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Characterization of pneumococcal isolates before and after PCV7 vaccine introduction in Peruvian children

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An abstract of

A thesis submitted to the Faculty of the

Rollins School of Public Health of Emory University

in partial fulfillment of the requirements for the degree of

Master of Public Health

in Global Epidemiology

2014

Abstract

Characterization of pneumococcal isolates before and after PCV7 vaccine introduction in Peruvian children

By Caitlin Shay Law

Infection caused by *Streptococcus pneumoniae* is a major preventable global public health issue severely affecting young children, especially those in developing countries. The purpose of this project is to examine the effect of the rollout of the 7-valent pneumococcal conjugate vaccine (PCV7) on pneumococcal strains in response to vaccine introduction in developing countries, specifically isolates from Peru. This was done via comparison of serotypes and clonal complexes of 525 carriage isolates and 215 invasive isolates before and after vaccination.

We found a pre-PCV7 carriage rate of 24.73% (525/2123) and predominant serotypes for the carriage population were 19F (18.7%), followed by 6B (14.7%), 23F (9.1%), and 14 (6.9%). Antibiotic resistance was seen in mostly vaccine-type serotypes and 108 of 525 (20.6%) carriage isolates were multidrug resistant.Out of 214 isolates from cases of IPD, the predominant serotypes were 14 (20.9%), 6B (14.9%), and 19F (12.6%). Vaccine-type serotypes accounted for 66.95% of the invasive pneumococcal population. For the 2 years and under age group, the predominant serotype before PCV7 introduction was 14 and remained dominant after vaccination. In the over 2 age group, 23F was the predominant serotype before PCV introduction, and 19F became prevalent post-vaccination. Oxacillin non-susceptibility significantly changed ($p=0.001$) after vaccination in the under 2 age group, but there was no substantial change with resistance in any other antimicrobial class in either age group. Sequence type 156 was prevalent in both the carriage and invasive populations. 82 out of 208 (39.4%) isolates in the invasive population were global resistance-conferring PMEN clones, and 21.9% (114/521) of carriage isolates were PMEN clones.

Molecular epidemiology is an extremely useful tool for understanding population dynamics following the use of vaccines for control and prevention of *S. pneumoniae* infection. Future studies using whole genome sequencing would provide an increased level of sensitivity to further investigate these changes for vaccine evasion at a population level.

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Background

Infection caused by *Streptococcus pneumoniae* is major preventable global public health issue severely affecting young children, especially those in developing countries. *Streptococcus pneumoniae*, often referred to as "the pneumococcus," is a Gram-positive, extracellular, alpha-hemolytic, diplococcal pathogen responsible for some of the highest rates of morbidity and mortality worldwide (1). The pathogen is the leading cause of bacterial pneumonia, which drives an enormity of the disease's mortality, especially in the developing world (2). Globally, *S. pneumoniae* infections disproportionately affect children under 5 years of age. An enormous amount of cases of severe disease and death is seen in children under 2 years of age. In 2000, an estimated 14.5 million episodes of severe pneumococcal infections in this age group were diagnosed worldwide; around 826,000 of these were fatal (1). Currently in Latin America, it is estimated that 2 children die from pneumococcal infection per hour (3).

Host risk factors for invasive pneumococcal disease include age, nasopharyngeal carriage, respiratory virus co-infection, positive HIV status, immunodeficiencies, and presence of other chronic diseases (4). Environmental factors also pose risk for pneumococcal infection, such as winter season and crowding, while some evidence also points to attendance at day care centers as a risk factor in children under 5 (5). Pneumococcal infection is also more frequent in urban areas and low socioeconomic demographics (4).

The majority of pneumococcal disease manifests as pneumonia, meningitis, or bacteremia, although cases of otitis media, fasciitis, and peritonitis are often seen. Although the number of organisms required to generate infection or infectious dose is currently unknown given the bacteria's ability to be asymptomatic, pneumococcal disease can be acquired via droplet transmission and direct oral contact (4). Person-to-person transmission is also common, but severe illness from this pathway is infrequent (6). Once *S. pneumoniae* enters and colonizes the nasopharynx, it may remain in the nasal cavity as an asymptomatic infection or traverse the mucosal barrier and spread to other organs (Figure 1). A high percentage of people are asymptomatic carriers of *S. pneumoniae,* although nasopharyngeal carriage is a prerequisite for invasive pneumococcal disease (IPD) (4). Carriage is generally higher in children compared to other age groups (7). Current studies estimate up to 75% of children located in the Andes region of Latin America carry *S. pneumoniae* in their nasopharynx (8).

Severity of *S. pneumoniae* infection depends on a variety of virulence factors. Among these are surface proteins that enhance cell lysis and invasion, such as pneumolysin and autolysin. Perhaps the most important virulence factor is the polysaccharide capsule surrounding the bacterium. The capsule, which protects the bacterium from phagocytosis by host cells may be classified as any one of over 92 known serotypes, depending on its reaction with antibody (9). Evidence supports the involvement of the *S. pneumoniae* capsule in determining whether the strain is invasive or asymptomatic (10). The capsule may or may not be

expressed, which determines whether the colonies are smooth and transparent or rough and opaque. Smooth colonies express the capsule, whereas rough colonies do not. In experiments, these rough colonies have little to no virulence, confirming the role the capsule plays in severity of disease (11). Studies also show a higher incidence of certain serotypes involved in invasive disease, while others are implicated in mostly nasopharyngeal carriage (9, 12).

Another factor that contributes to the infectivity and virulence of *Streptococcus pneumoniae* is the ability to easily uptake genetic material from the environment and other bacterial species via the process of natural transformation. *S. pneumoniae* has an extensive 2.16 mbp genome capable of constant change (9). During the process of transformation, exogenous DNA in the environment of the bacterium is absorbed by the pneumococcus and integrated into its own DNA, leading to frequent genetic alterations, many of which are beneficial to the success of the pneumococcus. Through transformation, *S. pneumoniae* has acquired a variety of advantageous genes from bacteria such as *Escherichia coli* and *Staphylococcus aureus*. This process is credited not only with the increasing antimicrobial resistance of many pneumococcal strains, but also the ability to evade vaccine response.

Resistance to *S. pneumoniae* is widespread, and has been shown throughout the world, including South America (13, 14). Evidence historically points to an established association between serotype and resistance, with almost all resistance seen in serogroups 6, 9, 14, 19 and 23 (14, 15). The increased

prevalence of resistance globally may be attributed to the spread of the pediatric serotypes 6A, 6B, 9V, 14, 15A, 19F, 19A, and 23F, although a new study suggests a closer association between antibiotic resistance and genotype as opposed to serotype (16). Recent data from children under 16 with IPD in Peru, where our study lays its focus, shows high resistance rates for multiple antibiotics, particularly in serotypes 14, 6B, 19F, 19A, and 23F (3).

Current evidence shows that, while effective against most cases of invasive pneumococcal disease (IPD), the pneumococcal conjugate vaccines (PCV) have forced the *S. pneumoniae* population to evolve genetically (17). Upon success of the PCV to eliminate the invasive vaccine-type serotypes, less infective serotypes have filled the open niche in the nasopharynx and became able to cause IPD, most likely due to the process of capsular switching (18). Genetic material taken in by the pneumococcus allows the bacteria to change expression of the capsular polysaccharide, effectively altering the serotype, and enhancing or decreasing virulence. Strains that possess this capability are called 'vaccine escape variants.'

The aforementioned genetic processes can be and are currently being monitored through molecular surveillance on both a country and global-level scale. Multilocus sequence type (MLST) analyses are used in order to determine sequence types based on allelic variations in seven housekeeping genes and track the molecular patterns of the disease (19). Clonal complex (CC) analyses are also used to map the linkages between these sequence types, giving researchers a more comprehensible picture of the evolution of the multiple strains of the

pathogen. Antimicrobial resistance is also monitored through many of these surveillance programs, as resistance of the pneumococcus has significantly increased due in part to the spread of multidrug resistant clones. More recent efforts are being undertaken to evaluate pneumococcal strains through whole genome sequencing as well, which will ultimately produce a thorough representation of the molecular patterns of the pneumococcal population.

Surveillance has shown that the PMEN1 clone in particular is a major driver of *S. pneumoniae* antimicrobial resistance (14). Commonly circulating as a serotype 23F, multilocus sequence type 81 clone, PMEN1 belongs to clonal complex 81, which features substantial genetic diversity. Although this serotype is included in the pneumococcal conjugate vaccine formulation, this genetic diversity implies that the clone is capable of rapid genetic evolution, contributing to its multidrug resistance (2). This capability has been shown to also contribute to the increasing antimicrobial resistance of other clones, again due to natural transformation.

The 7-valent pneumococcal conjugate (PCV7) was licensed in 2001, but introduced into the routine childhood vaccination schedule in Peru on 1 January 2009 at 2, 4, and 12-month dosing intervals (20). In 2006, post-licensure but before introduction into the national vaccination schedule, the predominant serotypes in Lima, Peru were 14, 23F, 6B, 6A, 5, 19F, 4, 9N, 11A, and 15A in children under 6 years of age (20). The major serotypes particularly for invasive disease were 14, 6B, 19F, 5, and 23F for the pediatric population under 14 years (20). Previous studies from Lima, Peru report different results for the dominant

serotype, depending on the time period, geographic region, and resistance profile. One study reports 23F as the most prevalent antimicrobial resistant serotype, while another describes 14 as the predominant invasive serotype in children, which corroborates the previously mentioned study (21, 3). Another indicates serotype 19 as the predominant nasopharyngeal carriage serotype under 2 years of age before introduction of PCV7 to the vaccination schedule (22).

PCV7 was originally formulated for the most prevalent pediatric pneumococcal serotypes in the United States: 4, 6B, 9V, 14, 18C, 19F, and 23F (23). These particular vaccine-type (VT) serotypes are not necessarily the most prevalent and invasive serotypes in other countries, although based on previous reports, they cover most of the established prevalent serotypes in Peru. There is limited data from Peru, but recent estimated vaccine coverage as of 2010 in Peru is 56-73% (3).

This investigation evaluates 215 random invasive and 525 carriage isolates of *S. pneumoniae* from various individuals in Peru. The invasive isolates were collected from 2006 to 2011, allowing us to determine changes in the pneumococcal population before and after introduction of PCV7 to the national vaccination schedule. Data regarding the serotype prevalence of IPD are essential to inform policy around the use of pneumococcal conjugate vaccines. Information that aids in understanding the genetic background of pneumococci associated with IPD is also important, as the invasive potential of individual clones to succeed and spread in a population is associated with genotype. Carriage isolates

evaluated in this study were only obtained in the period before vaccine introduction, but data extracted from these isolates serves as both a baseline prevalence of the pneumococcal population in the area as well as a comparison to the invasive isolate population before vaccine introduction. Some antimicrobial resistance values were obtained via the disk diffusion method. This data is also evaluated in order to further characterize the pneumococcal population in Peru and help address the increasing problem of pneumococcal resistance. This study aims to contribute to limited knowledge on serotype and genotype of isolates from Peru and to provide information on vaccine coverage and circulating clonal types, pre- and post-vaccine introduction.

