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Bacterial Density, Serotype Distribution and Antibiotic Resistance of Pneumococcal Strains from the Nasopharynx of Peruvian Children Before and After Pneumococcal Conjugate Vaccine 7

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Abstract

Background—Pneumococcal conjugate vaccines (PCV) have decreased nasopharyngeal carriage of vaccine-types but little data exists from rural areas. We investigated bacterial density, serotype distribution and antibiotic resistance of pneumococcal strains within the nasopharynx of young children in the Peruvian Andes, two years after PCV7 was introduced.

Methods—Pneumococcal strains were isolated from a subset of 125 children from our Peruvian cohort, who entered the study in 2009 and had pneumococcus detected in the nasopharynx in both 2009 and during follow-up in 2011. Strains were quellung-serotyped and tested for susceptibility to antibiotics. Bacterial density was determined by qPCR.

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Results—The prevalence of PCV7 strains decreased from 48% in 2009 to 28.8% in 2011, whereas non-PCV7 types increased from 52% to 71.2% ($p=0.002$). There was a 3.5-fold increase in carriage of serotype 6C in 2011 ($p=0.026$). Vaccination with PCV7 did not affect pneumococcal density in children colonized by a PCV7 type but did increase density in those colonized with a non-PCV7 type. Antibiotic resistance did not change after vaccine introduction; strains were non-susceptible to tetracycline (97.2%), trimethoprim-sulfamethoxazole (56.4%), penicillin (34%), erythromycin (22.4%), chloramphenicol (18.8%) and clindamycin (12.4%).

Conclusions—Serotype replacement was observed post-PCV7 vaccination with a concomitant, not previously recognized, increased nasopharyngeal density.

Keywords

nasopharyngeal colonization; *S. pneumoniae*; PCV7; antibiotic resistance; serotype replacement

Background

Streptococcus pneumoniae (pneumococcus) causes a broad spectrum of diseases including acute otitis media, pneumonia, septicemia, and meningitis. According to the World Health Organization, approximately 800,000 children under five years old die each year due to pneumococcal pneumonia (1, 2). Thus, pneumococcal infections are a leading cause of death in children under five years of age, primarily in developing countries (3, 4). Pneumococcal carriage is a prerequisite for pneumococcal disease (PD) and for the subsequent spread to others (5, 6). Nasopharyngeal (NP) colonization is most prevalent during the first two years of life (7-9).

Pneumococcal conjugate vaccines (PCV) directly reduce the burden of PD in vaccinated children and indirectly among non-vaccinated (10-13). Multiple studies have reported a significant decrease in nasopharyngeal carriage of PCV vaccine types and an increase in non-PCV serotypes in vaccinated children, a phenomenon known as serotype replacement (14, 15).

The replacement of serotypes colonizing the nasopharynx may produce a change in the prevalence of antibiotic-resistant strains of vaccine types (11). To assist in implementing and sustaining effective strategies for treatment and prevention of PD, it is important to understand colonization and antibiotic resistance patterns of *S. pneumoniae* strains, particularly in PCV vaccinated populations (5, 16).

The seven-valent pneumococcal conjugate vaccine (PCV7) was introduced into the national infant immunization program in Peru by July 9th 2009. The current study analyzed a subset of a cohort of young Andean children from whom strains were isolated before and after PCV7 was introduced. We investigated serotype prevalence and antibiotic resistance of strains, and whether PCV7 had an additional effect on nasopharyngeal densities of pneumococcal serotypes.

Materials and Methods

Study population

This cross-sectional study was nested within the prospective study of respiratory infections in Peruvian Andean children (RESPIRA-PERU). Households with potentially eligible children were identified and after informed consent was obtained, the children were enrolled (16). Demographic information and data on health services utilization patterns, known risk factors for acute respiratory infections, and history of vaccination was obtained. Consent was obtained to access medical and vaccination records during the duration of study participation. Detailed data of the study population (i.e., demographic, socio-economic, environmental and geographic information, and health care related characteristics including vaccination history) was published earlier (16). The enrolled children and their families lived in rural communities in the San Marcos Province, Department of Cajamarca, located in the northern highlands of Peru in the Andes Mountains (16). NP swabs from children were selected for this study if they had *S. pneumoniae* detected in a routine monthly NP swab in both the pre PCV7 period (May to September 2009) and the post PCV7 period (May to September 2011). 125 eligible children were identified with two NP swabs each; all 250 samples had a pneumococcus detected.

