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Impact of crowding on the antibiotic resistance of carriage isolates of *Streptococcus pneumoniae* from healthy Peruvian children in the pre-PCV7 period

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2015

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Abstract

Impact of crowding on the antibiotic resistance of carriage isolates of *Streptococcus pneumoniae* from healthy Peruvian children in the pre-PCV7 period

By Zachary J Matson

Background: *Streptococcus pneumoniae* is a highly transmissible upper respiratory pathogen that is a major contributor to child mortality, especially within developing countries. Conjugate vaccines, such as PCV7, have been effective in reducing pneumococcal burden worldwide, but vaccine-type replacement, growing antimicrobial resistance, and poor vaccine access in developing areas indicate that additional solutions are needed. Understanding how environmental factors influence pneumococcal dynamics in carriers may be useful in creating effective interventions that complement vaccination. The purpose of this study was (1) to describe molecular aspects and invasive disease potential of *S. pneumoniae* carriage isolates collected from healthy young children between 2007-2009 and (2) determine how exposure to crowded environments and molecular factors promote multidrug-resistant *S. pneumoniae* carriage in healthy young children in Peru.

Methods: This cross-sectional study utilized whole genome sequencing to describe the serotype distribution, genetic lineages, and antibiotic resistance profiles of carriage *S. pneumoniae* isolates (n=508) collected from healthy children aged 2-24 months in seven Peruvian cities between 2007-2009. The invasive disease potential (IDP) of each serotype was quantified using a subset of the carriage isolates from Lima (n=259) and invasive-disease isolates from a separate study conducted in Lima (n=133) [2]. Risk factors for the carriage of multidrug-resistant *S. pneumoniae* were assessed using logistic regression models that dichotomized the number of antibiotic classes a strain was resistant into ≥ 3 classes and <3 classes.

Results: Among 508 carriage isolates, 52.6% had a PCV7 serotype, 40.4% could be linked to a PMEN clonal complex, and 22.4% were resistant to at least three classes of antibiotics. Serotypes 14 and 6B were found to have an IDP>1.0 at $\alpha=0.05$ and represented 21.5% (n=109) of all carriage isolates. Multivariate logistic regression determined that being a PCV7 serotype carrier (OR=11.83, $p<0.0001$), daycare attendance (OR=4.92, $p=0.031$), and living in a highly urban area (OR=2.86, $p=0.008$) were significant risk factors for children being carriers of multidrug-resistant *S. pneumoniae*.

Conclusions: *S. pneumoniae* carriage strains in Peruvian children before PCV7 implementation were genetically diverse and highly drug-resistant. Carriage of multidrug-resistant *S. pneumoniae* in young children can be partly attributable to exposure to crowded environments and carrying a vaccine-type strain.

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Finally, I would like to dedicate this thesis to my soon-to-be born nephew Griffin Alexander in hopes that he will live a healthy, purposeful life. I'm looking forward to meeting you!

Table of Contents

I. INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is the leading preventable cause of pneumonia, septicemia and meningitis and was estimated by the World Health Organization in 2010 to be responsible for approximately 800,000 deaths in young children each year [1]. *S. pneumoniae* is a Gram-positive, alpha-hemolytic bacterium that is a contributor to normal nasopharyngeal flora. Alongside other commensal upper respiratory tract organisms such as *Moraxella catarrhalis*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Staphylococcus aureus*, *S. pneumoniae* colonizes the nasopharynx in an asymptomatic carriage state [24]. For children, the rate of nasopharyngeal carriage is highly dependent on age with those under 5 years old being at an elevated risk for becoming carriers [24-27]. The first year of life is marked by rapid nasopharyngeal colonization. A 2001 Finnish study demonstrated this as they found that the pneumococcal carriage rate rapidly increased from 13% for children under 6 months old to 43% in children over 19 months old [24, 28]. Certain studies have indicated that peak pneumococcal carriage occurs in children 3 years of age with an estimated 55% colonization incidence that declines with age [24, 26]. Pneumococcal colonization rates are highly variable among geographic regions, however. A 2006 a cross-sectional study conducted in western Gambia villages estimated that 72% of participants of all ages were pneumococcal carriers and that the carriage rate for infants under 1 year of age was 97% [31]. For comparison, in 2004 it was estimated that 41% of children in Peru under the age of 2 were *S. pneumoniae* carriers [6].

Typically, *S. pneumoniae* is capable of becoming pathogenic in carriers with weaker immune systems (ie. the elderly, children, and the immunocompromised) [6]. Transition from a carriage state to normally sterile sites (e.g. blood and cerebrospinal fluid) results in often fatal invasive pneumococcal disease (IPD) such as bacteremia and meningitis. Pneumococcal carriage

does not always translate to the development of IPD, however. The pathogenic capabilities of pneumococcal strains are highly contingent on a strain's polysaccharide capsule that prevents phagocytosis by host immune cells. Variability among capsular virulence factors exist with each strain possessing a unique serotype dictating capsule composition. Currently, there are over 95 known serotypes ranging in their virulence and propensity to cause IPD [1,2]. In addition to cell capsules, pili are also considered to be notable virulence factors. Pili enhance the adhesion to host upper respiratory tract epithelial cells required to initiate IPD. Two types of pili in particular have been shown to contribute to host adhesion: type 1 (PI-1) and type 2 (PI-2) [53-54]. Other virulence factors include pneumolysin production, pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC), and pneumococcal surface adhesin A (PsaA) [14]. However, these virulence factors are not considered to have as significant of a role in the pathogenesis of IPD as polysaccharide capsules and pili [14].

To address the global pneumococcal disease burden, the 7-valent pneumococcal conjugate vaccine (PCV7) was licensed in the United States in 2000 and introduced to Peru in 2009. Before the implementation of PCV7, 12,000-18,000 annual deaths among children <5 years of age in Latin America were attributable to IPD [2, 5]. Introduction of the PCV7 vaccine has been effective in decreasing pneumococcal mortality, but only protects individuals from 7 of the pathogen's >90 highly diverse serotypes: 4, 6B, 9V, 14, 18C, 19F, and 23F [1-2, 15]. These 7 serotypes were determined based on the most prevalent serotypes found in the United States, not internationally. In 2010, it was estimated that PCV7 coverage ranged from 56%-73% of all isolates in Latin America [42]. Given the dynamic and rapid nature of natural pneumococcal acquisition and clearance, alongside vaccine pressure, changes in nasopharyngeal colonization have evolved over time. Studies have shown that immunization with PCV7 did not affect the

overall nasopharyngeal colonization rate in study cohorts; however, vaccination did reduce overall colonization with vaccine serotypes and increases were seen in non-vaccine serotypes [7, 57-58, 64]. For example, following implementation in Peru, carriage rates of PCV7 serotypes in healthy Andean Peruvian children were found to decline from 48% in 2009 to 28.8% in 2011, while non-PCV7 serotype carriage increased from 52% to 71.2% [7]. The implementation of newer conjugate vaccines in Peru, such as PCV10 in 2011, expanded immunity to additional serotypes, but are also vulnerable to non-vaccine serotype replacement [2]. Due to serotype replacement rather than eradication occurring in response to conjugate vaccines, PCV optimization is an ongoing process. Development of new conjugate vaccines requires a steady input of data capable of detecting the most burdensome and prevalent invasive serotypes.

Studies guiding the development and evaluation of conjugate vaccines involve a significant emphasis on serotype distribution within populations of interest. Early studies utilized the rank order prevalence of invasive disease serotypes to determine the most suitable conjugate vaccine composition [11]. Rank order prevalence approaches are not optimized to detect a serotype's tendency to cause disease, however. Rather, this approach favors detection of serotypes that children are frequently exposed to. It is likely that serotypes that are more virulent, but that children are less frequently exposed to are not emphasized using this method [11]. Because of this shortcoming, the invasive disease potential (IDP) metric gained traction in the early-mid 2000s as a complement to rank order prevalence in consideration of serotype inclusion in PCV [11]. IDP is an empirical odds ratio that controls for serotype exposure frequency by comparing the odds that a given serotype is going to cause invasive disease compared to the odds of being found in carriage. Based on previous studies, IDP = (ad)/(bc) where a is the frequency of invasive isolates of the serotype of interest, b is the frequency of carriage isolates of the

serotype of interest, *c* is the frequency of all other invasive isolates, and *d* is the frequency of all other carriage isolates [11-14]. Serotypes with an IDP > 1.0 are invasive, while those with IDP < 1.0 are generally non-invasive. In addition to communicating vaccine development needs, IDP has the advantage of assessing post-vaccine invasive serotype replacement [13-14, 16]. However, a caveat to this application is that investigating invasive serotype replacement requires possessing both carriage and invasive data with the same sampling ages within a geographic region.