Methods

Hypotheses

Using data obtained from a cross-sectional surveillance study in Peru, we will be evaluating the following hypothesis questions:

(A) Is a change in pneumococcal serotypes seen in invasive disease 2 years after PCV7 rollout in Peru?

(B) Does the clonal structure change after vaccine introduction?

(C) Do clones of vaccine serotypes identified before vaccine introduction persist through the acquisition of non-VT capsules by capsular switching?

(D) Do patterns of resistance change after vaccination?

(E) Are their differences in serotype and genotype between invasive and carriage isolates pre-PCV7 introduction?

Since the majority of this study is descriptive, most data is presented as percentages. We assume the proportion of vaccine-type serotypes will be significantly reduced in the invasive population after the rollout of the 7-valent pneumococcal conjugate vaccine, as well as the clonal structure. Chi-square analyses are used to compare changes in proportion of serotypes before and after vaccine introduction. Clonal variation of the invasive population is assessed mainly by visualizing the clonal structures in clonal complex generation software. Resistance is associated with vaccine-type serotypes, as they are targets of the PCV and should evolve under vaccine pressure. Modifications in antimicrobial and multidrug resistance patterns before and after vaccination in the invasive

population were examined similarly to serotype changes, also using Chi-square or Fisher exact analyses to determine any statistical differences.

Isolates

Strains included in the project were acquired through the Global Strain Bank as part of a previous Gates Foundation-funded PATH proposal at the Centers for Disease Control and Prevention and Emory University. 215 strains from invasive pneumococcal disease (IPD) in children under 16 years of age from a passive surveillance study in 16 public and private hospitals in Peru from 2006 to 2010 were analyzed. Included with the serotype, MLST, and resistance data for these strains were demographic and other clinical variables: age, gender, clinical manifestation, and year of isolation. Nasopharyngeal swab samples were collected from 2,123 healthy children 2 to 24 months of age in pediatric hospitals and medical centers in 7 cities in Peru from 2007 to 2009, contributing the 525 pneumococcal carriage isolates available for inclusion in the study. No additional demographic data were obtained for carriage isolates. Bacterial strains were checked for purity and screened by optochin susceptibility and bile solubility. Isolates were then subcultured onto 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, MD) and incubated overnight at 35°C with 5% CO_2 prior to DNA extraction and serotyping.

DNA Extraction

Extractions were completed using the QIAmp DNA Mini kit or DNeasy Blood and Tissue kit (Qiagen, West Sussex, United Kingdom) per the manufacturer's instructions with a pre-lysis step. Final DNA concentrations were obtained using Qubit Assay kit (Life Technologies, Carlsbad, CA).

Serotyping and Molecular Typing

Serotype was previously determined by Quellung reaction. MLST analyses were conducted using a method described by Enright and Spratt, with modified primers detailed at http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm (19). An allelic profile of each strain was determined by PCR and sequencing each of 7 housekeeping genes, aroe, gdh, gki, recp, spi, xpt, and ddl. Allele number and sequence types (STs) were assigned using the pneumococcal MLST web site (http://spneumoniae.mlst.net/) (24). Strains with identical profiles or varying at a single locus were considered related.

Statistical and Clonal Complex Analysis

Collected data was imported to SAS 9.3 (Cary, NC). Data was analyzed for descriptive statistics. The invasive population was stratified into two age groups: 2 years and under and over 2, based on the high burden of disease in the children under 2. We further divided these groups into 2 time periods: pre-vaccine (2006-2009) and post-vaccine (2010-2011), representing the introduction of PCV7 into the Peruvian national vaccine schedule. Since 2009 was the first year of nation-wide vaccination in a 3-dose series at 2, 6, and 12-month intervals, we assumed

the pneumococcal population had not begun to concede to evolutionary pressure in this year and included strains isolated in 2009 in the “pre-vaccine” group. For this study, vaccine-types refer to those serotypes in PCV7, i.e. 4, 6B, 9V, 18C, 19F and 23F; non-vaccine types refer to all serotypes other than those in PCV7. Sequence type and MLST data were imported to eBURSTv3 program (Imperial College London) and analyzed to determine clonal complex group and group linkages (25).

Antimicrobial Resistance

Resistance profiles were determined by disc diffusion method. Antibiotics tested were: oxacillin, erythromycin, tetracycline, and chloramphenicol. Each of these represents a different class of antimicrobial, resulting in a more complete profile of resistance. After growth of isolates with various antibiotic discs, zones of clearing were measured and non-susceptibility was determined using Clinical and Laboratory Standards Institute 2013 cut points (Appendix B) (26). Multidrug resistance was determined based on resistance to 3 or more antimicrobials in the screen.

Results

Serotypes among carriage and IPD isolates

We found a pre-PCV7 carriage rate of 24.73% (525/2123). Of the 525 samples, 274 (52.2%) were PCV7 vaccine-type serotypes. We identified a total of 40 serotypes. The predominant serotypes for the carriage population were 19F (18.7%), followed by 6B (14.7%), 23F (9.1%), and 14 (6.9%) (Figure 2).

Since carriage isolates were collected prior to nation-wide PCV7 introduction, we were able to compare the carriage population to the population of invasive isolates also collected before vaccine introduction (Table 1). We found a significant difference between the distributions of vaccine-type and non-vaccine-type serotypes ($p=0.003$).

One isolate from the invasive group was omitted from analysis due to missing data. Of 214 isolates from cases of IPD, we determined 35 different serotypes. The predominant serotypes were 14 (20.9%), 6B (14.9%), and 19F (12.6%) (Figure 3). 130 (60.5%) of these isolates were vaccine-type serotypes (Figure 3). 81.4% of the isolates were from children 2 years of age and younger and 56.7% were males (Table 2). Pneumonia was the leading clinical manifestation, presenting in 44.7% of cases, followed by meningitis (29.8%) (Table 3). Invasive isolates were stratified by both age and vaccine introduction time period for further analysis.

In the 2 years and under age group, the predominant serotype before PCV7 introduction was 14 (26.5%), followed by 6B (20.7%), 19F (9.9%), 19A (6.6%), and 23F (5.8%) (Figure 4). Vaccine-type serotypes accounted for 66.95% of the invasive pneumococcal population. During the time period examined after PCV7 introduction (2010-2011), 14 (20.8%) remained the predominant serotype in this age group, followed by 19F (15.1%), 19A (9.5%), and 6B, 23F, and 10A (7.6%) (Figure 4). Only 3 serotypes showed statistically significant changes in distribution before and after vaccine introduction: 6B (p=0.03, 13.1% reduction), 3 (p=0.03, 3.8% increase), and 10A (p=0.002, 7.6% increase). Four new non-vaccine serotypes were seen following PCV7 introduction: 3, 7C, 10A, and 35F.

The serotype distribution was slightly different in the over 2 years age group, with the predominant serotypes before PCV introduction being 23F (21.4%), 6B, 6C, and 19F, (14.3%) (Figure 5). Vaccine-type serotypes accounted for 64.3% of the population. Post-vaccine introduction, 19F (19.2%) became the prevailing serotype, followed by 23F, 15B, 15C, and 16F (7.7%), and fourteen new non-vaccine type serotypes were seen: 3, 6A, 6C, 12F, 15B, 15C, 16F, 19A, 19B, 23A, 24F, 35F, 38, and 39. However, there was no statistically significant change in any serotype. Vaccine-type serotypes accounted for 34.6% of the population. We also tested to determine changes in the vaccine-type group as a whole before and after vaccination within both age groups, but neither age group showed a statistically significant change in vaccine types.

Antimicrobial susceptibility and multidrug resistance

Oxacillin non-susceptible isolates comprised 48.7% of the carriage population, while 42.3% of isolates were tetracycline-resistant (Table 3). Table 3 also shows that only 22.5% and 4.8% of isolates were erythromycin and chloramphenicol resistant, respectively. Oxacillin non-susceptibility was seen mostly in vaccine-type serotypes (74.7%), and predominantly in serotypes 19F (29.2%) and 6B (21.4%). Similarly, resistance to erythromycin (63.6%), tetracycline (61.7%), and chloramphenicol (100%) was primarily vaccine-type serotypes. 108 of 525 (20.6%) carriage isolates were multidrug resistant, driven by resistance in serotypes 19F (48.1%), 23F (17.6%) and 6B (16.7%).

Antimicrobial susceptibility in the invasive population overall was high, although oxacillin non-susceptibility exceeded 50% (68.8%) (Table 4). Overall, most oxacillin non-susceptibility and erythromycin resistance were serotype 14, while tetracycline and chloramphenicol resistant isolates were 6B and 19F. Resistance was a notable problem, however, with vaccine-type serotypes in both age groups. For the 2 years and younger age group, 83.1% of oxacillin non-susceptible isolates, 80.7% of erythromycin resistant isolates, 75.7% of tetracycline resistant isolates, and 100% of chloramphenicol resistant isolates were vaccine type (Table 5). Post-vaccination, oxacillin non-susceptibility significantly changed from 83.1% to 61.9% ($p=0.001$), a 21.2% reduction, while there was no substantial change with resistance in any other antimicrobial class. For children over 2 years, vaccine type resistance was over 50% for each antimicrobial class prior to

vaccination. Although resistance in vaccine types was reduced for all but one antimicrobial (chloramphenicol), none were significantly reduced.

Multidrug resistance seemed to be an issue only in the pre-vaccine invasive population. Out of 214 isolates with resistance data, 31 (14.5%) showed resistance or non-susceptibility to 3 or more antimicrobials. 29 (21.5%) of these multidrug resistant isolates were in the pre-vaccine population (N=135), while only 2 (2.5%) were in the post-vaccination population (N=79), resulting in a 19% reduction in proportion of multidrug resistant isolates. Chi-square analysis showed a significant difference ($p<0.001$) between these proportions. Further investigation into multidrug resistance led to a comparison of multidrug resistance proportions in carriage and invasive isolates, but this was not statistically significant.

Population genetic structure

Of 208 invasive isolates available for clonal complex analysis with complete MLST data, 79 different STs were observed, with 10 new alleles and 40 new STs identified. 82 (39.4%) belonged to one of the 43 described PMEN clones. The predominant sequence type was ST156. 38 isolates belonged to this ST group, with 37 of these presenting as serotype 14, and one was serotype 23F (Table 6). 521 carriage isolates were available for clonal complex analysis. 170 different STs were observed, with 41 new alleles 113 new STs identified. 21.9% (114/521) were PMEN clones. Dominant sequence types for the carriage group were ST156, 81, 1421, 242. 26 of the 27 ST156 isolates were serotype 14, and two were serotype 15B and 19F. Serotypes represented by these other STs were 19F (81, 1421), 23B

(242), and 23F (81, 242) (Table 7). Figure 6 shows an eBURST comparison between the carriage population and the invasive population prior to PCV introduction. This conveys that the bulk of this study population belongs to 5 main sequence types: ST156, 81, 242, 1421, and 646, three of which are global PMEN clones, except ST1421 and 646 (Figure 6). Figure 7 illustrates a similar comparison between the pre and post-PCV7 invasive population, which further supports that the majority of invasive clones belong to ST156 and 81 (Figure 7).