Nasopharyngeal specimens

For the assessment of pneumococcal colonization, NP samples were collected following recommendations from the World Health Organization (17) with a deep NP swab, using rayon polyester swabs and were immediately placed in 2.0 ml cryogenic vials with 1 ml of transport medium, a mixture containing skim milk-tryptone-glucose-glycerin (18). Specimens were maintained in cold packs, transported to the local headquarters within 8 h of collection, vortexed for 15 s to disperse bacteria and then stored at -70°C .

DNA extraction and quantitative PCR (qPCR)

DNA was extracted using a QIAamp DNA mini kit (Qiagen, Valencia, CA) (19, 20). Bacterial density was quantified by qPCR reactions in a total of 25 μl volume. Reactions contained 1 $\times$ Platinum qPCR superMix (Invitrogen, Carlsbad, CA), 200 nm each of primers and probe, and 2.5 μl of NP purified DNA. The nucleotide sequence of primers and probe were published elsewhere (21). No template controls were run with each set of samples. qPCR reactions were carried out using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The following amplification parameters were utilized: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. qPCR standards using genomic DNA from reference strain TIGR4 were run in parallel to construct a standard curve utilized to calculate cfu/ml using the software Bio-Rad CFX manager (Bio-Rad, Hercules CA)

Isolation and identification of *S. pneumoniae* strains

In order to isolate *S. pneumoniae* strains, NP swabs were thawed, vortexed for 15 s and 200 μl of the specimen transferred to a 5 ml Todd-Hewitt broth containing 0.5% of yeast extract and 1 ml of rabbit serum (Gibco® by Life Technologies, Carlsbad, CA) (22). This enriched culture was incubated for 6 h at 37°C in a 5% CO_2 atmosphere and then inoculated onto

blood agar plates [(BAP), tryptic soy agar plates supplemented with 5% sheep blood] and incubated for 18-24 h at 37°C in a 5% CO₂ atmosphere. The predominant strains (i.e., colonies which are morphologically similar in culture and more abundant), were isolated and identified with the optochin test (Remel, Lenexa, KS) and bile solubility test as previously described (22). *S. pneumoniae* strains (N=250) were serotyped by the Quellung reaction.

Antibiotic susceptibility testing

The Kirby–Bauer disc diffusion method was used to investigate susceptibility to antibiotics, according to the Clinical and Laboratory Standards Institute guidelines (CLSI) (23). The inoculum was prepared from fresh cultures on BAP by making a suspension of colonies, in sterile saline (0.85% NaCl), and adjusted to a turbidity equivalent to the 0.5 McFarland standard (SiemensMicroScan Turbidity Meter, Washington, DC). A BAP was then inoculated with a swab that had been immersed in the suspension. Antibiotic discs were placed on the inoculated plate using a BBL Sensi-Disc Dispenser (Becton-Dickinson, East Rutherford, NJ). The culture was incubated overnight at 37°C in a 5% CO₂ atmosphere. The following antibiotic discs (Becton-Dickinson, East Rutherford, NJ) were used: oxacillin (1 µg), erythromycin (15 µg), clindamycin (2 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), and tetracycline (30 µg). Isolates were regarded as susceptible, intermediate, or resistant using following breakpoints set by the CLSI (23): Oxacillin susceptible (S), 20 mm, Erythromycin S, 21 mm, resistant (R) 15 mm; Clindamycin S, 19 mm, R, 15 mm; Chloramphenicol S, 21 mm, R, 20 mm; Trimethoprim-Sulfamethoxazole S, 19 mm, R, 15 mm; Tetracycline S, 28 mm, R, 24 mm. Strains non-susceptible to oxacillin (diameter 19 mm) were investigated for resistance to penicillin using Penicillin G E-test (bioMérieux, Durham, NC). Isolates with a penicillin minimal inhibitory concentration (MIC) 1µg/ml were further investigated for resistance to ceftriaxone using Ceftriaxone E-test (bioMérieux, Durham, NC). The MICs of penicillin and ceftriaxone were determined by breakpoints described by the CLSI (23): Penicillin (oral penicillin V) S, 0.06 µg/ml, R, 2 µg/ml; Ceftriaxon (nonmeningitis) S, 1 µg/ml, R, 4 µg/ml. Inoculum was prepared in the same way for this E-Test. E-Test strips were placed across the plates and incubated overnight at 37°C in a 5% CO₂ atmosphere. The MIC of penicillin was determined based on the oral penicillin breakpoint and the ceftriaxone MIC based on non-meningitis breakpoints (23). Quality control was done with *S. pneumoniae* reference strain ATCC 49619 which was inoculated in parallel with each disc diffusion test or E-Test.