It is important to consider that IDP is not a definitive answer as to whether a particular serotype found in carriage will transition into causing invasive disease. Rather, this measure estimates the magnitude of an individual serotype's proclivity to become invasive or remain in a carriage state. Meta-analysis of international pre-PCV7 IDP studies with study periods between 1994-2009 have generated some consensus on the invasive potential of certain serotypes. It was found that the following serotypes were universally highly invasive: 1, 4, 5, 7F, 8, 12F, 14, 18C, and 19A [11,14,17-22]. Serotypes that were considered universally non-invasive include 6A, 6B, 11A, 15B/C, and 23F [11,14,17-22]. However, variability in a serotype's IDP can exist regionally due to geographic differences and confounding factors. For example, Hanage et al. found serotype 6B to be significantly invasive (IDP= 1.643 [$CI_{95\%}$: 1.01-2.67] with n=84) in Finland between 1995-1999, yet similar studies conducted before PCV7 implementation in the United Kingdom and Portugal found 6B to be significantly non-invasive [18-19, 23]. For this reason, discretion must be used when assessing a serotype using international IDP measures. Because of regional variations, IDP is most useful when considered in the context of the country a serotype is isolated from. Even then, geographic and socioeconomic differences within

countries such as urban-rural divides could hypothetically result in differing IDP values for a serotype.

Vaccines are a critical intervention that emphasize prevention rather than direct treatment. However, PCV vaccines are often not immediately accessible; 90% of the annual 800,000 deaths among young children each year occur in developing countries with low access to these serotype-based vaccines [1]. The use of antibiotics to treat IPD have been an effective direct intervention, but growing global resistance to multiple classes of antibiotics such as β-lactams, macrolides, lincosamides, tetracycline, and co-trimoxazole (TMP/SMX) complicates the treatment of pneumococcal infections [8, 32]. Much like carriage and serotype invasive disease potential, pneumococcal resistance rates vary by region [32]. Select resistant pre-PCV7 serotypes have been shown to be universally more prevalent than others: 6B (PCV7), 9V (PCV7), 14 (PCV7), 19F (PCV7), 23F (PCV7), 6A (non-PCV7), and 19A (non-PCV7) [32-40]. Data on resistant serotype distribution across Peru before PCV7 implementation are limited, but various studies conducted in Lima have indicated that 23F, 14, and 19 were the predominant carriage antimicrobial resistant serotypes in the pre-PCV7 period [41-43]. A 2006-2008 prospective surveillance study conducted in Lima found especially high rates of resistance to TMP/SMX (76.2%), erythromycin (24.8%), and penicillin (22.8%) using minimal inhibitory concentrations (MIC) of invasive *S. pneumoniae* isolates from hospitalized children under the age of 16 [42].

Investigating environmental factors that encourage transmission may prove useful in developing preventative interventions that can complement vaccination and mitigate emerging antibiotic resistance. One such factor is crowding in the home and daycare attendance, which has been determined to be associated with increased pneumococcal transmission and carriage in young children [3-4, 29]. More specifically, infants living with at least two siblings under the age

of 5 have been shown to have a significantly higher risk of carriage by the age of 4 months [45]. With *S. pneumoniae* being an upper respiratory organism, crowded environments increase the rate of transmission by offering more opportunities for carriers to spread the organism via nose and mouth bioaerosols. Increased transmission among carriers creates ample opportunities for horizontal gene transfer among nasopharyngeal flora. This process is largely responsible for the genetic diversity of *S. pneumoniae* that allows it to adapt to environmental pressures such as antibiotic exposure. Evidence demonstrating this has shown that siblings with acute otitis media (AOM), a common childhood disease caused by *S. pneumoniae*, tend to have genetically homologous pneumococcal strains including those that are multidrug-resistant [46]. Also, studies investigating the spread of multidrug-resistant *S. pneumoniae* in the United States have found that children in daycares are carriers of highly diverse strains that have an elevated prevalence of antibiotic resistance [47-48]. Although the effect crowding has on carriage and resistance has been well established, how crowded environments affect the distribution and prevalence of high IDP carriage serotypes in children remains unclear.

Additional socioeconomic and environmental factors may influence pneumococcal carriage and mortality in children. Socioeconomic disadvantage, which can result in poor and/or crowded housing conditions, has been shown by Huang et al. to increase the risk of pneumococcal carriage by nearly 3-fold in children from households below the median income in the United States even when controlling for daycare attendance [3]. A 2015 meta-analysis on risk factors for acute lower respiratory infections (ALRI) in 198,359 children under five years old in 29 low and middle-income countries identified several environmental factors significantly associated with increased mortality: lack of sewage/latrine infrastructure (OR=1.82 [$CI_{95\%}$: 1.30-2.54]), low drinking water quality (OR=2.85 [$CI_{95\%}$: 1.28-6.36]), second-hand smoke exposure

(OR=1.52 [$CI_{95\%}$: 1.20-1.93]), and indoor air pollution (OR=3.02 [$CI_{95\%}$: 2.11-4.31]) [49]. Crowding was not a significant risk factor for ALRI childhood mortality (OR=1.33 [$CI_{95\%}$: 0.86-2.05]) [49]. Although somewhat generalizable to IPD, the results of this systemic review apply to a broad range of respiratory pathogens and focus solely on mortality. More environmental studies are needed to refine the role that WASH (water, sanitation, and hygiene) and air quality have on pneumococcal carriage, invasiveness, and antibiotic resistance.

II. METHODS

Project aims

Using data obtained from a 2007-2009 cross-sectional surveillance study of Peruvian children 2 years of age or younger, this project aimed to achieve the following:

1. Utilize whole genome sequencing (WGS) to describe the serotype distribution, genetic lineages, and antibiotic resistance profiles of carriage *S. pneumoniae* isolates in the pre-PCV7 period.
2. Use carriage and invasive-disease *S. pneumoniae* isolates from Lima to determine and describe the invasive disease potential (IDP) of pre-PCV7 carriage *S. pneumoniae* serotypes found in Peru.
3. Address exposure to crowded conditions in the home, number of siblings, daycare attendance, and living in a highly urban city as potential environmental determinants of multidrug-resistant *S. pneumoniae* carriage.
4. Address serotype and pili type as potential predictors of multidrug-resistant (MDR) *S. pneumoniae* carriage.

Aims 1-2 are descriptive in nature and were displayed using mostly frequencies and relative frequencies. For aim 1, genetic relatedness of carriage isolates was determined by assessing the presence of sequence types that indicated membership to clonal complexes. In aim 2, we assumed that IDP values obtained from observing Lima isolates were generalizable to all Peru serotypes. Aims 3-4 assessed the hypotheses of "Are children aged 2 or younger that are exposed to crowded conditions more likely to carry multidrug-resistant *S. pneumoniae* than those who are not exposed to crowded conditions?" and "Are children carriers of PCV7 serotypes and/or type 1 pili at increased odds of carrying a multidrug-resistant serotype?", respectively. These hypotheses were addressed using logistic regression with the following potential predictors: number of people in the home, cohabitating with at least 2 siblings, daycare attendance, living in a highly urban city, being a carrier of a PCV7 serotype, and being a carrier of an isolate with a type 1 pilus.

Carriage isolate acquisition

S. pneumoniae isolates used for this study were collected by a collaborative research group in Peru that partners with Centers for Disease Control and Prevention (CDC) National Center for Immunization and Respiratory Diseases (NCIRD) and includes data from Torres et al. [5]. Nasopharyngeal swabs were obtained from 2,123 healthy children aged 2-24 months between 2007-2009 at outpatient facilities in seven Peruvian cities: Lima, Piura, Cusco, Abancay, Huancayo, Arequipa, and Iquitos. In addition to nasopharyngeal samples, information on the number of children in the home, number of individuals in the home, daycare attendance, age, sex, previous antibiotic use, and medical facility of isolation for each child was collected.

Collected swabs were transferred in TSB to a central laboratory in Lima that plated all the samples on TSA with 5% sheep blood [5]. Isolation of *S. pneumoniae* was performed based on the following identification criteria: colony morphology, α-hemolysis, Gram staining, solubility in bile, and optochin sensitivity [5]. 508 *S. pneumoniae* samples were isolated and then transported to the CDC's *Streptococcus* laboratory (Atlanta, GA, USA) for serotype analysis using latex agglutination and conventional Quellung reaction methods [5].