Discussion

Vaccine evasion mechanisms, restricted access to vaccines, and lack of data from developing countries are just a few of the many barriers to effective pneumococcal control that currently exist. The ongoing development of pneumococcal vaccines covering an increasing number of serotypes is a start at addressing the pneumococcal disease problem, but varied geographic distribution of serotypes, minimal data from countries with high pneumococcal burden, and especially limited vaccine availability continue to impede progress towards effective control and prevention. The purpose of this study was to assess serotype prevalence and genetic structure of pneumococcal isolates from children in Peru and to evaluate the effects of vaccination on this population and its antimicrobial susceptibilities. The available data from Peru allowed us to examine the evolution of strains at a molecular level in a defined pneumococcal population and to contribute to limited data from this region of the world.

Previously, serotypes 14, 6B, 19F, 5, and 23F were described as the dominating pre-PCV invasive serotypes in the pediatric population of Peru (3, 20). The dominant serotypes for invasive isolates in this study were 14 (20.9%), 6B (14.9%), and 19F (12.6%), confirming the previous data. Contrary to previous studies, we did not see a significant reduction in vaccine-type invasive disease overall in the two years after the introduction of the pneumococcal conjugate vaccine, although the majority of other studies evaluate a longer post-vaccination time period (27-29). There are currently no data regarding vaccine uptake in Peru, but low uptake could also be a contributor to the non-significant overall

reduction two years post vaccine introduction. We did, however see a 13.1% reduction in vaccine type serotype 6B in the 2 years old and younger group, which was statistically significant (p=0.003).

Potentially supporting the theory of capsular switching and non-vaccine types filling open niches after vaccine introduction, we also saw significant increases in 2 non-vaccine type serotypes, 3 and 10A, in the 2 years and younger age group. Additionally, 14 non-vaccine type serotype strains were isolated from invasive disease cases after vaccination in children above 2 years. We also showed a significant difference in distribution of vaccine type serotypes between invasive isolates and carriage isolates, with more PCV types among IPD cases, and this was expected since PCV was formulated specifically for serotypes causing the majority of invasive disease (23). There were also far more cases of IPD in the 2 years and younger group (N=174) than the over 2 age group (N=40), as well as more cases in males (N=122) than females (N=83), both of which are expected (3, 4, 9). Interestingly, little to no data regarding *S. pneumoniae* peritonitis exist; we found 9 cases of peritonitis in our population, caused by multiple serotypes: 3, 6A, 10A, 15C, 19F (2), 23F, 24F, and 25F.

Antimicrobial resistance is also a byproduct of natural transformation, the same mechanism by which capsular switching operates. Historically, surveillance of resistance in carriage isolates can be used to predict resistance in invasive populations in the same defined geographic area (30). Our carriage resistance is an excellent indicator for erythromycin and chloramphenicol resistance in the

invasive population before PCV introduction, as these rates are similar. Carriage resistance rates for oxacillin and tetracycline, however, are lower than in the invasive population pre-PCV7, but might be an acceptable conservative comparison. Resistance rates have been found to be higher in vaccine-types, and the high rates of resistance in vaccine-type invasive isolates from this study were confirmatory (2). Some studies have shown an overall increase in penicillin non-susceptibility after PCV introduction (28, 31, 32). However, studies evaluating penicillin non-susceptible pneumococci (PNSP) in children in short time intervals found an initial decrease in PNSP with the introduction, followed by a significant increase (23, 33). This suggests that there will most likely be a surge of resistance in invasive disease in Peru in the future. The significant reduction of 19% in multidrug resistance in the invasive population post-PCV ($p<0.001$) could be an indication that the vaccine is effective in reducing multidrug resistant types (23).

Genetic diversity in the carriage strains seems to be higher than the invasive strains, as not many of the invasive strains were linked to each other. Children, especially those under 5, have a higher prevalence and length of nasopharyngeal carriage; since this is where the majority of genetic exchange occurs, this supports previous findings (12, 34-36). The resistance data shows that most antibiotic resistance originates from vaccine type serotype isolates, most of which belong to the dominant PMEN sequence type clones. As mentioned in the background, PMEN clones have been identified as global contributors to antimicrobial resistance, which also seems to be supported by this study (2, 14). A

capsular switch is indicated by isolates of different serotypes within the same genotype (ST) (11). Based on this information, only one capsular switch event is evident in the invasive population 2 years out of vaccine introduction; pre-PCV7, ST5673 was represented by serotype 9N, while post-PCV7, it was represented by serotype 16F (Table 8). However, these serotypes are both non-PCV types, which suggests this particular switch is not necessarily due to vaccine pressure, which would have been expected (11, 12). In addition, there were 4 STs related to vaccine-type serotypes pre-PCV7 with serotypes that disappeared post-PCV7: ST66 (9N) ST156 (9V, 23F), ST5475 (19F), and ST5625 (6A) (Table 8).

Strengths and Weaknesses

Weaknesses

A few limitations were encountered in this investigation. Stratifying the IPD cases by both age and year of PCV introduction resulted in small sample sizes, which could negatively affect the statistical power of the analyses. Data in this study were also only collected for 2 years after the pneumococcal conjugate vaccine was fully integrated into the Peruvian national vaccine schedule. Although we can still see changes, we may be able to see a more marked reduction in vaccine-type invasive disease with continued surveillance data.

Our antimicrobial resistance data was obtained via disc diffusion. A limitation of this method is that it does not provide a defined minimum inhibitory concentration (MIC) breakpoint. We also used the oxacillin disc screen to infer penicillin non-susceptibility. However, CLSI guidelines requires an MIC to be determined for those isolates with oxacillin zone diameter of $\leq$19mm because zones of $\leq$19mm occur with penicillin-resistant, intermediate, or certain susceptible strains. So, in this study, the penicillin resistance prevalence as defined by oxacillin zones $\leq$19mm may have been higher than the true prevalence if MICs were determined to penicillin.

For carriage analyses we only had pre-PCV7 isolates available and this limited our comparisons for determining impact of vaccination on carriage. These isolates were included to determine serotype and genotype differences that might be occurring within children in Peru to better understand the pneumococcal

population within both children presenting to hospitals and children with IPD. This initial carriage data will provide valuable baseline information for future post-PCV carriage studies in Peru.

Strengths

For this study and analyses, we were able to include both a collection of isolates from IPD in children within Peru as well as isolates from a carriage study from children presenting to hospitals within the same time period. This allowed for at least a comparison between circulating serotypes within the community and those causing severe disease within children. The carriage study will provide baseline data for continued surveillance for impact of vaccine on carriage. Comparisons of serotype in IPD strains allowed for generation of valuable baseline data prior to vaccine introduction and two years post PCV introduction. This will assist in the continued monitoring of vaccine impact in IPD in Peru.

There is limited data from Peru on serotype prevalence and no published data on genetic structure of pneumococci. This study included MLST and clonal complex analyses and provided a snapshot of the existing circulating clones within Peru. This will allow for continued analyses to monitor characteristics of specific successful clones that might persist through vaccination.

Future Directions

Molecular technologies are becoming increasingly advanced and more readily available. These advanced technologies are an important tool for understanding the rapid evolution and adaptations that *S. pneumoniae* possess. The evolution of the pneumococcal population can be more rapidly identified and studied with the use of whole genome sequencing. All isolates from this study have now been whole genome sequenced through a continued funded project to sequence 20,000 pneumococcal genomes to study changes associated with antibiotic use and vaccine introduction in both the developed and developing world. Continued surveillance in Peru and ongoing collaborations with investigators will allow for further monitoring of vaccine impacts on both serotype disease and carriage changes within children in this country post vaccine introduction.

Additionally, PCV7 is no longer manufactured, having been replaced by PCV10 and PCV13, both of which cover more serotypes, and are thus more efficacious. The introduction of PCVs is relatively recent in Peru, emphasizing the need for increased access to life-saving vaccines. Programs such as those administered by the Gates Foundation and the International Vaccine Access Center (IVAC) are making access to these newer formulations easier for developing countries. PCV10 was officially introduced to the national immunization program (NIP) of Peru in 2011, but the effects of this recent introduction have not yet been evaluated (Teresa J Ochoa, University of Texas School of Public Health-Houston, “personal communication,” 2014).

Continued pneumococcal surveillance is required for managing the ever-changing *S. pneumoniae* population. As the pneumococcal vaccines provide coverage against the previously dominant disease-causing strains, strains that were previously unable to cause disease have acquired the ability. Thus far, this has proved to be one of many burdens in pneumococcal control. However, other studies similar to the one conducted can add to the known molecular epidemiology of the pneumococcus and assist in better control and prevention of the disease.

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Tables

Table 1. Comparison of vaccine-type serotypes between invasive and carraige populations before PCV7 rollout in Peru (N=660)					
	VT serotypes		**Non-VT Serotypes**		**p-value***
	N	%	N	%	
Carriage	274	52.2	251	47.8	0.0026
Invasive	90	66.7	45	33.3	
*based on Chi-square analysis to compare differences between proportions, alpha=0.05					

Table 2. Characteristics of invasive pneumococcal disease (IPD) bacterial isolates from Peru (N=215)		
VARIABLE	**N**	**%**
Age		
≤2	175	81.4
>2	40	18.6
Sex		
Female	83	38.6
Male	122	56.7
Not Specified	10	4.7
Clinical Manifestation		
Fascitis	1	0.45
Sepsis	7	3.3
Bacteremia	22	10.2
Meningitis	64	29.8
Other	5	2.3
Peritonitis	9	4.2
Pneumonia	96	44.7
Septic arthritis	2	0.9
Unknown	9	4.2
Vaccine-Type Serotype		
	130	60.5

Table 3. Antimicrobial susceptibility by disc diffusion in 525 carriage strains of *S. pneumoniae,* Peru

	Susceptible		Intermediate		Resistant*	
Antibiotic	N	%	N	%	N	%
Oxacillin	268	51.05	-	-	257	48.95
Erythromycin	397	75.62	10	1.9	118	22.48
Tetracycline	116	22.1	187	35.62	222	42.29
Chloramphenicol	479	91.24	21	4	25	4.76

*Oxacillin non-susceptibilty is referred to as 'resistance' in this table

Table 4. Antimicrobial susceptibility by disc diffusion in 214 invasive strains of *S. pneumoniae*, Peru.