Statistical analysis

Statistical analyses were done using IBM SPSS Statistics for Windows Version 20 (Armonk, NY) and STATA 13 (StataCorp, College Station, TX). Graphs were drawn using Sigma Plot software. Frequency distributions were calculated for age, gender, serotypes, antibiotic resistance status, possession of a vaccination card, PCV7 doses, number of individuals living in the same household, and attendance at a daycare center. Chi-square statistics or Fisher's exact test were used as appropriate to compare proportions. A *p*-value 0.05 was considered statistically significant. The antibiotic resistance status of each tested antibiotic was combined to a new variable in order to investigate the non-susceptibility to 1 antibiotic as well as multidrug resistance [(MDR), resistance to 3 antibiotics]. Subsequently, the

antibiotic resistance status was transformed into a binary variable of either susceptible or non-susceptible (resistant or intermediate susceptibility). The variable vaccine serotype was binary coded according to PCV-types (PCV7-type and Non-PCV7-type). Also 6A (not part of PCV7 serotypes) was coded as a PCV7 serotype, due to the observed cross protection from PCV7 (24). Children who had received 2 or more doses of PCV7 at least 14 days before the time of sample collection were considered vaccinated. Multivariate logistic regression models were used to explore factors associated with non-susceptibility to the antibiotics.

Results

Study Population

The study population consisted of a subset of 125 children for whom qPCR studies demonstrated the presence of *S. pneumoniae* in the nasopharynx and a pneumococcal strain was isolated in both 2009 and 2011 (16). The number of males [51.2% (n=64)] and females [48.8% (n=61)] was similar and the median age of children was 8.2 months in 2009 (interquartile range (IQR) 5.6, 11.2 months) and 30.9 months in 2011 (IQR 28.5, 34.1 months). 99.2% of the children (n=124) had a vaccination card available. In 2009 80.8% of these children (n=101) had not received any doses of PCV7, whereas 11.2% (n=14) had received one dose and 8% (n=10) had received two doses. In 2011, 76 children (60.8%) had received 2 doses, 8.8% (n=11) only one and 30.4% (n=38) none (Table 1).

Serotype replacement in PCV7-vaccinated children

32 and 31 different serotypes were isolated in 2009 and 2011, respectively. In comparison to the strains isolated in 2009, most children carried a different pneumococcal serotype in the NP in 2011. The prevalence of individual *S. pneumoniae* serotypes in each year is shown in Figure 1.

In 2009, PCV7 strains were isolated from 48% of children, while non-PCV7 types were isolated from 52% (Fig. 1, inset). Carriage of PCV7-types significantly decreased in 2011 to 28.8% ($p=0.002$), whereas non-PCV7 strains significantly increased to 71.2% ($p=0.002$) (Fig. 1, inset). Carriage of PCV7 serotypes was similar in vaccinated and unvaccinated children in 2009 (40% and 48.7%, $p=0.598$). Overall carriage of PCV7 serotypes was also similar in vaccinated and unvaccinated children in 2011 (30.3% and 26.5%, $p=0.653$). However, carriage of vaccine serotypes was lower among vaccinated children in 2011 compared with unvaccinated children in 2009 ($p=0.014$). Similarly, carriage of vaccine serotypes was lower among unvaccinated children in 2011 compared with unvaccinated children in 2009 ($p=0.008$).

