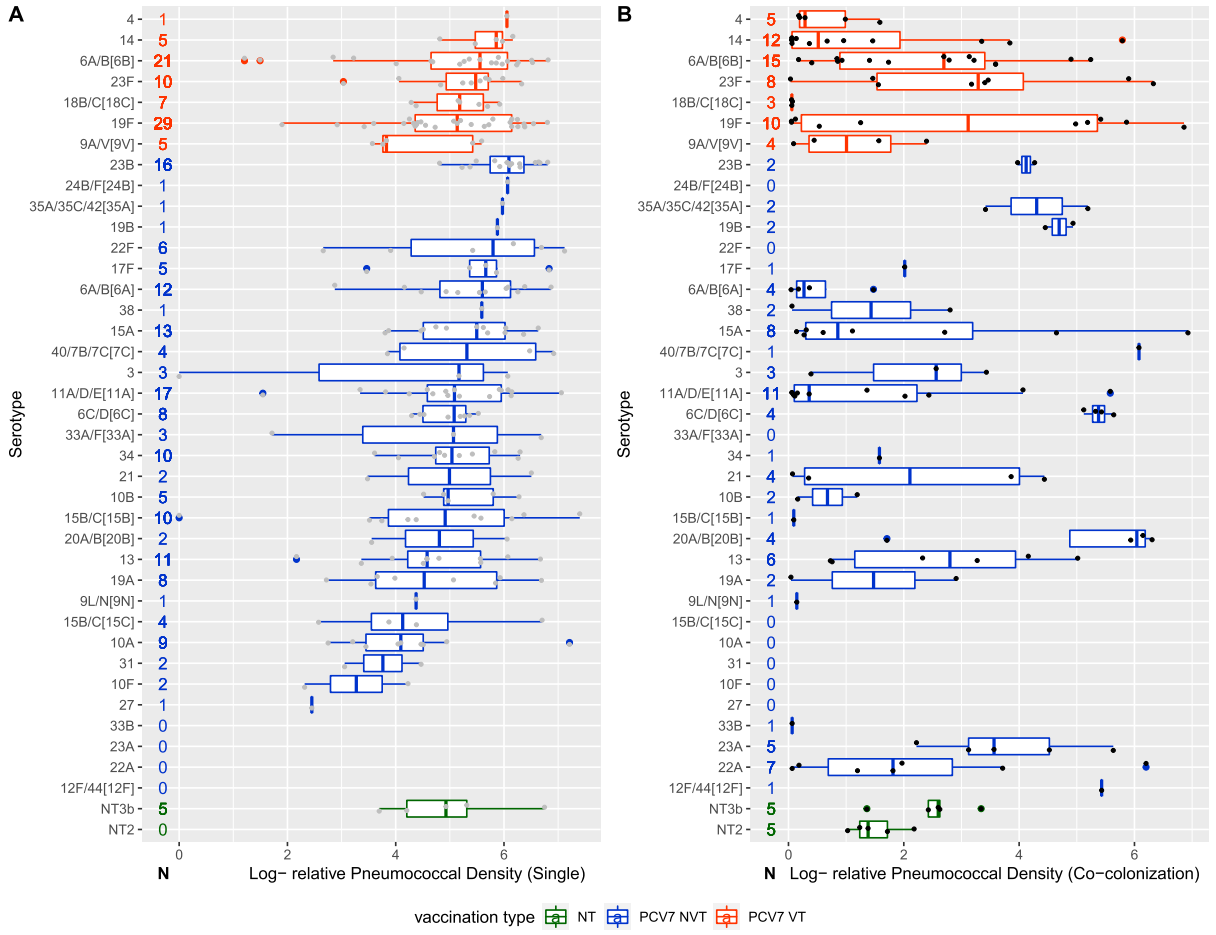

Figure 4. Boxplots representing the observed absolute density of individual serotype detections. Serotypes are categorized by PCV7 (VT; red), (NVT; blue), (NT; green) and ordered by descending median density according to VT, NVT, NT. Dots denote individual measurements of density (Panel a [gray dots]: single-serotype colonization, Panel b [black dots]: co-colonization). Central lines indicate the median of the distribution; box edges denote upper and lower interquartile ranges. Colored dots indicate outliers. NVT, nonvaccine serotypes; PCV7, 7-valent pneumococcal conjugate vaccine; VT, vaccine serotypes.

viral detections on pneumococcal density in asymptomatic children. It is well recognized that viral infections influence pneumococcal density and other colonization dynamics [4,28]. In addition, there were some differences in the subset of children included in this study relative to the overall RESPIRA-Peru study population regarding age and PCV7 status, which may need to be considered in interpreting the findings. Finally, these observations were derived from a subset of children from rural communities in the Peruvian Andes; thus, our observations may not be directly applicable to other populations and settings.

In conclusion, co-colonization with more than one pneumococcal serotype was common in this household-based cohort of children aged <3 years in rural Peru. Distinct pneumococcal serotypes appear to have varying propensities for co-colonization. No significant association between co-colonization and pneumococcal density was detected; larger sample sizes may reveal associations we were unable to detect. Nevertheless, our study highlights the importance of the use of highly sensitive detection methods to elucidate the complexities of pneumococcal colonization dynamics. Further studies are needed to assess whether pneumococcal co-colonization dynamics may impact ARI pathogenesis.

Declarations of Competing Interest

L. M. H. has received grant support from Pfizer for unrelated work. K. M. E. reports grant funding from the National Institutes of Health and Centers for Disease Control and Prevention; has served as a consultant to Bionet and IBM; and has served as a member of Data Safety and Monitoring Boards for Sanofi, X-4 Pharma, Seqirus, Moderna, Pfizer, Merck, and Roche. C. F. L. is a member of the World Health Organization COVID-19 vaccine effectiveness working group and reports grant funding from CureVac AG, PATH, and HilleVax from work not related to the current work. C. G. G. has served as a consultant to Pfizer, Merck, and Sanofi-Pasteur for unrelated work and received research support from Sanofi-Pasteur. All other authors report no potential conflicts of interest.

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Ethical approval

The study was approved by the institutional review boards of Vanderbilt University Medical Center and the Instituto de Investigacion Nutricional in Peru.

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Author contributions

All authors made substantial contributions to all of the following: (1) the conception and design of the study, acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.07.007.

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