	Susceptible		Intermediate		Resistant*	
Antibiotic	N	%	N	%	N	%
Oxacillin	67	31.16	-	-	148	68.84
Erythromycin	150	69.77	3	1.4	62	28.84
Tetracycline	79	36.74	65	30.23	71	33.02
Chloramphenicol	193	89.77	4	1.86	18	8.37

*Oxacillin non-susceptibilty is referred to as 'resistance' in this table

Table 5. Percentage of vaccine-type serotype invasive isolates by resistance and comparison of resistant proportions

		Pre-PCV7			Post-PCV7			p-value**
Age Group	**Antimicrobial**	**S**	**I**	**R***	**S**	**I**	**R***	
≤2	Oxacillin	38.6	-	83.1	45.5	-	61.9	0.001***
	Erythromycin	62.2	0	80.7	51.5	33.3	76.5	0.733
	Tetracycline	58.4	75.0	75.7	50.0	58.3	63.2	0.326
	Chloramphenicol	64.0	0	100.0	57.5	0	100.0	0.000
>2	Oxacillin	50.0	0	70.0	25.0	0	38.9	0.237
	Erythromycin	70.0	0	50.0	25.0	0	50.0	1.000
	Tetracycline	71.5	0	100.0	0	7.1	72.7	0.517
	Chloramphenicol	58.3	0	100.0	27.3	50.0	100.0	0.000

*Oxacillin is considered 'non-susceptible' as opposed to 'resistant', but refered to as 'resistant' here

**Based on Chi-square tests for differences between *resistant* proportions

***Significant at alpha=0.05

Table 6. Sequence type by serotype for 215 invasive *S. pneumoniae* isolates, Peru	
Serotype	**Sequence Type**
1	615 (3)
3	505 (2), unknown (1)
4	206 (2), 5465* (2)
5	289 (8)
6A	273 (1), 1876 (1), 5623* (1), 5635* (1)
6B	90 (6), 135 (3), 315 (2), 1121 (3), 1624 (1), 1662 (1), 2757 (1), 4720 (1), 5449* (4), 5450* (1), 5619* (1), 5625* (6), 5626* (1), 5628* (1)
6C	1292 (5)
7C	5468* (1), 6809* (1)
7F	5455* (1)
9N	66 (2), 5616* (1), 5673* (1)
9V	156 (1), 280 (3)
10A	5472* (3), unknown* (1)
11A	62 (1), 193 (1), 4063* (1)
12F	218 (4)
13	5593* (1)
14	15 (3), 25 (2), 156 (36), 5458* (1), 6144* (1), 7432* (1), unknown*(1)
15A	5448* (1), 5453* (1)
15B	3669 (2)
15C	3669 (2)
16F	5673* (1), 6149 (2)
18C	5451* (1), 6143* (1)
18F	5456* (1)
19A	66 (2), 276 (2), 320 (4), 1131 (2), 5452* (1), 5454* (1), 5460* (1), 6048* (1)
19B	199 (1)
19F	81 (3), 646 (3), 1203 (1), 1421 (14), 1591 (1), 5459* (1), 5676* (1), 7132* (1), unknown (2)
23A	338 (1), 439 (1), unknown* (1)
23B	6140* (1)
23F	81 (5), 156 (1), 242 (9), 5475* (1)
24F	5581* (1), 6139* (1), 6741* (1)
28A	494 (1)
34	1902 (1), 5447* (1), 5457* (1)
35F	2991 (1), 5600* (1)
38	5475* (3)
39	unknown* (1)
NT	5593* (1)

*Novel sequence types

Table 7. Sequence type by serotype in 525 carriage *S. pneumoniae* isolates, Peru

Serotype	**Sequence Types**
4	206 (1), 5465* (1)
6A	273 (1), 5467* (1), 5471* (2), 5610* (1), 5625* (5), 5628* (1), 6348* (5), 7417* (1), 7418* (1), 7455* (1), 7460* (2)
6B	90 (5), 135 (7), 138 (1), 146 (1), 315 (3), 748 (1), 1092 (3), 1121 (9), 1624 (1), 1662 (7), 2757 (1), 5449* (5), 5464* (1), 5625* (5), 5626* (1), 5628* (12), 6348* (1), 6731* (1), 7415* (1), 7416* (2), 7417* (1), 7419* (1), 7421* (1), 7430* (1), 7433* (1), 7434* (1), 7454* (1), 7457* (1), 8008* (1)
6C	1292 (1), 2777 (1), 5464* (3), 5470* (5), 5611* (6), 7423* (1), 8003* (1), 8004* (1), unknown (1)
6D	6148* (2)
7C	5468* (3), 8006* (1)
7F	191 (1)
9A	156 (1)
9N	66 (2)
9V	156 (5), 280 (5), 7440* (1)
10A	5466* (3), 5472* (2), 5658* (1), 7422* (1), 7449* (1), 7450* (1)
10F	7451* (2)
11A	62 (7), 193 (3), 3000 (2), 4063* (4), 5667* (1), 6345* (1), 6444* (1), 7414* (1)
13	4818 (2), 5593* (6)
14	15 (3), 25 (1), 156 (26), 379 (1), 7432* (2), 7437* (1), 7448* (1), 7456* (1)
15A	3669 (2), 5448* (8), 5453* (1), 7429* (1)
15B	156* (1), 3669 (8)
15C	3669 (7), 5461* (3)
16F	6142* (1), 6336* (1), 7438* (2)
17F	392 (2)
18A	241 (1), 7442* (1)
18B	6346* (1)
18C	6143* (1), 6346* (1)
19A	66 (1), 276 (6), 320 (1), 1131 (1), 5460* (5), 5662* (1)
19F	81 (12), 87 (1), 88 (2), 156 (1), 236 (6), 240 (1), 271 (2), 320 (1), 646 (19), 1421 (26), 1428 (1), 2033 (1), 5460* (1), 5462* (3), 5676* (5), 5677* (1), 6141* (1), 6584* (1), 6585* (1), 7431* (1), 7443* (2), 7444* (2), 7445* (4), 7446* (1), 7453* (1), 7459* (1)
20	5474* (3)
21	193 (2), 7435* (1), 7436* (1)

22F	5675* (2), 7447* (1), 8005* (1)
23A	42 (1), 338 (4), 439 (5), 5473* (1), 5675* (3), 7461* (1)
23B	242 (2), 387 (3), 775 (1), 2911 (3), 7424* (2)
23F	81 (15), 242 (23), 1012 (1), 5463* (3), 6200 (1), 6344* (1), 7413* (2), 7427* (1), 7452* (1)
24F	230 (1), 5468* (1), 5581* (1), 6139* (1), 6741* (1)
28A	546 (1)
33B/D	7428* (1)
33F	1012 (5)
34	1439 (1), 1902 (3), 6347 (1), 7412* (1), 7441* (1)
35A	5469* (2)
35B	7425* (2), 7426* (1)
35F	2991 (7), 5600* (3), 8007* (1)
39	7439* (1)
NT	448 (3), 2315 (1), 5475* (1), 5636* (10), 5637* (1), 5638* (8), 5639* (2), 6564* (1), 6587* (1), 7420* (1), 7458* (1), unknown (3)
*Novel sequence types	

Table 8. Serotypes associated with STs common to both pre-PCV and post-PCV isolates

	Serotype (n)	
ST	**Pre-PCV7**	**Post-PCV7**
66	9N (2), 19A (1)	19A (1)
81	19F (2), 23F (2)	19F (1), 23F (3)
90	6B (1)	6B (1)
156	9V (1), 14 (26), 23F (1)	14 (10)
218	12F (1)	12F (3)
242	23F (6)	23F (3)
280	9V (1)	9V (2)
289	5 (6)	5 (2)
315	6B (1)	6B (1)
320	19A (2)	19A (2)
615	1 (2)	1 (1)
1292	6C (3)	6C (2)
1421	19F (6)	19F (8)
5449	6B (1)	6B (1)
5475	23F (1), 38 (2)	38 (1)
5625	6A (1), 6B (5)	6B (1)
5673*	9N (1)	16F (1)
6149	16F (1)	16F (1)
*Potential capsular switch event		

Figures

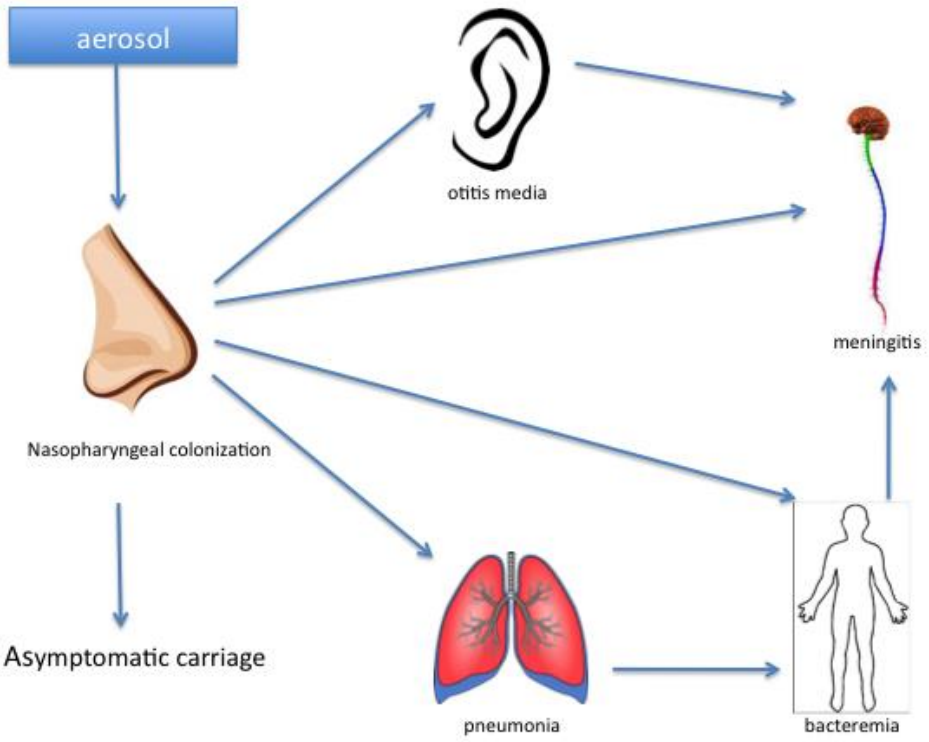


Figure 1. Potential modes of transmission for *Streptococcus pneumoniae*

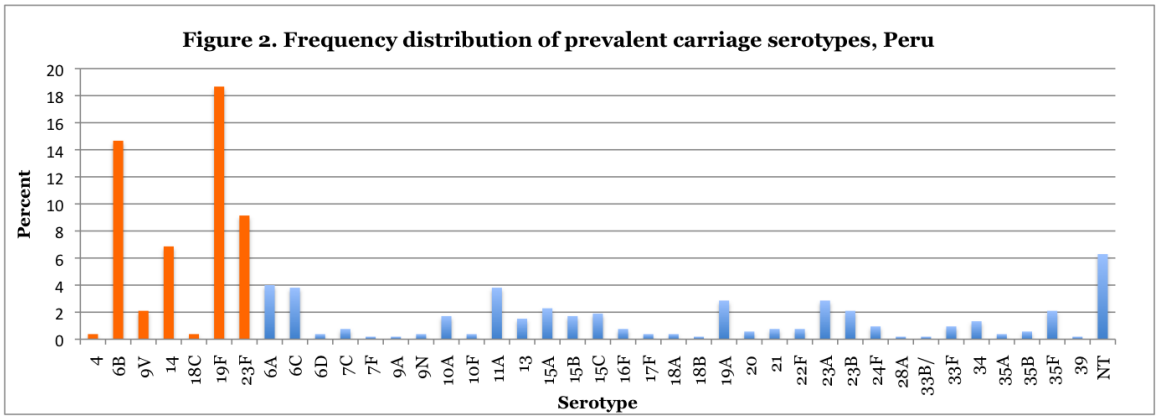


Figure 2. Frequency distribution of prevalent carriage serotypes before PCV introduction, Peru