The most prevalent serotypes identified in 2009 were vaccine types 19F, 23F, and 6B (13.6%, 12.8%, and 12%, respectively), whereas serotypes 19F, 6C, and 11A (11.2%, 11.2%, 9.6%, respectively) were more prevalent in 2011. Nasopharyngeal carriage of non-vaccine serotype 6C increased ($p=0.025$) and PCV7 serotype 23F decreased ($p=0.026$), both significantly.

Antibiotic susceptibility of *S. pneumoniae* strains

Studies on antibiotic susceptibility for all strains (n=250) showed that 69.6% of pneumococcal strains were completely resistant to tetracycline, and 97.2% were non-susceptible (resistant plus intermediate susceptibility). Pneumococcal resistance to trimethoprim-sulfamethoxazole was 49.2% (56.4% non-susceptible), chloramphenicol resistance was 18.8%, and erythromycin resistance was 18.0% (22.4% non-susceptible). Strains isolated with resistance to penicillin were 10.0% (34% non-susceptible) and only 8% were resistant to clindamycin with 12.4% with non-susceptibility (Fig. 2, see Table, Supplemental Digital Content 1). Only 3% (n=1) of strains were resistant to ceftriaxone among those with non-susceptibility to penicillin MIC $\geq 1 \mu\text{g/ml}$ (n=31) (not shown).

Overall, resistance to ≥ 1 antibiotic was observed in 86% of strains isolated in 2009 with 21% displaying MDR. Similarly, 79% of strains isolated in 2011 were resistant to ≥ 1 antibiotic with 22% MDR. Due to the observed high prevalence of strains non-susceptible to tetracycline (97%), only 1% of strains were susceptible to all tested antibiotics. No statistically significant difference in antibiotic resistance, or non-susceptibility, was observed between strains isolated in 2009 or 2011 (Fig. 2). Although the prevalence of strains resistant to tetracycline decreased from 2009 (75.5%) to 2011 (64.0%), this difference was not significant ($p=0.054$).

Association between PCV7 types and susceptibility to antibiotics

We used logistic regression models to assess the association of vaccine types with non-susceptibility to ≥ 1 antibiotic (except tetracycline, where resistance was highly prevalent) or non-susceptibility to individual antibiotics as dependent variables (Table 2). The models accounted for PCV7 doses, age, gender, electricity at home, cooking with stove, attending to daycare, and living with smokers. Compared with carriage of PCV7 serotypes, carriage of non-PCV7 types was negatively associated with non-susceptibility to ≥ 1 antibiotic (OR 0.30 [CI 95% 0.16-0.54], $p<0.001$). Similarly, when individual antibiotics were modeled, we found a negative association between carrying non-PCV7 types and non-susceptibility to trimethoprim-sulfamethoxazole (OR 0.30 [CI 95% 0.17-0.53], $p<0.001$). Furthermore, compared with vaccination with PCV7 (2 or more PCV7 doses), lack of vaccination (<2 PCV7 doses) was positively associated with carriage of strains non-susceptible to chloramphenicol (OR 3.47 [CI 95% 1.29-9.38], $p=0.014$).

Changes in the prevalence of non-susceptible PCV7 and non-PCV7 types were also assessed. Excluding strains not susceptible to tetracycline, the prevalence of PCV7 strains not susceptible to ≥ 1 antibiotic was similar in 2009 (76.7%) and 2011 (86.1%) ($p=0.261$). Similarly, carriage of non-PCV7 types non-susceptible to ≥ 1 antibiotic did not significantly change; from 61.5% in 2009 to 53.9% in 2011 ($p=0.410$).