Whole genome sequencing

S. pneumoniae isolates were cultured on TSA (5% sheep blood supplemented) and incubated overnight in a 37°C and 5% CO_2 environment. Manual DNA extraction was then performed using a modified QIAmp DNA mini kit protocol (Qiagen, Inc., Valencia, CA, USA). Extracted nucleic acid concentrations were determined via Invitrogen Qubit assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA samples were then transferred to sealed 96-well plates and shipped to the Sanger Institute (Hinxton, Saffron Walden CB10 1SA, United Kingdom) to be sequenced as a part of the Global Pneumococcal Sequencing (GPS) project (www.pneumogen.net).

WGS of each sample was performed at the Sanger Institute using the Illumina HiSeq 2500 system. Raw nucleotide sequences were then evaluated for the presence of relevant genetic determinants using the CDC's *Streptococcus* lab pneumococcal typing bioinformatics pipeline. Using this pipeline, information on serotype, sequence type (ST), pilus genes, transpeptidase domain amino acid sequences from penicillin-binding proteins (PBPs), and various resistance features of each isolate were determined (https://github.com/BenJamesMetcalf/Spn_Scripts_Reference) [50]. Genetic relatedness among each isolate was determined using multilocus sequence typing (MLST) and clonal complexes (CC) were identified using ST eBURST analysis (eburst.mlst.net). CCs were defined as groups of STs sharing ≥5 MLST alleles with one or more members of the set. The presence of Pneumococcal Molecular Epidemiology Network (PMEN) clonal complexes was also assessed (www.pneumogen.net/pmen). Minimum spanning trees estimating the evolutionary relationships between STs, serotypes, and multi-drug resistance (MDR) were constructed using PhyloVIZ ((http://www.phyloviz.net/) [67].

Antibiotic resistance determination

Whole genome sequencing and the CDC's pneumococcal bioinformatics pipeline were used to predict the resistance profile of each carriage isolate. Resistance to 6 common β-lactam drugs were evaluated using previously described methods [50-51]. Each isolate's PBP type was correlated with phenotypically measured minimum inhibitory concentrations (MIC) previously determined in isolates with the same PBP type [50-52]. Clinical Laboratory Standards Institute (CLSI) MIC cutoffs were then used to assess whether an isolate was susceptible, intermediately-resistant, or fully resistant to each β-lactam antibiotic (**Appendix Table 1**) [52]. Susceptibility to the following non-β-lactam antibiotics were also predicted using similar whole genome analysis approach correlating resistance determinants with phenotypic resistance: erythromycin, clindamycin, chloramphenicol, aminoglycoside, rifampin, co-trimoxizole, and tetracycline. Pipeline queries used to detect non-β-lactam resistance determinants are described in Metcalf et al. (2016) [50].

The extent of drug resistance for each isolate was determined by summing the number of antibiotic classes each strain was fully resistant to. The following classes were evaluated: β-lactams, macrolides (erythromycin), lincosamides (clindamycin), amphenicols (chloramphenicol), aminoglycosides (aminoglycoside), rifamycins (rifampin), tetracyclines (tetracycline), and folate inhibitors (TMP/SMX). Each isolate was then assigned one of the following: non-resistant (susceptible to all classes of antibiotics), resistant (non-susceptible to at least 1 class of antibiotics), or multidrug-resistant (resistant to ≥3 classes of antibiotics).

Invasive disease potential determination

To determine serotype IDP, we used the subset of the 508 carriage isolates (n=259) that were collected in Lima, since invasive isolates were available from this location only. The invasive isolates used in the analysis (n=133) were collected between 2006-2009 (before PCV7 introduction) and have been previously described in Hawkins et al. [2]. The Lima IDP results were then generalized to all 508 carriage isolates including those not originating from Lima.

The equations below describe the layout for IDP and confidence interval calculations where *A* is the frequency of invasive isolates of the serotype of interest, *B* is the frequency of carriage isolates of the serotype of interest, *C* is the frequency of all other invasive isolates, and *D* is the frequency of all other carriage isolates. Confidence intervals were used to assess statistical significance of IDP≠ 1.0 at α= 0.05 for each serotype. IDP was not calculated for serotypes that were not found (n=0) in carriage or invasive-disease isolates.

$$IDP = \frac{odds\ of\ invasive}{odds\ of\ carriage} = \frac{(A)(D)}{(B)(C)}$$

$$CI_{95\%} = e^{\ln(IDP) \pm 1.96\sqrt{\frac{1}{A}+\frac{1}{B}+\frac{1}{C}+\frac{1}{D}}}$$

Statistical analysis

Statistical analyses were performed using SAS v9.4 (SAS Institute Inc., Cary, NC) statistical software. Univariate analysis revealed that the distribution of persons per home was right skewed (skewness= 1.82; kurtosis= 5.25). Logarithmic transformation of persons per home was applied to approximate a normal distribution (skewness= 0.02; kurtosis= 0.10). Cities of isolation were reassigned into a binary "urban region" variable in accordance with their 2009-2016 % urban population [55]. Lima (98% urban) and Arequipa (90% urban) were categorized as more urban while Piura (77.5% urban), Iquitos (67.3% urban), Huancayo (65.5% urban), Cusco (55.5% urban), and Abancay (40.2% urban) were categorized as less urban [55].

Logistic regression model was used to determine the odds ratio of the outcome of possessing a MDR (resistant ≥3 drug classes) carriage isolate. The predictor variables in this model included type 1 pilus possession, PCV7 serotype, persons per home, having at least 2 siblings six years of age or younger in the home, daycare attendance, antibiotic use within 3 months of isolate collection, previous hospitalization for an upper respiratory infection, age, sex, and urban region isolation. Multicollinearity among variables was assessed and statistical significance was defined as $p < 0.05$. To determine the most optimal model predicting MDR pneumococcal carriage, a multivariate model was constructed using stepwise selection with an entry criterion of $\alpha=0.10$ and retention criterion of $\alpha=0.05$. A Hosmer-Lemeshow (HL) χ^2 test was used to assess the goodness of fit for the final model, while the receiver operator characteristic (ROC) area was used to describe its prediction capabilities (sensitivity/specificity).

III. RESULTS

ST and serotype prevalence

Of the 508 carriage isolates used in this study, 257 (50.6%) were from Lima, 55 (10.8%) from Iquitos, 47 (9.3%) from Cusco, 46 (9.1%) from Piura, 43 (8.4%) from Abancay, 30 (5.9%) from Huancayo, and 30 (5.9%) from Arequipa (**Figure 1**). 163 unique sequence types were identified among all carriage isolates with ST156 (n=34 [6.8%]), ST81 (n=26 [5.2%]), ST242 (n=23 [4.6%]), and ST1421 (n=20 [4.0%]) being the most common. The isolates that belong to PMEN clones accounted for 40.4% (n=205) of all isolates with PMEN14 (n=55 [10.8%]), PMEN3 (n=38 [7.5%]), PMEN1 (n=27 [5.3%], and PMEN15 (n=27 [5.3%]) were the most frequently identified (**Figure 2, Table 1**). Non-PMEN clonal complexes accounted for 27.0% (n=137) of all isolates with CC5638 (n=23 [4.5%]) and CC5625 (n=19 [3.7%]) being the most frequently identified (**Figure 2, Table 1**). Non-CC singletons composed 31.7% of all isolates with ST3669 (n=33 [6.5%]) and ST5628 (n=17 [3.3%]) being the most commonly found (**Figure 2, Table 1**).

Among all carriage isolates, 266 (52.6%) possessed a PCV7 serotype (4, 6B, 9V, 14, 18C, 19F, or 23F) and 27 (5.3%) were non-typeable. The most common serotypes among all carriage isolates were 19F (n=95 [18.8%]), 6B (n=74 [14.6%]), and 23F (n=48 [9.5%]) (**Figure 3**). Serotypes 6B was a contributing serotype to three focal ancestors: ST5449, ST5625, and ST5464 (**Figure 4**).

Antibiotic resistance prediction

Of the 508 carriage isolates, 208 (40.9%) were predicted as resistant to at least one class of antibiotics and 114 (22.4%) were predicted as resistant to at least three classes (**Table 2**). Among all classes, the highest rate of resistance was observed against folate inhibitors (TMP/SMX) (n=260; 51.4%) while the lowest rates of resistances were observed in aminoglycosides (aminoglycoside) and rifamycins (rifampin) (n=3 [0.6%] and n=4 [0.8%], respectively) (**Table 2**). All β-lactam resistant isolates were fully resistant to penicillin 138 (27.3%) and none were fully resistant to cefuroxime (**Table 2**). The serotypes with the highest proportion of carriage isolates with resistance to at least one antibiotic class were 14 (97.1%), 23F (91.7%), 6B (89.2%), and 19F (87.4%). The serotypes with the highest proportion of carriage isolates with resistance to at least 3 antibiotic classes were 19F (54.7%), 23F (50.0%), 19A (42.9%), and 6B (31.1%).