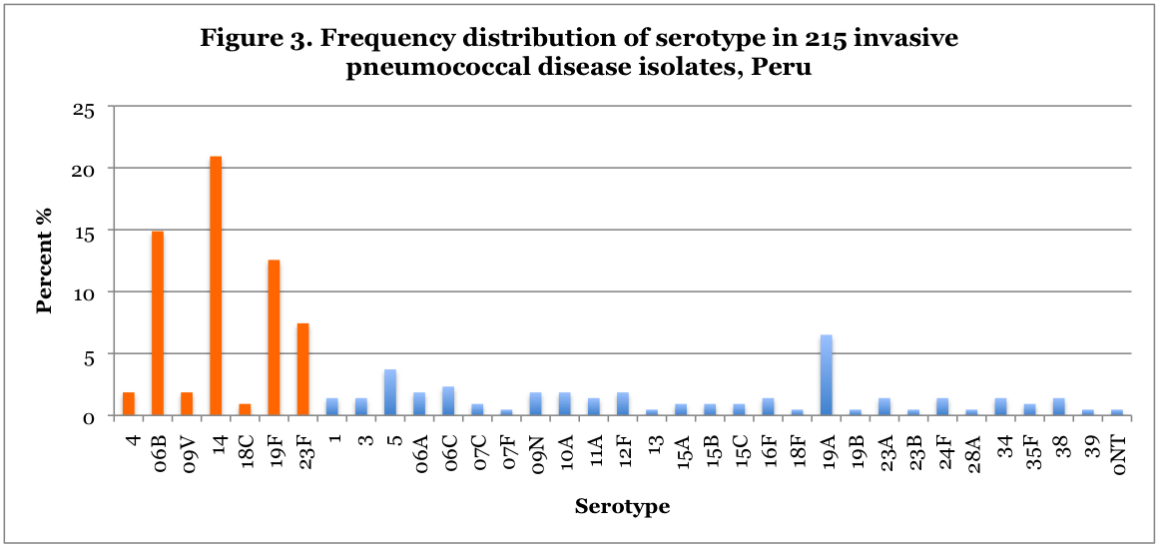


Figure 3. Frequency distribution of serotypes in 215 invasive pneumococcal disease isolates, Peru

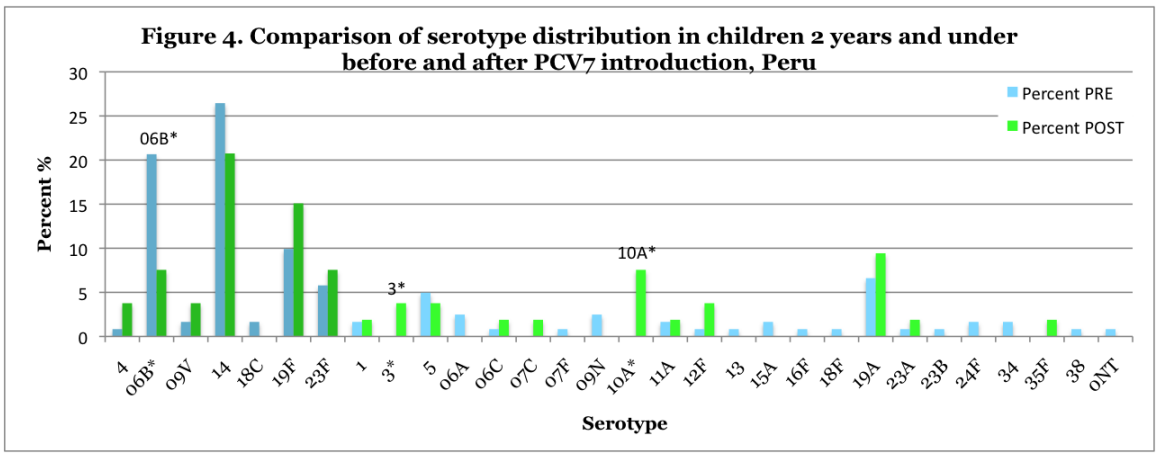


Figure 4. Comparison of serotype distribution in children 2 years and under before and after PCV7 introduction, Peru

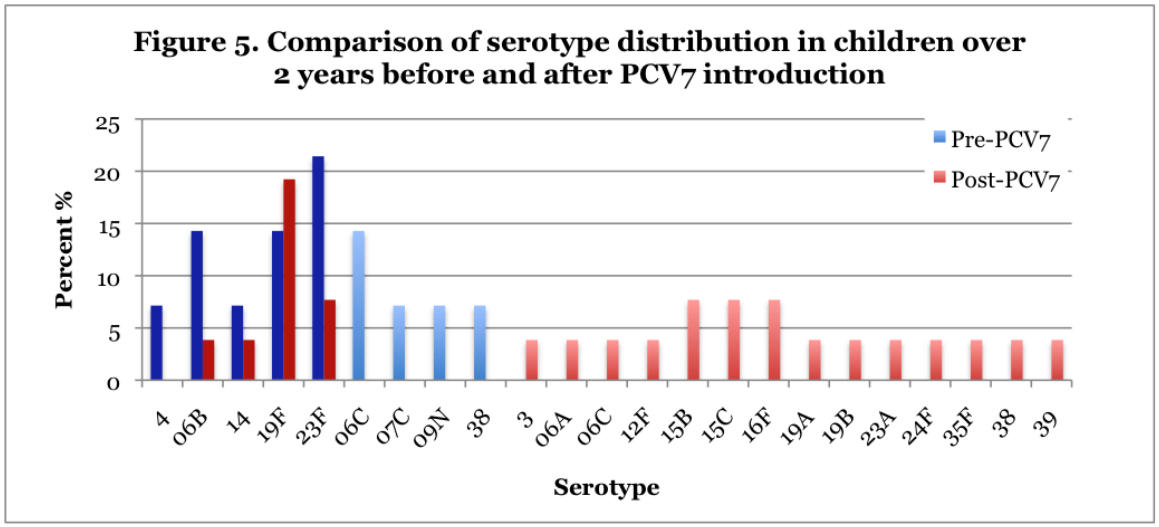


Figure 5. Comparison of serotype distribution in children over 2 years before and after PCV7 introduction, Peru

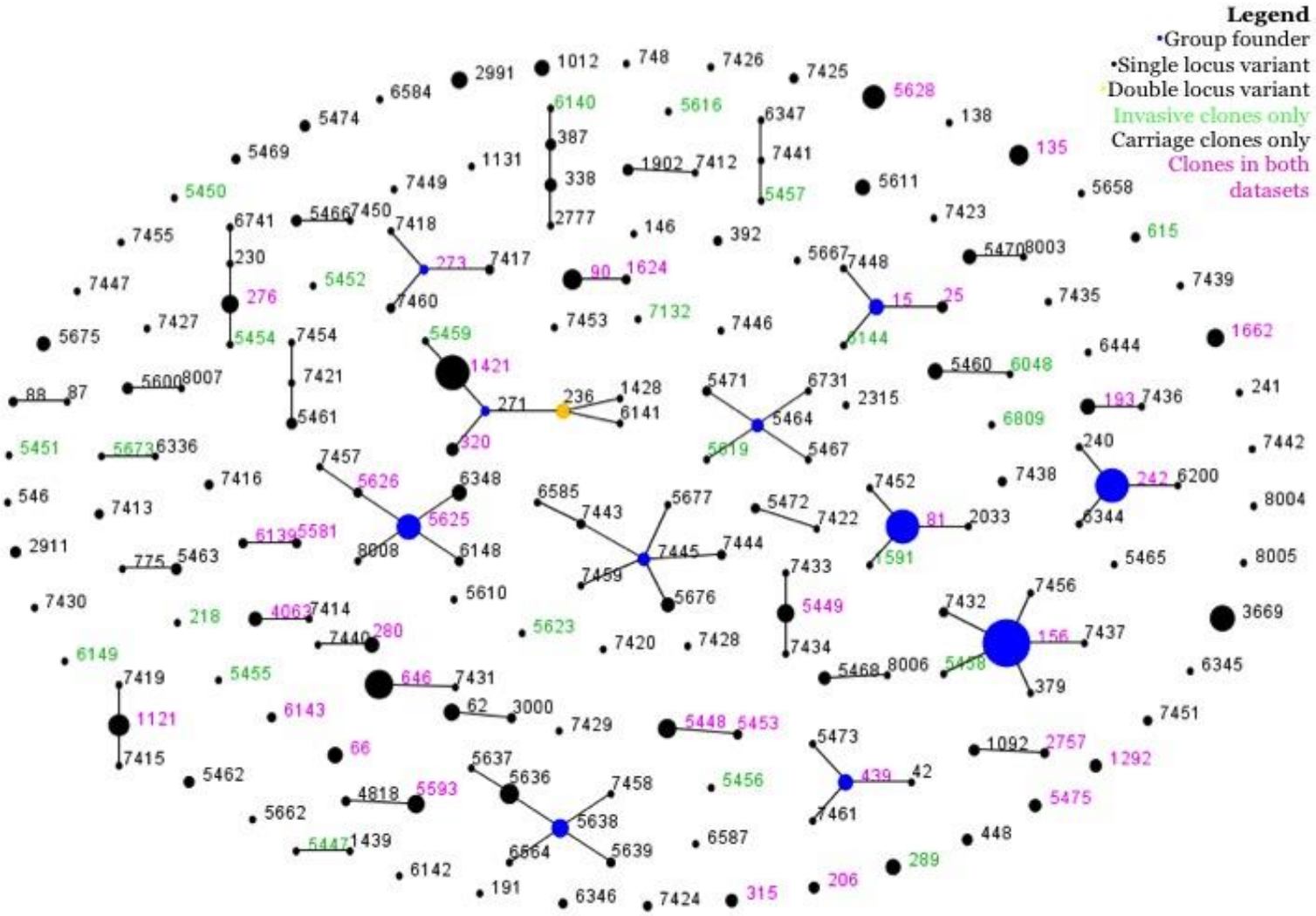


Figure 6. Clonal complex map comparing 135 invasive pre-PCV isolates and 525 carriage isolates