Non-susceptibility to antibiotics of the most prevalent PCV7 and non-PCV7 types is presented in Table 3. Excluding tetracycline, a high prevalence of non-susceptibility to ≥ 1 antibiotic was observed in serotypes 11A (90%), 19F (87.1%), 23F (86.4%), 6B (81.8%), 6C (72.2%), and 19A (70.0%). Although the overall prevalence of strains non-susceptible to penicillin was low in both years (Fig. 2), 50% of pneumococci belonging to serotypes 6B,

6C, 11A, 19A and 23B were non-susceptible to this beta-lactam antibiotic (Table 3). In contrast, *S. pneumoniae* serotype 6A strains were all susceptible to penicillin. Serotype 19A strains were resistant to almost all tested drugs (Table 3). Finally, most prevalent MDR strains belonged to serotypes 19A, 19F and 6C, with a prevalence of 70%, 45.2% and 38.9% respectively.

Associations between vaccine serotypes, antibiotic susceptibility, and pneumococcal nasopharyngeal density

Nasopharyngeal densities obtained by qPCR analyses from all samples that indicated pneumococcal colonization were stratified by increasing density (cfu/ml). As shown in Table 4, the distribution of samples within all density categories was similar in both years. No significant overall differences were observed when we compared pneumococcal nasopharyngeal density of samples obtained in 2009 vs. those in 2011 ($p=0.649$) (Fig. 3, see Table, Supplemental Digital Content 3). Pneumococcal density in children colonized only by PCV7 types in 2009 vs 2011 was also similar ($p=0.210$). Significant differences in nasopharyngeal density, however, were observed in children carrying a non-PCV7 type in 2009 (median 1.9×10^5 cfu/ml) vs those in 2011 (median 5.5×10^5 cfu/ml), ($p=0.046$) (Fig. 3, see Table, Supplemental Digital Content 3).

Pneumococcal density in children carrying susceptible or non-susceptible strains was assessed next. No significant differences were seen in children colonized in 2009 and/or 2011 by either a strain not susceptible to 1 antibiotic (excluding tetracycline) ($p=0.969$) or those carrying a strain susceptible to all antibiotics (excluding tetracycline) ($p=0.458$) (see Table, Supplemental Digital Content 3). When nasopharyngeal densities were compared within the same year, no significant differences were observed for strains non-susceptible to 1 antibiotic vs those susceptible, $p=0.575$ or $p=0.161$, respectively (see Table, Supplemental Digital Content 3). Nasopharyngeal densities between those children carrying MDR strains and those carrying only completely susceptible strains (not even tetracycline resistance) were similar.

Discussion

This study demonstrates reduction in PCV7 serotypes, herd protection, and replacement of PCV7 types by non-PCV7 types in the nasopharynx of Andean children two years after the introduction of PCV7 in Peru. Interestingly, among isolated pneumococci, resistance to selected antibiotics, including tetracycline and trimethoprim-sulfamethoxazole, was remarkably high and did not change significantly after introduction of PCV7.

The observed decrease in nasopharyngeal carriage of PCV7 strains in young Andean children (Fig. 1, inset) that had received 2 or more doses of PCV7 is similar to observations in other settings within a few years post vaccination with 3 doses of pneumococcal conjugate vaccines (15, 25-27). For example, in Massachusetts, US, the prevalence of NP carriage of PCV7 types decreased ~3 years after PCV7 was licensed in the US from 22% to 2%, whereas non-PCV7 types increased from 7% to 16% (15). In that study, 66.5% of children had received 3 doses of PCV7 whereas 60.8% of children from the Peruvian cohort studied here had received 2 doses of PCV7. A similar study in South African

children, from rural villages with high prevalence of HIV positivity, showed a marked decrease in PCV7 types 2 years after the vaccine was introduced (28). Longer exposure to pneumococcal vaccines has reduced nasopharyngeal carriage of vaccine types further. A recent study showed that the nasopharyngeal prevalence of vaccine types isolated from Korean children decreased from 62% to 24%, seven years post-PCV7 introduction (25).

Evidence of herd protection was observed in non-vaccinated children as those carrying PCV7 types in 2011 significantly decreased in comparison to those non-vaccinated children colonized by vaccine types in 2009 ($p < 0.008$). Similar observations following the introduction of PCV7 have been reported elsewhere (26, 29, 30).