Invasive disease potential

The results of the IDP analysis on isolates from Lima identified nine serotypes with high invasive potential, i.e. that are more likely to be found in invasive disease isolates than in carriage isolates: 1, 12F, 14, 18F, 3, 38, 5, 6B, and 7F (**Table 3**). Of these nine, two serotypes (14 and 6B) were observed both among carriage and invasive isolates and had an IPD > 1.0 at α=0.05 while the other seven were only found among invasive disease isolates. Among the remaining 34 Lima serotypes, 19F was the only serotype found both in invasive disease and carriage isolates to have an IDP < 1.0 at α=0.05, while the remaining 33 serotypes were only observed among carriage isolates. Among all Peru carriage isolates used in this study, 41 unique

serotypes were present two of which had statistically significant IDP > 1.0: 6B (n=74) and 14 (n=35). In total, invasive isolates represented 21.5% (n=109) of all the Peru carriage isolates.

Model results

Simple logistic regression of the outcome of possessing a carriage isolate resistant to at least 3 classes of antibiotics (multi-resistant) revealed 3 significant independent risk factors: possessing an isolate with a type 1 pilus (cOR=2.77 [$CI_{95\%}$: 1.80-4.27]), possessing an isolate with a PCV7 serotype (cOR=10.69 [$CI_{95\%}$: 5.80-19.70]), daycare attendance (cOR=5.92 [$CI_{95\%}$: 1.69-20.75]), and being a carrier in an urban region (cOR=2.02 [$CI_{95\%}$: 1.29-3.15]) (**Table 5**). The full multivariate logistic regression used 330 of the 508 total observations and found possession of an isolate with a PCV7 serotype (aOR=11.87 [$CI_{95\%}$: 5.24-26.90]), daycare attendance (aOR=7.70 [$CI_{95\%}$:1.43-41.45]), and being a carrier in an urban region (aOR=3.00 [$CI_{95\%}$: 1.33-6.77]) to be significant risk factors, but not possession of an isolate with a type 1 pilus (aOR=1.32 [$CI_{95\%}$: 0.71-2.46]) (**Table 5**). No statistically significant interaction was found for possessing a PCV7 serotype and being a carrier in a highly urban city (p=0.837) or possessing a PCV7 serotype and possessing an isolate with a type 1 pilus (p=0.367).

Stepwise model selection with an entry criterion of α=0.10 and retention criterion of α=0.05 determined that possession of an isolate with a PCV7 serotype (p<0.0001), daycare attendance (p=0.011), and being a carrier in an urban region (p=0.007) were the only significant predictors of possessing a multi-resistant isolate at α=0.05 (**Table 6**). The overall fit of this multivariate model was good (HL χ^2= 1.16 [df=3], p=0.764) with above-average predictive capabilities (ROC area= 0.7915).

IV. DISCUSSION

Serotypes and lineages

All PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) were present and widespread in this study population. These seven serotypes comprised 52.6% of all carriage isolates in this study, while 34 non-PCV7 serotypes were attributed to the remaining carriage isolates. Carriage serotype replacement in rural Andean Peruvian children following PCV7 implementation has been demonstrated in two separate cohorts, but studies evaluating carriage PCV7 serotype replacement in Peru as a whole are scarce [7, 64]. Additionally, it has been shown that PCV7 implementation in 2009 resulted in the proportion of PCV7 serotypes in invasive disease isolates to decrease from 66.9% to 50.6% in Lima by 2010-2011 [2]. Although unclear how these results translate to pneumococcal carriers in Peru as a whole, it is likely that PCV7 serotype carriage proportions have also declined following PCV7 implementation. Unfortunately, post-PCV7 follow-up studies on children in this study area did not occur until recently (2018) which leaves the status of national serotype replacement in years 2009-2017 largely unknown.

Identifying 163 unique sequence-types (ST) among all 508 isolates in this study highlights the immense genetic diversity of *S. pneumoniae*. 50/163 identified STs were associated with 1 of 10 PMEN CCs, while 51/163 STs were linked to non-PMEN clones. These CCs represented 40.4% and 27.0% of all carriage isolates in this study, respectively. The clonal types found in this study are common in Peru, as a previous study identifying the lineages of pre-PCV7 invasive-disease isolates in Lima reported the presence of similar clones: PMEN1, PMEN2, PMEN3, PMEN14, PMEN15, PMEN26, and PMEN 32 [2]. Like serotype replacement, lack of follow-up data prevented us from determining if clonal and sequence type distributions were altered in carriage isolates following PCV7 implementation. The previously described

study in Lima did not find a significant clonal shift in invasive-disease isolates following PCV7 implementation, but these results may not generalizable to Peru as a whole due to small sample sizes and an emphasis on Lima invasive-disease isolates [2]. PCV10 replaced PCV7 in Peru in 2011 and carriage studies currently being conducted in these same geographic regions in 2018 will allow for assessment of PCV impact on serotype and clonal lineages

Antibiotic resistance

A significant portion of isolates (40.9%) in this study possessed resistance determinants to at least one class of antibiotics, while 22.4% possessed resistance to at least 3 classes of antibiotics. Overall, the resistance rates found in this study are comparable to previous reports. The elevated resistance rates TMP/SMX (folate inhibitors), macrolides, β-lactams, and tetracycline found in this study parallel with previously reported resistance rates in Peru for both pre-PCV7 carriage and invasive disease pneumococcal isolates, [2, 7, 42].

Minimum spanning trees revealed that CC81 (PMEN1), CC90 (PMEN2), CC1421 (PMEN14), CC276 (PMEN32), and CC5638 predominantly contained MDR STs (**Figure 5**). Interestingly, the majority of CC5638 isolates were non-typeable (**Table 1**). Serotypes 19F, 23F, 19A, and 6B were found to have the highest proportion of MDR isolates, which aligns with previously reported resistant pre-PCV7 serotypes both globally and in Peru [32-43]. These four serotypes composed the majority of PMEN1, PMEN2, PMEN14, and PMEN32 clones indicating that MDR carriage isolates are genetically related (**Table 1**). Of these four highly resistant serotypes, only 19A is not protected against by PCV7 or PCV10 which is the current vaccine in use in Peru (it is included in the PCV13 formulation, however). PCV13 has not yet been implemented in Peru, so it will be of interest to determine if 19A resistance has continued to

increase in both carriage and invasive isolates. Although PCV7 serotype replacement has been demonstrated in invasive-disease isolates in Lima and carriage isolates in Andean children, both studies found that drug-resistance proportions did not significantly change following PCV7 introduction [2, 7]. However, this may be attributable to low PCV7 coverage (37.9% in 2009) in 2010-2011 [2, 56].

Invasive disease potential

Serotypes 14 (IDP=5.78 [$CI_{95\%}$: 2.96-11.25]) and 6B (IDP=1.87 [$CI_{95\%}$:1.06-3.29]) were found to have a statistically significant high propensity to cause invasive disease, while 19F (IDP=0.42 [$CI_{95\%}$: 0.22-0.78]) was found to have a statistically significant low propensity to cause invasive disease. Pre-PCV7 serotype 14 and 19F are commonly reported to have IDP > 1.0 and IDP < 1.0, respectively, whereas 6B is often reported as having both low and high IDP depending on region of isolation [11,14,17-22]. Interestingly, 19F had the highest proportion of MDR isolates (54.7%) and 6B had the 4th highest proportion of MDR isolates (31.1%), suggesting an unclear relationship between propensity to cause invasive disease and MDR. Elevated drug-resistance in 19F can be attributed to its tendency to remain in a carriage state where ample opportunities for horizontal gene transfer among nasopharyngeal flora can occur. Measurement uncertainty in our study (6B IDP $CI_{95\%}$=1.06-3.29) and high 6B frequencies indicate that this serotype is found in a carriage state enough to have similar gene transfer opportunities.