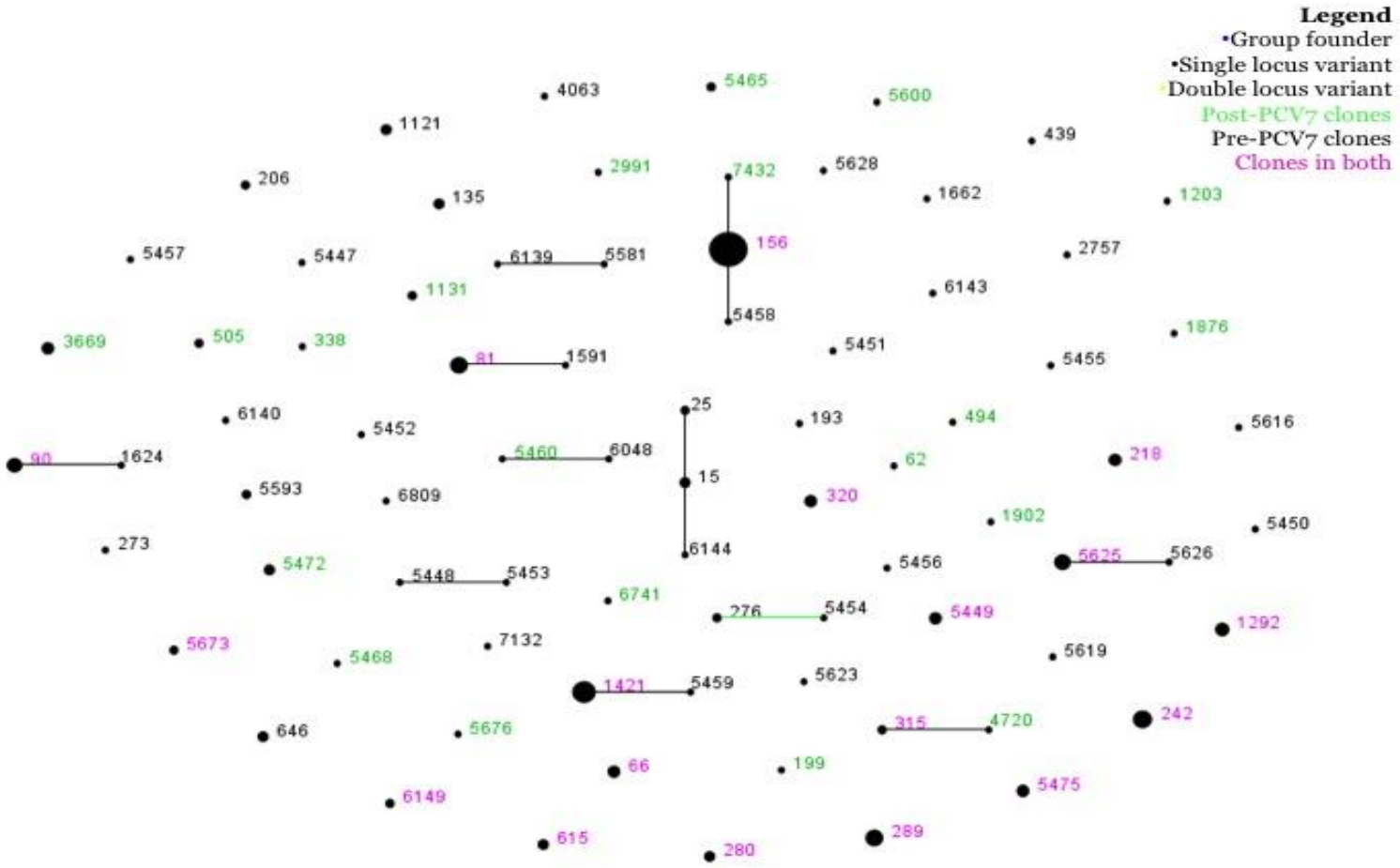


Figure 7. Clonal complex map comparing 135 pre-PCV and 74 post-PCV invasive isolates, Peru

Appendices

Appendix A. Institutional review board letter of exemption from review.

EMORY UNIVERSITY | Institutional Review Board

March 17, 2014

Lesley McGee, P.h.D
Respiratory Disease Branch
Division Bacterial Diseases
National Center for Immunization and Respiratory Diseases
Centers for Disease Control and Prevention
Building 18 - Room 133 Mailstop G-03
1600 Clifton Rd, N.E.
Atlanta GA 30333, USA

RE: Determination: No IRB Review Required
Bacterial Isolates of *S.pneumoniae* from Peru
PI: Lesley McGee

Dear Dr. McGee:

Thank you for requesting a determination from our office about the above-referenced project. Based on our review of the materials you provided, we have determined that it does not require IRB review because it does not meet the definition of research involving "human subjects" or the definition of "clinical investigation" as set forth in Emory policies and procedures and federal rules, if applicable. Specifically, in this project, you have been given coded samples of baceterial isolates for the purposes of research. The samples have been have been completely de-identified and the study team does not have access to the key that would allow them to identify subjects.

This determination could be affected by substantive changes in the study design, subject populations, or identifiability of data. If the project changes in any substantive way, please contact our office for clarification.

Thank you for consulting the IRB.

Sincerely,

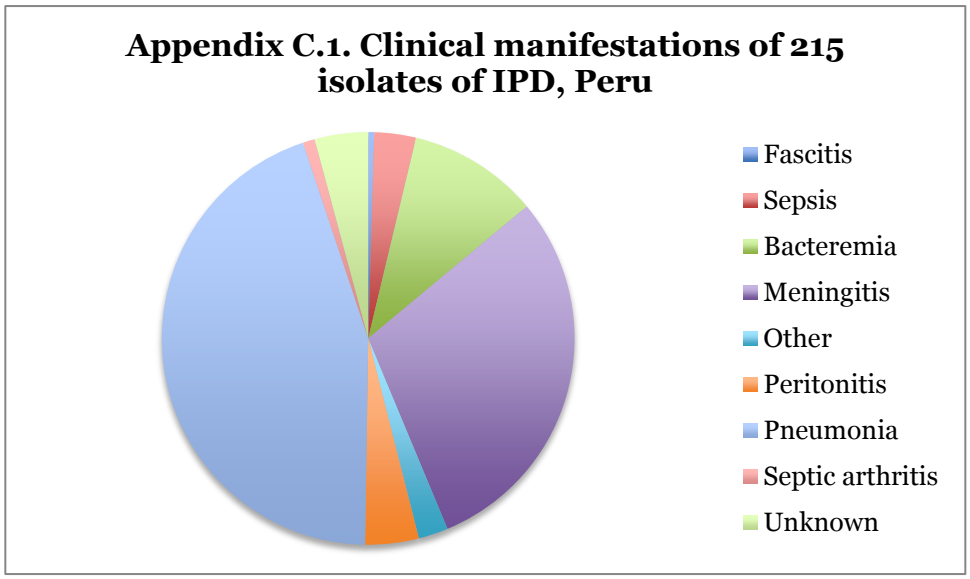
Steven J. Anzalone, M.S.
Research Protocol Analyst
This letter has been digitally signed

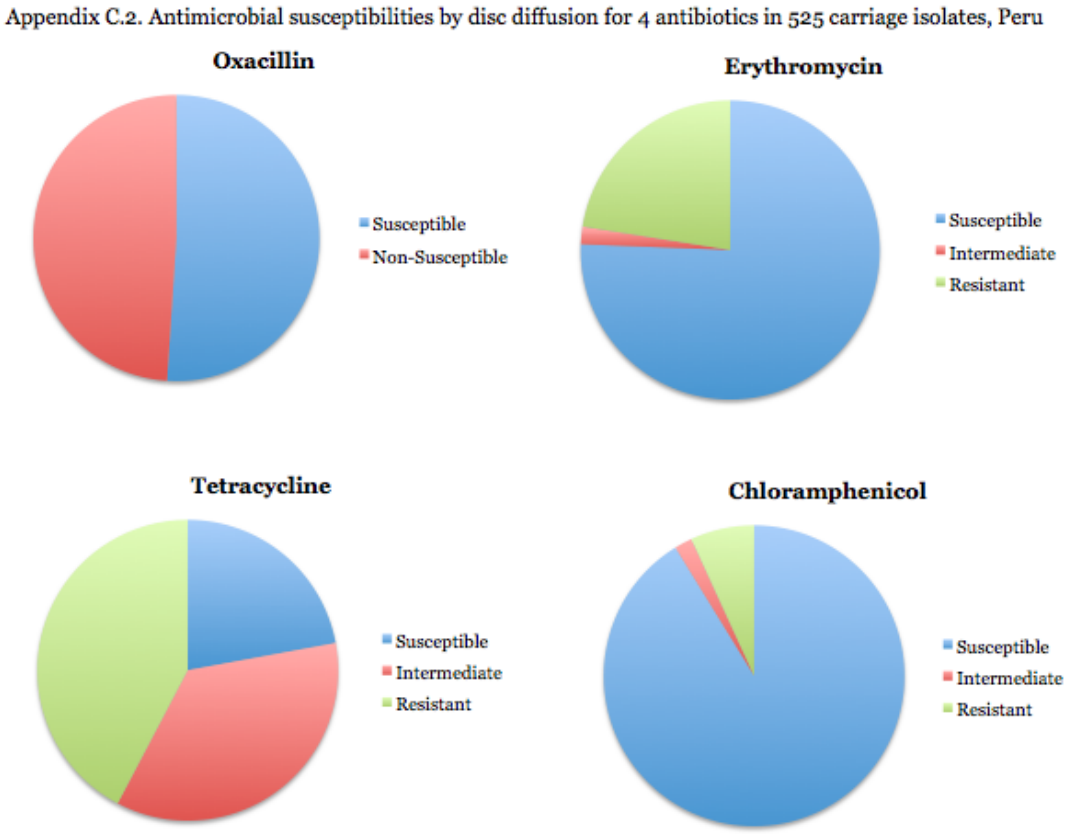
Emory University
1599 Clifton Road, 5th Floor - Atlanta, Georgia 30322
Tel: 404.712.0720 - Fax: 404.727.1358 - Email: irb@emory.edu - Web: http://www.irb.emory.edu