Regarding individual serotypes, a decrease in carriage of serotype 6B strains was observed with significantly increased nasopharyngeal carriage of strains belonging to serotype 6C. Serotype 6C was first described in 2007 as a subtype of 6A that differed in reactivity with monoclonal antibodies from the majority of 6A strains (31). PCV7 contains polysaccharide from 6B and protects against 6A and 6B but does not protect against serotype 6C (25, 32). A worrisome increase in the carriage and incidence of IPD due to serotype 6C has been observed post PCV7 introduction, and is confirmed in rural Peru in this study (32-36). PCV13, which includes serotype 6A, offers cross protection against serotype 6C and it has decreased following introduction of PCV13, supporting its future introduction in Peru (37, 38).

No significant changes in nasopharyngeal carriage of serotype 19A strains (non-PCV7 serotype) were seen in this study, suggesting that vaccination did not impact carriage of this strain. Similar observations were reported in Norway and Colombia [29, 37]. Importantly, as in other studies (39, 40), most 19A strains in our study were resistant to three or more antibiotics.

We found in this study that the prevalence of 19F strains post-PCV7 did not change. While other carriage studies have reported a significant reduction of serotype 19F post PCV7 usage (26, 29, 30, 41), no decrease has also been observed in countries such as Norway, Greece and Korea (25, 26, 29, 30, 42). It has been hypothesized that persistence of 19F in vaccinated populations might be due to a weaker vaccine-induced mucosal immunity against serotype 19F strains (26, 29, 30).

Two main conclusions can be drawn concerning antibiotic resistance: 1) most strains were not susceptible to tetracycline and trimethoprim-sulfamethoxazole; 2) no changes in antibiotic resistance in strains isolated from vaccinated children, suggesting significant antibiotic resistance in non-PCV7 strains. Our observations are in line with studies conducted in Colombia, Norway and Portugal showing no overall changes in antibiotic resistance of pneumococci after country-wide vaccination with pneumococcal conjugate vaccines (27, 29, 30).

The observed high prevalence of pneumococci not susceptible to tetracycline and trimethoprim-sulfamethoxazole needs to be highlighted. Tetracycline has been comprehensively used in veterinary medicine in both developed and developing nations to stimulate growth of livestock (43-45). Cajamarca is a major dairy-producing region in Peru.

More than 80% of the farms in Cajamarca utilize oxytetracycline to treat infections in their livestock (46). Presence of tetracycline in ~60% of milk utilized for human consumption has been reported in some regions of the country (47). Oxytetracycline is also widely available for human use due to low cost, but is not recommended for use in children. Importantly, the observed resistance to tetracycline appears to be regional. Unlike the strains isolated in this study, Torres et al. (48) demonstrated a lower prevalence (29.1%) of pneumococci resistant to tetracycline but a similar (58%) resistance to trimethoprim-sulfamethoxazole of pneumococci isolated from healthy Peruvian children from urban settings. The authors pointed out that trimethoprim-sulfamethoxazole is the most widely used antibiotic for respiratory infections in children in Peru (48).

For >90% of children in the study the pneumococcal nasopharyngeal density was 10^4 cfu/ml. However, carrying a non-PCV7 type in the nasopharynx was associated with a higher nasopharyngeal density. This finding could be due to change in age of the children or other factors not analyzed. It also could have been influenced by the vaccination campaign. We hypothesize that vaccine pressure on the pneumococcal nasopharyngeal density of vaccine types (i.e., reduction in acquiring carriage of vaccine types with subsequent serotype replacement) allowed the increase in nasopharyngeal density of non-PCV7 types. As nasopharyngeal carriage of 2 serotypes is as common as that of one strain (49, 50), it is possible that children were colonized by a mixture of vaccine and non-vaccine types. Thus, a nasopharyngeal density of vaccine types might be higher than their counterparts when both are carried together. Once this competition is removed (or decreased) by PCV, non-PCV7 types might increase in prevalence and density.