IDP analysis of carriage and invasive-disease isolates from Lima only found 3 of 43 serotypes to have IDP $\neq$ 1.0 at α=0.05. Small sample sizes (n=257 for invasive and n=133 for

carriage) and the high number of serotypes present were limiting factors in estimating statistically significant IDP values for each Lima serotype. In addition, geographic, cultural, and biological differences may reduce the generalizability of IDP values found in Lima to the rest of Peru. For more robust IDP measures, future attempts must ensure a significant sample size that represents the distribution of carriage and invasive-disease serotypes in each region of Peru. However, it is important to consider that zero counts of serotypes in carriage isolate may be attributable to their rate of transition to an invasive state rather than the low sample sizes in this study. For example, serotype 1 is highly associated with meningitis and is rarely found in carriage [11,14,17-22]. The opposite of this is also true for zero counts in invasive-disease isolate, as non-typeable isolates are highly correlated with carriage and are not usually known to transition to an invasive state [11,14,17-22].

Crowded environments and molecular determinants of MDR

Daycare attendance (cOR=5.92, p=0.006; aOR=7.70, p=0.018) and living in an urban region of Peru (cOR=2.02, p=0.002; aOR=3.00, p=0.008) were found to be statistically significant crowded environment risk factors for MDR pneumococcal carriage in children aged 2-24 month (**Table 5**). The number of people living in the home (crude p=0.761, adjusted p=0.947) and having at least 2 siblings under the age of 6 years (crude p=0.413, adjusted p=0.797) were not found to be statistically significant (**Table 5**). Attending daycare and living in an urban environment indicates that a child is regularly exposed to crowded environments that facilitate *S. pneumoniae* transmission. High-transmission environments accelerate horizontal gene transfer, which increases the probability that drug-resistance genetic information is disseminated among nasopharyngeal flora. Studies have demonstrated that children who attend

daycare have an elevated risk of having a drug resistant isolate, which is consistent with our findings [47-48]. Having siblings is a known risk factor for carriage in general, but our findings indicate no relationship with MDR carriage and having at least two siblings 6 years of age or younger [45]. It is known that risk factors associated with carriage are more limited and that carriage rates overall are much lower in adults than in children, which could explain why we found no association with the number of people in the home and MDR carriage in children [59-62]. Also, measuring the density of people living in the home is likely a better approximation of a crowded home than a simple count.

Possessing an isolate with a type 1 pilus was a significant independent risk factor for MDR carriage (cOR=2.77, p<0.0001), but became non-significant (aOR= 1.40, p=0.294) in the multivariate model after adjusting for PCV7 serotype (**Table 5**). PCV7 serotype was a significant independent (cOR=10.69, p<0.0001) and multivariate (aOR=11.87, p<0.0001) risk factor for MDR carriage (**Table 5**). Our study and previously reported pre-PCV7 carriage and invasive disease studies in Peru have shown PCV7 serotypes to be disproportionately drug resistant compared to non-PCV7 serotypes [2,7]. Although promising that conjugate vaccines protected against the most drug resistant pneumococcal isolates in Peru, subsequent PCV7 vaccine serotype replacement does not necessarily translate to reduced MDR in carriage isolates [2, 7, 57-58]. Continued surveillance and identification of resistance drivers following vaccine implementation will be necessary to address the growing global threat of antimicrobial resistance.

The logistic regression models to identify MDR carriage risk factors in this study have several limitations. First, missing data on daycare attendance and sibling information reduced the amount of observations from n=508 to n=330 in the initial multivariate model and n=342 in the

stepwise multivariate model (**Tables 4-6)**. This not only diminishes the statistical power of the multivariate models, but may also introduce bias. Secondly, with this being a cross-sectional study, the odds ratio measures do not bear the causal inference capabilities of more sophisticated observational study designs like case-control or cohort studies. Thirdly, a lack of potential confounding factors in the models, such as air pollution exposure or seasonality, may have resulted in biased measures of association.

Conclusions and future recommendations

In this cross-sectional study we used WGS to describe the serotype distribution, genetic lineages, antibiotic resistance profiles, and serotype invasive disease potential of 508 carriage *S. pneumoniae* isolates obtained from healthy children aged 2-24 months from Peru during the pre-PCV7 period (2007-2009). Additionally, this study aimed to identify risk factors associated with the carriage of multidrug-resistant *S. pneumoniae* isolates in children aged 2-24 months in Peru during the pre-PCV7 period. Among all carriage isolates, 52.6% had a PCV7 serotype, 40.4% were members of 1 of 10 identifiable PMEN clonal complexes, 40.9% were resistant to at least one class of antibiotics, 22.4% were resistant to at least three classes of antibiotics, 51.4% were resistant to TMP/SMX, 27.3% were resistant to β-lactams, and 21.5% had a serotype with a significantly high propensity to cause disease (6B and 14). Multivariate logistic regression determined that attending daycare, living in a highly urban city, and being a PCV7 serotype carrier were significant risk factors for multidrug-resistant *S. pneumoniae* carriage in children aged 2-24 months in Peru before PCV7 implementation. Despite concerns with missing data and controlling for certain confounding factors, our results show that the odds of young children becoming carriers of multidrug-resistant *S. pneumoniae* are higher in those exposed to chronic crowded conditions and being a vaccine-type carriers than those who are not.

From this study, there are several recommendations to improve future pneumococcal surveillance and further understand environmental factors that influence pneumococcal carriage, invasive pneumococcal disease (IPD), and drug resistance. For better insight into serotype disease potential and improved vaccine development, more studies are needed in evaluating IDP before and after introducing new conjugate vaccines in Peru. Future studies addressing this must make sure to obtain carriage and invasive-disease pneumococcal for both pre-vaccine and post-vaccine periods with a sufficiently large sample size capable of representing the diversity of serotypes in Peru. For studies seeking to further elucidate the role of crowded environments in carriage, IPD, and drug resistance, it would be of interest to further refine what constitutes a crowded environment. For example, crowding in the home could be evaluated by the number of people (or siblings) per room (or area) to quantify how close in proximity at-risk carriers are to others. Finally, the role of additional environmental factors in carriage, IPD, and drug resistance should be considered. Air pollution in particular should be further explored. Exposure to indoor air pollution from second-hand cigarette smoke or cooking methods that result in incomplete fuel combustion have shown to increase pneumonia risk in children under 5 years of age [63]. Additionally, frequent use of indoor wood cook stoves in the Peruvian Andes has shown to increase the incidence of viral respiratory infections in children, which is a risk factor for pneumococcal carriage [64-66]. The effect of air pollution on MDR pneumococcal carriage is unknown, but chronic exposure to respiratory pollutants may alter nasopharyngeal flora population dynamics in a way that alters resistance gene transfer and horizontal gene transfer in general.

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VI. TABLES AND FIGURES

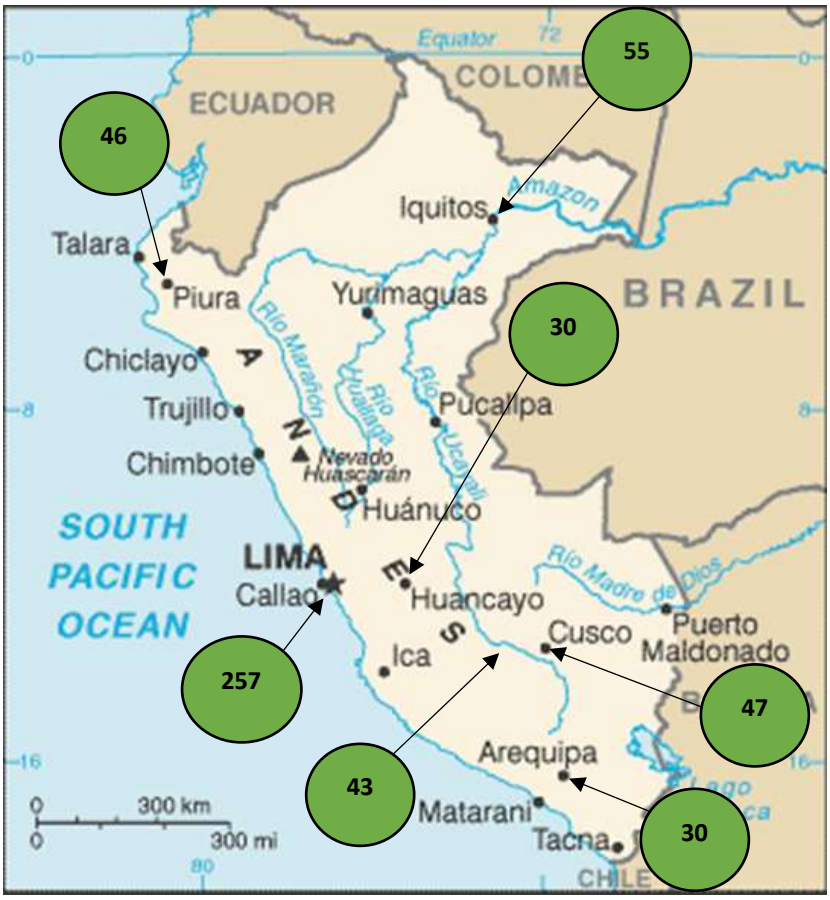

Figure 1. Frequencies of carriage isolates by city of isolation.