Appendix B. Circulating PMEN clones and sequence types

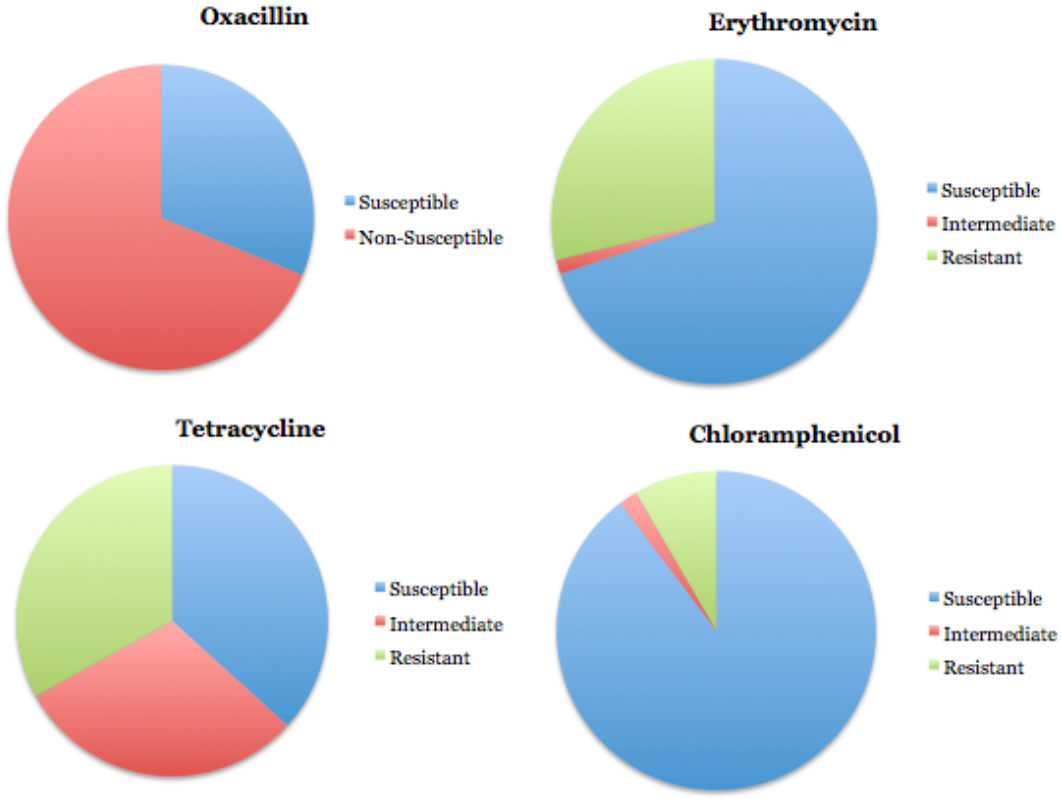
PMEN#	Name	ST
PMEN01	Spain^{23F} ST81	81
PMEN02	Spain^{6B} ST 90	90
PMEN03	Spain^{9V} ST156	156
PMEN04	Tenn^{23F} ST37	37
PMEN05	Sapin^{14} ST18	18
PMEN06	Hungary^{19A} ST268	268
PMEN07	S.Africa^{19A} ST75	75
PMEN08	S.Africa^{6B} ST185	185
PMEN09	England^{14} ST9	9
PMEN10	CSR^{14} ST20	20
PMEN11	CSR^{19A} ST175	175
PMEN12	Finland^{6B} ST270	270
PMEN13	S.Africa^{19A} ST41	41
PMEN14	Taiwan^{19F} ST236	236
PMEN15	Taiwan^{23F} ST242	242
PMEN16	Poland^{23F} ST173	173
PMEN17	Maryland^{6B} ST384	384
PMEN18	Tenn^{1}4 ST67	67
PMEN19	Colombia^{5} ST289	289
PMEN20	Poland^{6B} ST315	315
PMEN21	Portugal^{19F} ST177	177
PMEN22	Greece^{6B} ST273	273
PMEN23	N.Carolina^{6A} ST376	376
PMEN24	Utah^{35B} ST377	377
PMEN25	Sweden^{15A} ST63	63
PMEN26	Colombia^{23F} ST338	338
PMEN27	Sweden^{1} ST217	217
PMEN28	Sweden^{1} ST306	306
PMEN29	USA^{1} ST615	615
PMEN30	Greece^{21} ST193	193
PMEN31	Netherlands^{3} ST180	180
PMEN32	Denmark^{14} ST230	230
PMEN33	Netherlands^{8} ST53	53
PMEN34	Denmark^{12F} ST218	218
PMEN35	Netherlands^{14} ST124	124
PMEN36	Netherlands^{18C} ST113	113
PMEN37	Netherlands^{15B} ST199	199
PMEN38	Sweden^{4} ST205	205
PMEN39	Netherlands^{7F} ST191	191
PMEN40	Sweden^{1} ST304	304
PMEN41	Portuagal^{6A} ST327	327
PMEN42	Norway^{NT} ST344	344
PMEN43	USA^{NT} ST448	448

Appendix C. Additional Figures

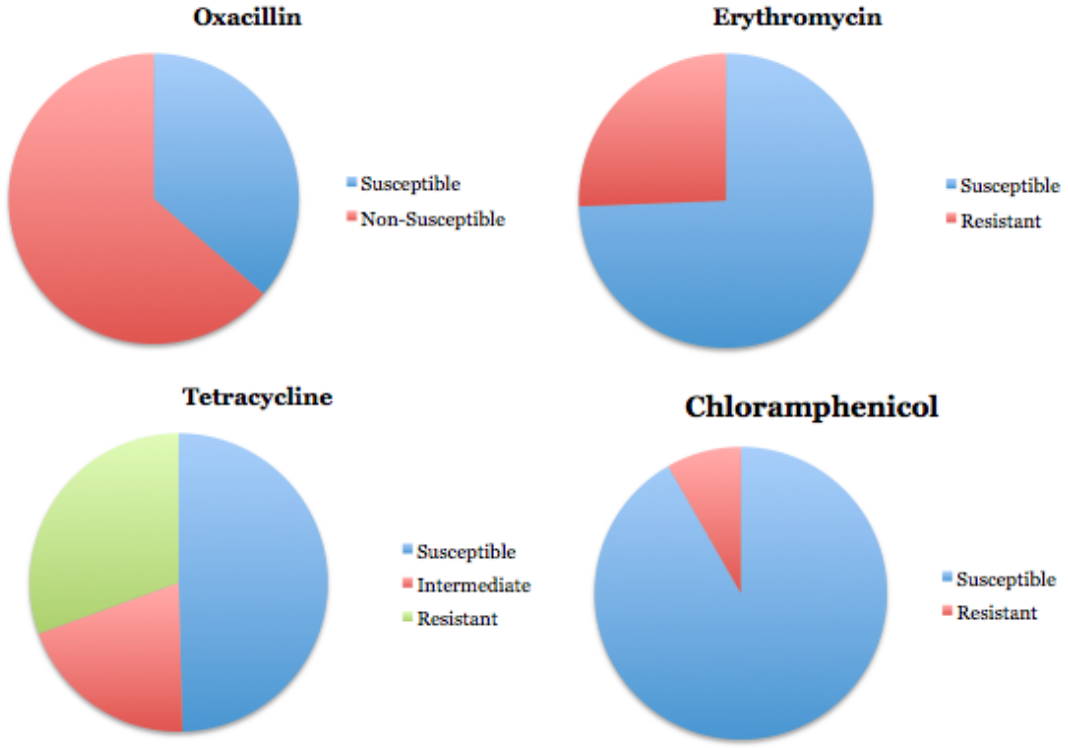




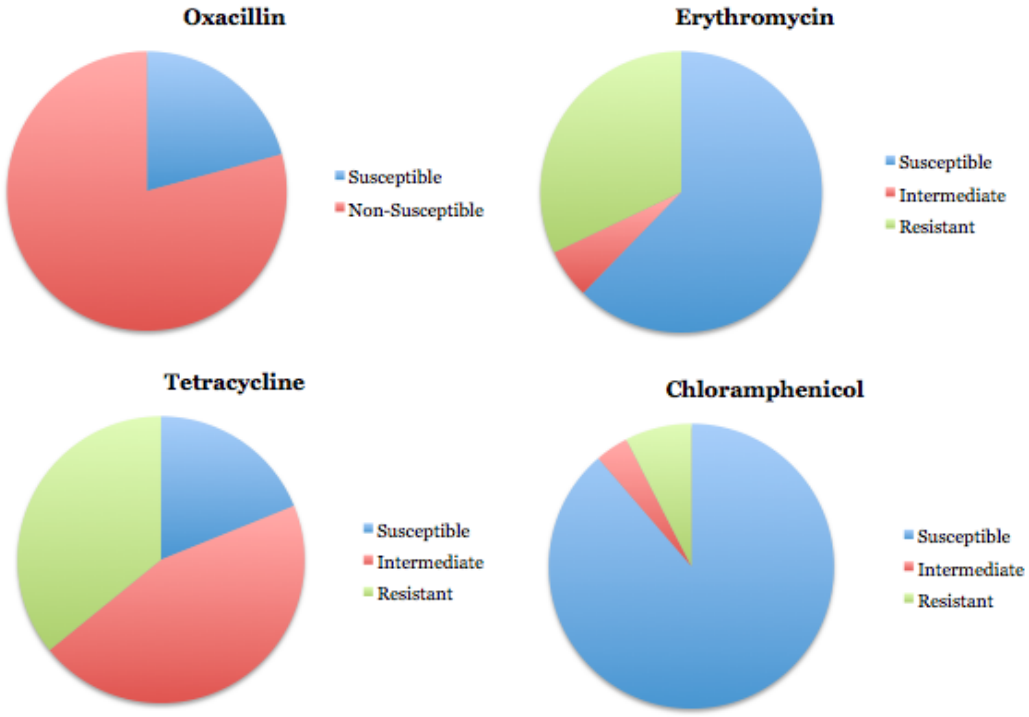
Appendix C.3. Antimicrobial susceptibilities by disc diffusion for 4 antibiotics in 215 invasive isolates, Peru



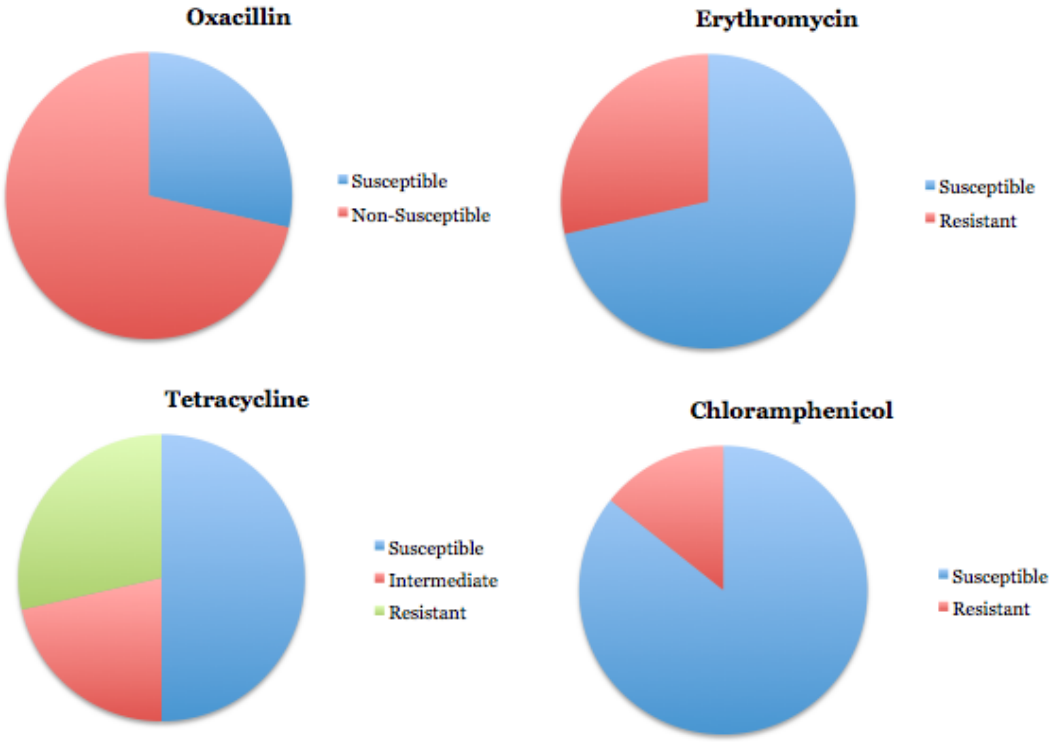
Appendix C.4. Antimicrobial susceptibilities by disc diffusion for 4 antibiotics in 121 invasive isolates from children 2 years and younger before PCV introduction, Peru