Some limitations need to be considered in our study. First, information on the predominant serotype was obtained from nasopharyngeal specimens thereby overlooking other serotypes potentially carried at lower densities that could not be recovered in culture. Second, although we studied the same children, their age differed by ~2 years between the time when the first specimen was collected in 2009 and the second one in 2011. Thus, comparing the same children over time can be seen as a potential confounder as children were older in 2011, and their serotypes may change with age. However, the serotype changes observed are most likely due to serotype replacement following PCV7 as, in the absence of the vaccine, prevalence of PCV7 serotypes increases with age (51). Third, this study only included a subset of children from the parent RESPIRA-Peru cohort study, (16) and only ~60% of children in this study had been vaccinated with PCV7. Fourth, individualized history of antibiotic exposures, which could affect pneumococcal carriage and antibiotic-resistance patterns, was not available for this study. Finally, our analyses of pneumococcal density were conducted in selected nasopharyngeal specimens in which we expected, due to their positivity in culture that predicts higher density, and later demonstrated (Table 4), high pneumococcal density (20, 52). Additional studies of serotype specific pneumococcal densities are warranted to draw final conclusions on the effects of PCV7 on nasopharyngeal densities.

In this study of young Andean children, we observed that after PCV7 introduction there was a significant decline in nasopharyngeal carriage of PCV7 types and an increase in carriage of non-PCV7 types. There was a statistically significant increase in pneumococcal

nasopharyngeal density among those carrying non-PCV7 types. We also noted that most isolated strains were resistant to tetracycline and trimethoprim-sulfamethoxazole and the prevalence of carriage of these resistant strains did not change post-PCV7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Prevalence of PCV7 and non-PCV7 serotypes isolated from Andean Peruvian children <3 years in 2009 and 2011

Inset shows the percentages of PCV7 and non-PCV7 types isolated in each year. * $p < 0.05$,

** $p < 0.002$.

Fig. 2. Antibiotic susceptibility of *S. pneumoniae* strains isolated in 2009 and 2011

Pneumococcal strains isolated in 2009 (N=125) or 2011 (N=125) were tested for susceptibility to antibiotics as specified in Material and Methods and classified as resistant, intermediate (with intermediate susceptibility) and susceptible.

Fig. 3. PCV7 induces an increased pneumococcal nasopharyngeal density in children colonized by non-PCV7 types

DNA purified from nasopharyngeal specimens was utilized as template in qPCR reactions and nasopharyngeal pneumococcal densities were obtained. Panels show nasopharyngeal densities of children colonized, in 2009 vs those colonized in 2011, by (A) PCV7-types and non-PCV7 types, (B) children colonized only by PCV7 types or (C) children colonized only by non-PCV7 types. Statistical analysis was performed with the Mann-Whitney U test. Dotted lines represent overall mean value.

Table 1

Characteristics of study participants

Characteristics	Number (%)
Sex	
Male	64 (51.2)
Female	61 (48.8)
Age (month)	
2009	8.2 (IQR 5.6, 11.2)
2011	30.9 (IQR 28.5, 34.1)
Vaccination card available	
Yes	124 (99.2)
No	1 (0.8)
At least one dose of PCV7	
2009	24 (19.2)
2011	87 (69.6)
2 or more doses of PCV7	
2009	10 (8.0)
2011	76 (60.8)
Number of people living in the same household *	
3-5	79 (63.2)
7-10	45 (36.0)
Attendance at a daycare-equivalent center *	
Yes	11 (8.8)
No	113 (90.4)

* Information for one child is missing

Table 2

Odds ratios with 95% confidence intervals for non-susceptibility to antibiotics^a