Table 1. Frequencies of serotypes and sequence-types associated with clonal complexes and PMEN clones. "Other" denotes singletons where n=1 and “NT” represents non-typeable isolates.

Clonal Complex	ST	ST Frequency (n)	ST Relative Frequency (%)	Serotype (n)
CC81 (PMEN1)	81	26	5.12	23F (15), 19F (11)
	7452	1	0.20	23F (1)
CC90 (PMEN2)	90	4	0.79	6B (4)
	1624	1	0.20	6B (1)
	7417	2	0.39	6A (1), 6B (1)
	7418	1	0.20	6A (1)
CC156 (PMEN3)	156	34	6.69	14 (26), 9V (6), 15B (1), 19F (1)
	7432	2	0.39	14 (2)
	7456	1	0.20	14 (1)
	379	1	0.20	14 (1)

CC439	439	5	0.98	23A (5)
(PMEN4)	5473	1	0.20	23A (1)
	42	1	0.20	23A (1)
	7461	1	0.20	23A (1)
CC1421	1421	20	3.94	19F (20)
(PMEN14)	236	6	1.18	19F (6)
	5676	5	0.98	19F (5)
	7445	4	0.79	19F (4)
	271	2	0.39	19F (2)
	320	2	0.39	19F (1), 19A (1)
	7444	2	0.39	19F (2)
	9906	2	0.39	19F (2)
	11396	2	0.39	19F (2)
	1428	1	0.20	19F (1)
	5677	1	0.20	19F (1)
	6141	1	0.20	19F (1)
	7443	1	0.20	19F (1)
	7446	1	0.20	19F (1)
	7459	1	0.20	19F (1)
	12185	1	0.20	19F (1)
	12186	1	0.20	19F (1)
	12189	1	0.20	19F (1)
	6585	1	0.20	16F (1)
CC242	242	23	4.53	23F (21), 23B (2)
(PMEN15)	240	1	0.20	19F (1)
	12405	1	0.20	23F (1)
	6200	1	0.20	23F (1)
	6344	1	0.20	23F (1)
CC646	646	19	3.74	19F (19)
(PMEN21)	7431	1	0.20	19F (1)
CC338	338	4	0.79	23A (4)
(PMEN26)	387	3	0.59	23B (3)
	2777	1	0.20	6C (1)
	1131	1	0.20	19A (1)
CC193	193	4	0.79	11A (3), 21 (1)
(PMEN30)	7436	1	0.20	21 (1)
	7435	1	0.20	21 (1)
	6444	1	0.20	11A (1)
CC276	276	5	0.98	19A (5)
(PMEN32)	230	1	0.20	24F (1)
CC5638	5638	7	1.38	NT (6), 23F (1)
	5636	11	2.17	NT (10), 19F (1)

	7458	1	0.20	NT (1)
	5639	2	0.39	NT (2)
	6564	1	0.20	NT (1)
	5637	1	0.20	NT (1)
CC5625	5625	9	1.77	6A (4), 6B (5)
	6348	6	1.18	6A (5), 6B (1)
	6148	2	0.39	6D (2)
	7457	1	0.20	6B (2)
	8008	1	0.20	6B (1)
CC5464	5464	4	0.79	6C (3), 6B (1)
	5471	2	0.39	6A (2)
	6731	1	0.20	6B (1)
	5467	1	0.20	6A (1)
CC7421	7421	1	0.20	6B (1)
	5461	3	0.59	15C (2), 15B (1)
	7454	1	0.20	6B (1)
CC15	15	3	0.59	14 (3)
	7448	1	0.20	14 (1)
	25	1	0.20	14 (1)
CC1902	1902	2	0.39	34 (2)
	12190	1	0.20	34 (1)
	7412	1	0.20	34 (1)
CC5466	5466	3	0.59	10F (3)
	7450	1	0.20	10F (1)
CC7441	7441	1	0.20	34 (1)
	6347	1	0.20	34 (1)
CC280	280	5	0.98	9V (5)
CC5449	5449	6	1.18	6B (6)
	7433	1	0.20	6B (1)
CC5472	5472	2	0.39	10A (2)
	7422	1	0.20	10A (1)
CC88	88	2	0.39	19F (2)
	87	1	0.20	19F (1)
CC4063	4063	4	0.79	11A (4)
	7414	1	0.20	11A (1)
CC1121	1121	10	1.97	6B (10)
	7415	1	0.20	6B (1)
CC5463	5463	3	0.59	23F (3)
	775	1	0.20	23B (1)
CC5470	5470	3	0.59	6C (3)
	8003	1	0.20	6C (1)

CC6139	6139	1	0.20	24F (1)
	5581	1	0.20	24F (1)
CC5468	5468	4	0.79	7C (4)
	8006	1	0.20	7C (1)
CC62	62	7	1.38	11A (7)
	3000	2	0.39	11A (2)
CC5448	5448	8	1.57	15A (8)
	5453	1	0.20	15A (1)
Singletons	Other	33	6.50	
	3669	17	3.35	15B (8), 15C (9)
	5628	12	2.36	6B (11), 6A (1)
	5593	8	1.57	13 (8)
	135	7	1.38	6B (7)
	1662	7	1.38	6B (7)
	2991	7	1.38	35F (7)
	1012	6	1.18	33A (3), 33F (2), 23F (1)
	5460	6	1.18	19A (5), 19F (1)
	5611	6	1.18	6C (6)
	5675	5	0.98	22F (2), 23A (3)
	5600	4	0.79	35F (4)
	7460	4	0.79	6A (4)
	315	3	0.59	6B (3)
	448	3	0.59	NT (3)
	1092	3	0.59	6B (3)
	2911	3	0.59	23B (3)
	5462	3	0.59	19F (3)
	5474	3	0.59	20 (3)
	7451	3	0.59	10F (3)
	66	2	0.39	19A (1), 9N (1)
	392	2	0.39	17F (2)
	5469	2	0.39	35A (2)
	6346	2	0.39	18B (1), 18C (1)
	7413	2	0.39	23F (2)
	7416	2	0.39	6B (2)
	7424	2	0.39	23B (2)
	7425	2	0.39	35B (2)
	7438	2	0.39	16F (2)
Missing Data		5	0.98	

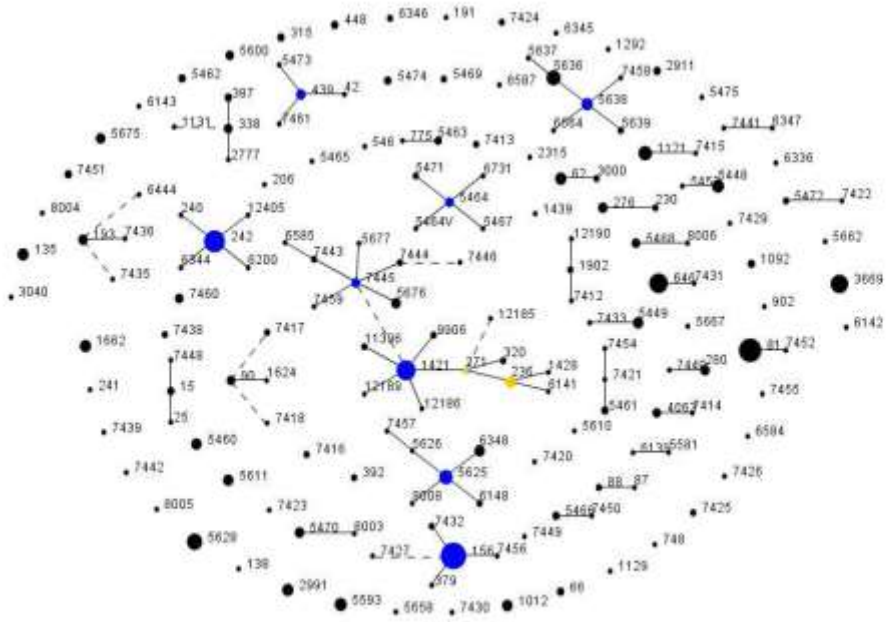

Figure 2. eBURST diagram of all identifiable clonal complexes. Solid lines connect single locus variants and dashed lines connect double locus variants. Dot size represents the number of carriage isolates that belong to each sequence type where larger dots = higher frequencies.

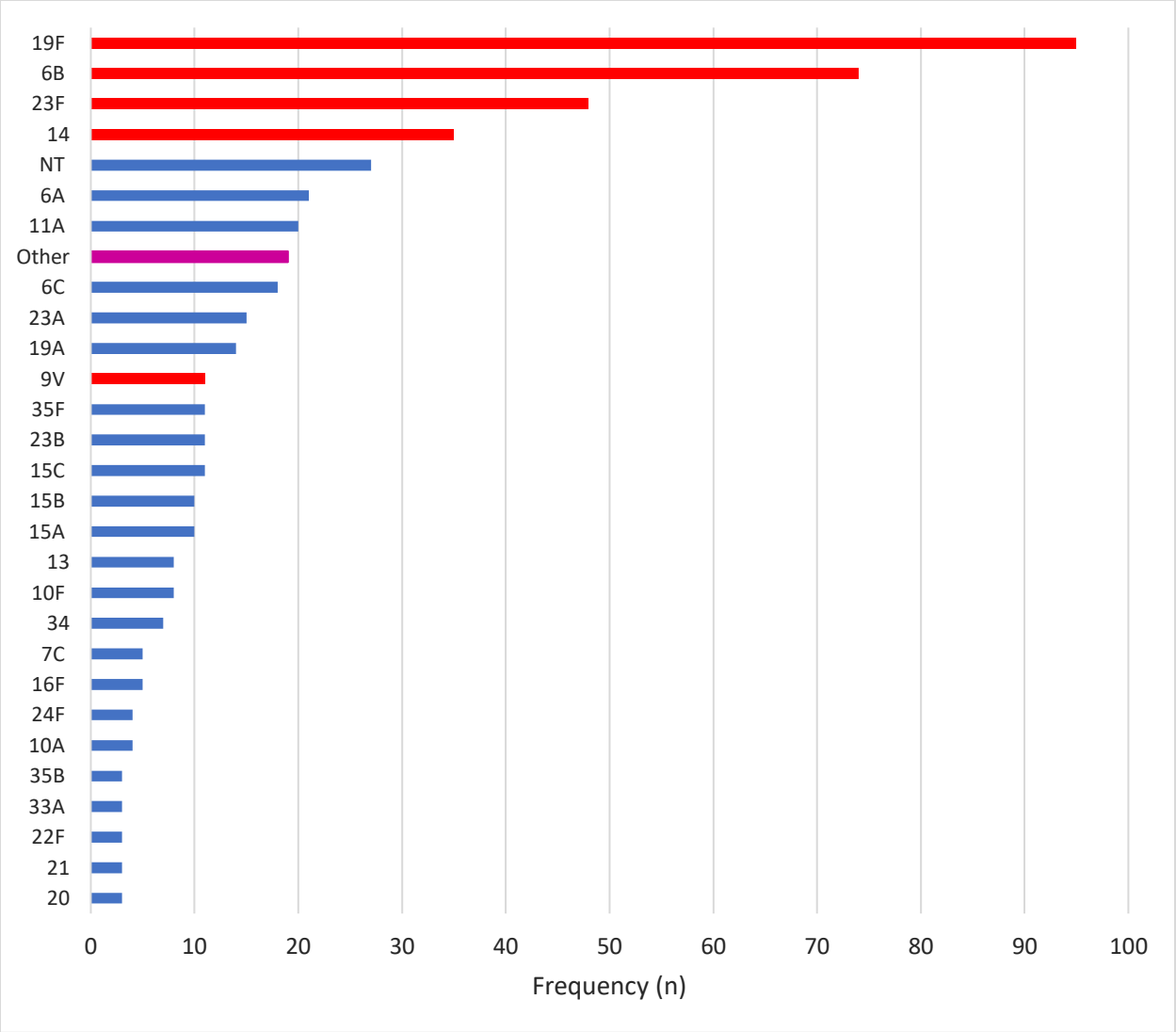

Figure 3. Carriage serotype frequencies. "Other" denotes serotypes where n ≤ 2 and "NT" represents non-typeable isolates.

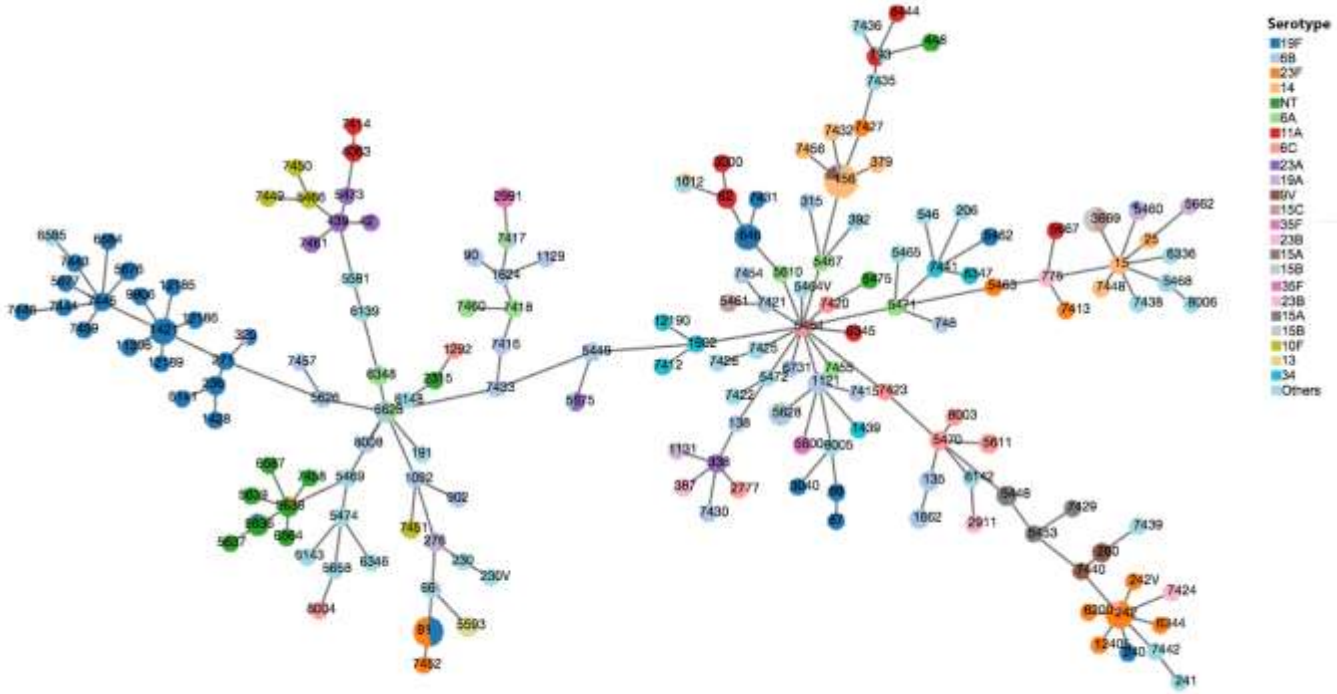

Figure 4. Minimum Spanning Tree representing evolutionary relationships between isolates, based on MLST data and associated serotypes; created with PhyloVIZ (http://www.phyloviz.net/).