	Non-susceptible to 1 antibiotic	Non-susceptible to Penicillin	Non-susceptible to Erythromycin	Non-susceptible to Clindamycin	Non-susceptible to Chloramphenicol	Non-susceptible to Trimethoprim-sulfamethoxazole	Non-susceptible to Tetracycline
Age							
Older age (above median) relative to younger age	0.8 (0.42-1.54)	0.93 (0.48-1.80)	1.74 (0.83-3.65)	0.97 (0.38-2.51)	0.48 (0.17-1.32)	1.00 (0.55-1.83)	0.07 (0.01-0.99)
PCV7 doses							
Less than 2 doses relative to 2+ doses	1.45 (0.73-2.88)	1.77 (0.89-3.54)	0.72 (0.33-1.59)	1.12 (0.41-3.06)	3.47 (1.29-9.38)	1.15 (0.55-1.83)	₁ ^b
Serotypes							
Non-PCV7 types relative to PCV7 types	0.30 (0.16-0.54)	0.74 (0.41-1.35)	0.99 (0.49-1.98)	1.19 (0.49-2.88)	1.05 (0.50-2.23)	0.30 (0.17-0.53)	0.43 (0.04-4.63)
Crowding							
Crowding relative to no crowding	0.86 (0.50-1.49)	1.18 (0.66-2.10)	1.54 (0.81-2.94)	1.15 (0.53-2.51)	0.88 (0.43-1.80)	0.92 (0.53-1.61)	0.75 (0.13-4.21)
Sex							
Female relative to Male	0.79 (0.45-1.35)	0.72 (0.42-1.25)	0.66 (.36-1.21)	0.52 (0.24-1.15)	0.93 (0.51-1.68)	0.71 (0.41-1.23)	0.13 (0.01-1.63)

Statistically significant associations are shown in bold.

^a adjusted for serotypes, PCV7 doses, year, age, sex, electricity, stove, attending daycare-equivalent, smokers at home, and crowding.

^b omitted because of collinearity.

Table 3

Non-susceptibility to antibiotics of more prevalent pneumococcal serotypes

Antibiotics	Serotypes									
	PCV7 serotypes % (N)					Non-PCV7 serotypes % (N)				
	6B (n=22)	19F (n=31)	23F (n=22)	6A (n=7)	6C (n=18)	11A (n=22)	19A (n=10)	23B (n=10)		
Penicillin	59.1 (13)	41.9 (13)	22.7 (5)	0.0 (0)	50.0 (9)	63.6 (14)	70.0 (7)	100.0 (10)		
Erythromycin	31.8 (7)	25.8 (8)	22.7 (5)	0.0 (0)	66.7 (12)	13.6 (3)	70.0 (7)	20.0 (2)		
Clindamycin	13.6 (3)	19.4 (6)	9.1 (2)	0.0 (0)	0.0 (0)	9.1 (2)	70.0 (7)	10.0 (1)		
Chloramphenicol	13.6 (3)	22.6 (7)	22.7 (5)	0.0 (0)	5.6 (1)	31.8 (7)	30.0 (3)	10.0 (1)		
Trimethoprim-Sulfame thoxazole	81.8 (18)	80.6 (25)	72.7 (16)	42.9 (3)	72.2 (13)	90.9 (20)	70.0 (7)	60.0 (6)		
Tetracycline	100.0 (22)	100.0 (31)	100.0 (22)	100.0 (7)	100.0 (18)	95.5 (21)	100 (10)	100.0 (10)		
Non-susceptible to at least one antibiotic ^a	81.8 (18)	87.1 (27)	86.4 (19)	42.9 (3)	72.2 (13)	90.0 (20)	70.0 (7)	100.0 (10)		
MDR ^b	22.7 (5)	45.2 (14)	22.7 (5)	0.0 (0)	38.9 (7)	27.3 (6)	70.0 (7)	10.0 (1)		

^aTetracycline was excluded.^bMultidrug resistant (resistant to three or more antibiotics).

Table 4

Pneumococcal nasopharyngeal density categorized by increasing bacterial load.

Density category (CFU/ml)	2009 ^{ab}		2011 ^a	
	n	%	n	%
10 ² <10 ³	0	0	1	0.8
10 ³ <10 ⁴	3	2.4	11	8.8
10 ⁴ <10 ⁵	32	25.6	25	20
10 ⁵ <10 ⁶	47	37.6	39	31.2
10 ⁶ <10 ⁷	33	26.4	38	30.4
10 ⁷ <10 ⁸	5	4	11	8.8
Total	120	100	125	100

^aYear when the sample was collected.

^bFive DNA preps were not available.