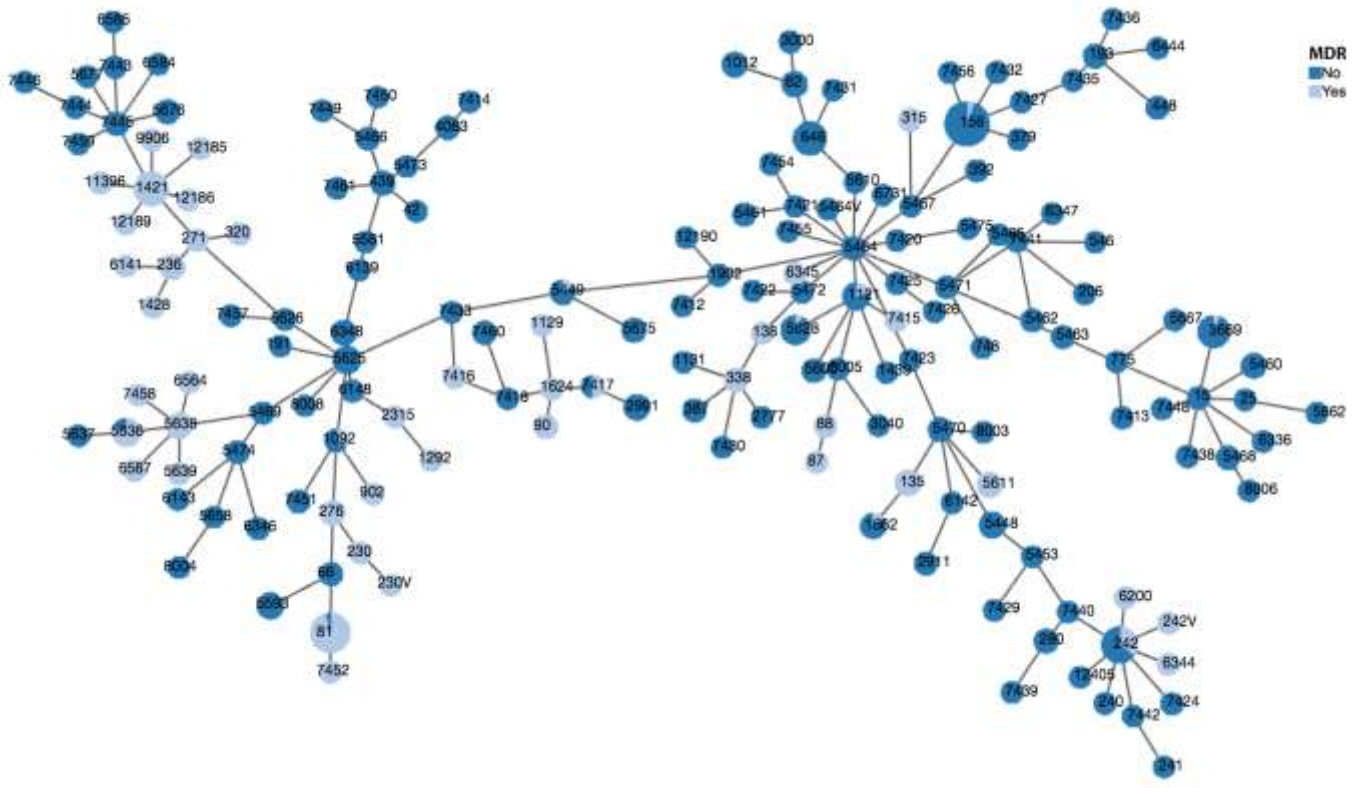

Figure 5. Minimum Spanning Tree representing evolutionary relationships between isolates, based on MLST data and associated multidrug resistance (MDR); created with PhyloVIZ (http://www.phyloviz.net/).

Table 2 . WGS antibiotic resistance prediction results

Resistance Class	**n (Relative %)**		
Non-resistant	186 (36.6%)		
Resistant (1-2 classes)	208 (40.9%)		
Multi-resistant (≥3 classes)	114 (22.4%)		
Antibiotic Class	**n Resistant (Relative %)**		
β-lactams	138 (27.3%)		
macrolides	125 (24.7%)		
lincosamides	68 (13.4%)		
amphenicols	43 (8.5%)		
aminoglycosides	3 (0.6%)		
rifamycins	4 (0.8%)		
tetracyclines	160 (31.6%)		
TMP/SMX	260 (51.4%)		
β-lactam	**n Intermediate (Relative %)**	**n Resistant (Relative %)**	**Predicted MIC50 and MIC90**
PEN	102 (20.2%)	138 (27.3%)	≤0.03 / 2.0
TAX	136 (26.9%)	1 (0.2%)	≤0.03 / 2.0
CFT	4 (0.8%)	5 (1.0%)	≤0.5 / 1.0
CFX	147 (29.1%)	0 (0%)	≤0.5 / >2.0
AMO	0 (0%)	12 (2.4%)	≤0.03 / 2.0
MER	122 (24.1%)	12 (2.4%)	≤0.06 / 0.5

PEN=penicillin; TAX=cefotaxime; CFT=ceftriaxone; CFX=cefuroxime; AMO=amoxicillin; MER=meropenem.

Table 3. Lima IDP analysis results

Serotype	**n Carriage**	**n Invasive**	**IDP**	**$CI_{95\%}$**
1	0	2		
12F	0	1		
14*	14	33	5.78	2.96-11.25
18F	0	1		
3	0	1		
38	0	3		
5	0	5		
6B*	31	27	1.87	1.06-3.29
7F	0	1		
10A	2	0		
10F	3	0		
11A	13	2	0.29	0.06-1.30
13	3	2	1.3	0.22-7.89
15A	8	2	0.48	0.10-2.29
15B	6	0		
15C	9	0		
16F	3	1	0.65	0.07-6.28
18B	1	0		

18C	1	2	3.94	0.35-43.84
19A	8	8	2.01	0.74-5.48
19F*	57	14	0.42	0.22-0.78
20	1	0		
21	1	0		
22F	1	0		
23A	12	1	0.16	0.02-1.21
23B	3	1	0.65	0.07-6.28
23F	25	9	0.68	0.31-1.50
24F	4	2	0.97	0.18-5.38
28A	1	0		
33A	2	0		
33B	1	0		
33F	1	0		
34	3	2	1.3	0.22-7.89
35A	1	0		
35B	1	0		
35F	5	0		
4	2	2	1.96	0.27-14.09
6A	8	3	0.72	0.19-2.78
6C	4	3	1.47	0.32-6.67
7C	1	1	1.95	0.12-31.50
9N	2	2	1.96	0.27-14.09
9V	4	2	0.97	0.18-5.38
NT	17	0		

** indicates IDP ≠ 1.0 at α=0.05*

Table 4. MDR risk factor univariate analyses

(a) Continuous variables							
Variable	**Observations (n)**	**Missing Data (n)**	**Mean**	**Median**	**SD**	**Min**	**Max**
Age (months)	508	0	11.89	12.0	6.0	2.0	24.0
log(people in the home)	347	161	1.44	1.39	0.52	0	2.94
(b) Binary variables							
Variable	**Yes (n)**	**No (n)**	**Missing Data (n)**				
PCV7 serotype	266	240	2				
Type 1 pilus	222	284	2				
Daycare attendance	11	333	164				
> 2 Sibling under 6yo at home	41	303	164				
Antibiotic use within 3mo of isolation	149	200	159				
Previous URI Hospitalization	80	261	167				
Male sex	268	240	0				
Highly urban city	287	221	0				

Table 5. Independent and multivariate risk factor analysis for MDR carriage

Variable	cOR	Wald $CI_{95\%}$	p-value	aOR	Wald $CI_{95\%}$	p-value
Type 1 pilus	2.77	1.80-4.27	<0.0001	1.40	0.75-2.61	0.294
PCV7 serotype	10.69	5.80-19.70	<0.0001	11.87	5.24-26.90	<0.0001
log(people in the home)	1.08	0.67-1.73	0.761	1.02	0.57-1.84	0.947
>2 Siblings at home	0.71	0.32-1.61	0.413	0.87	0.31-2.44	0.797
Daycare attendance	5.92	1.69-20.75	0.006	7.70	1.43-41.45	0.018
Antibiotic use within 3mo of isolation	1.53	0.93-2.49	0.092	0.88	0.48-1.61	0.681
Previous URI hospitalization	1.38	0.79-2.43	0.262	1.16	0.59-2.30	0.666
Age	1.04	1.00-1.07	0.049	1.01	0.96-1.06	0.858
Male vs Female sex	1.25	0.82-1.90	0.301	0.84	0.46-1.51	0.555
Highly urban vs. less urban	2.02	1.29-3.15	0.002	3.00	1.33-6.77	0.008

Table 6. Multivariate significant risk factors for MDR carriage

Variable	OR	Wald $CI_{95\%}$	p-value
PCV7 serotype	11.83	5.76-24.28	<0.0001
Daycare attendance	4.92	1.16-20.90	0.031
Highly urban vs. less urban	2.86	1.32-6.18	0.008

VII. APPENDIX

Appendix Table 1. CLSI pneumococcal β-lactam MIC cutoffs

β-lactam	Susceptible MIC (µg/mL)	Intermediate MIC (µg/mL)	Resistant MIC (µg/mL)
PEN	≤ 0.06	0.12-1.0	≥ 2.0
TAX	≤ 0.5	1.0	≥ 2.0
CFT	≤ 0.5	1.0	≥ 2.0
CFX	≤ 0.5	1.0	≥ 2.0
AMO	≤ 2.0	4.0	≥ 8.0
MER	≤ 0.25	0.5	≥ 1.0

PEN=penicillin; TAX=cefotaxime; CFT=ceftriaxone; CFX=cefuroxime; AMO=amoxicillin; MER=meropenem [52].