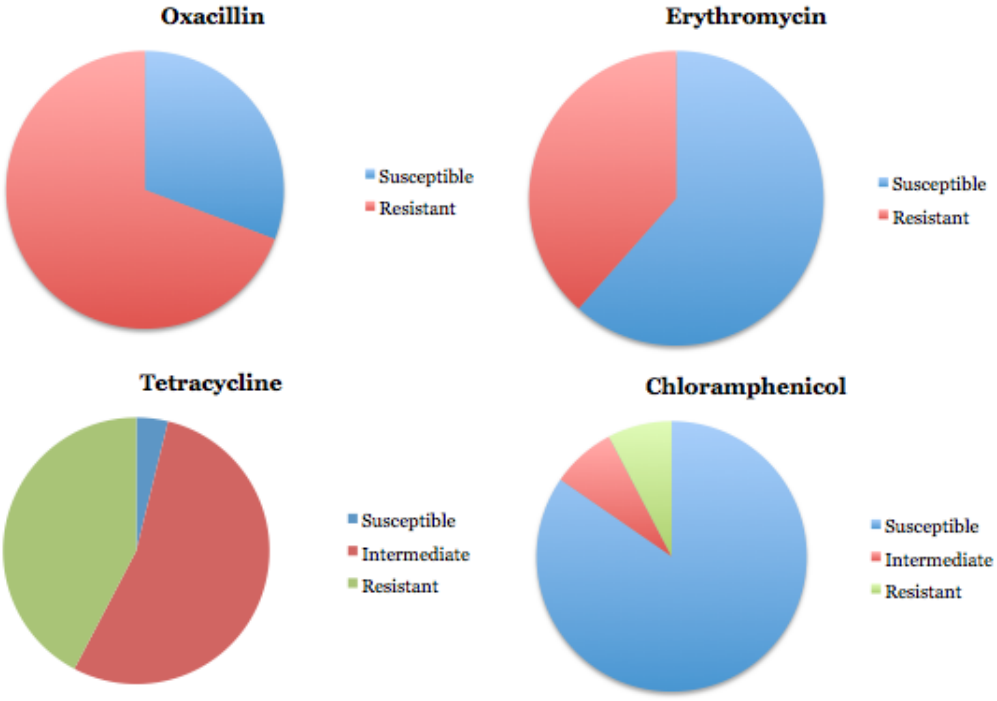
Appendix C.5. Antimicrobial susceptibilities by disc diffusion for 4 antibiotics in 53 invasive isolates from children 2 years and younger after PCV introduction, Peru

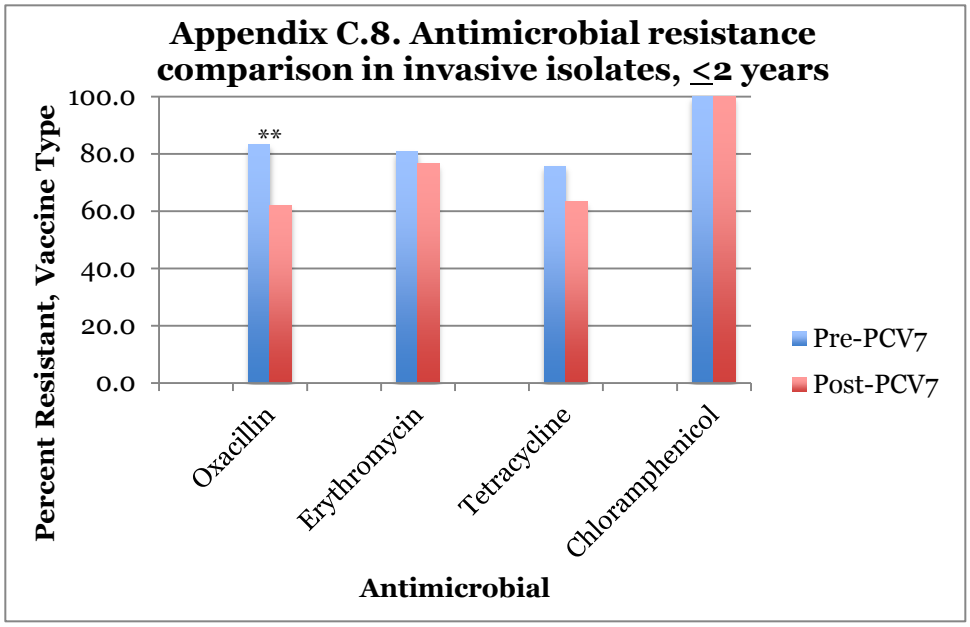


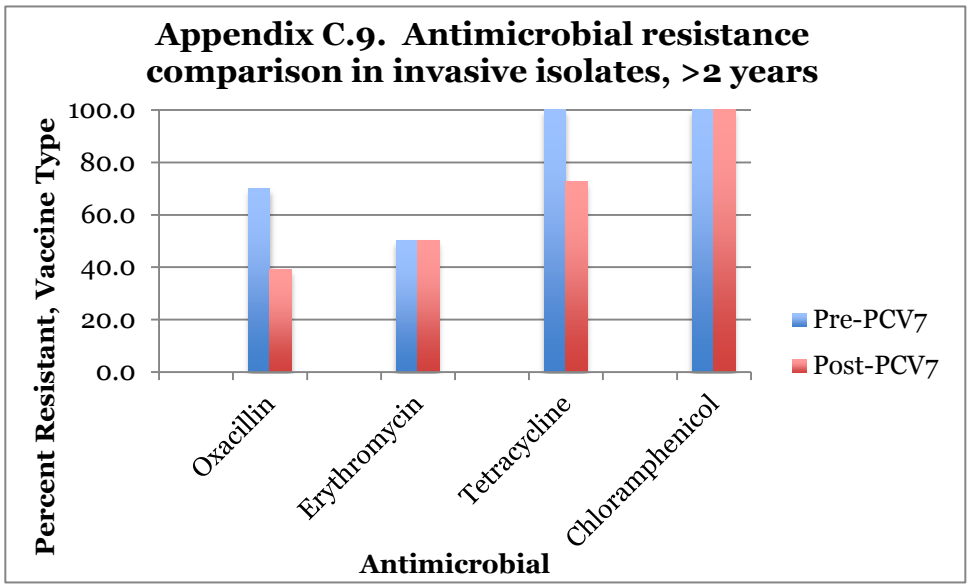
Appendix C.6. Antimicrobial susceptibilities by disc diffusion for 4 antibiotics in 14 invasive isolates from children over 2 years before PCV introduction, Peru



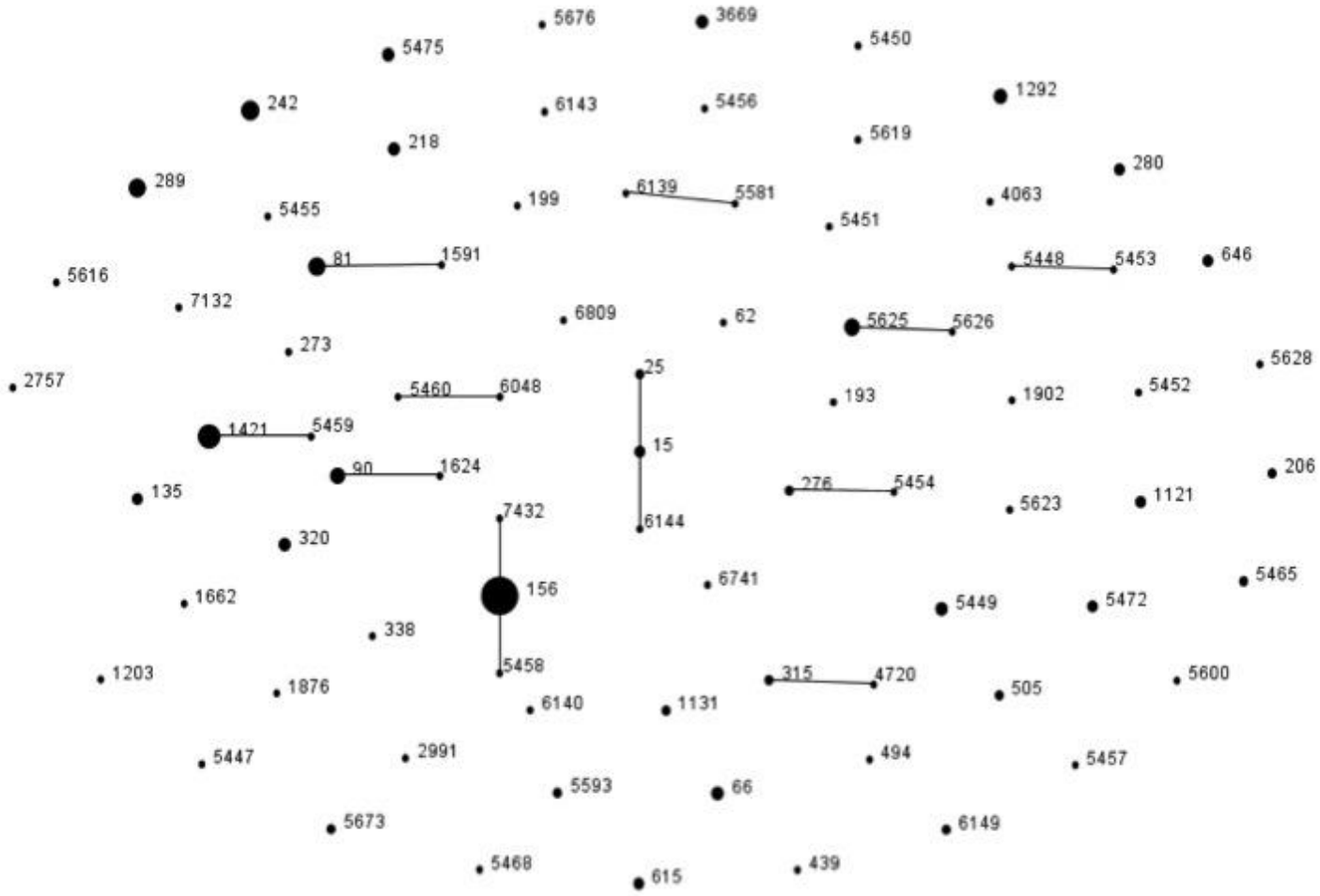
Appendix C.7. Antimicrobial susceptibilities by disc diffusion for 4 antibiotics in 26 invasive isolates from children over 2 years after PCV introduction, Peru




Appendix C.8. Antimicrobial resistance comparison in invasive isolates, ≤2 years
Percent Resistant, Vaccine Type
100.0
80.0
60.0
40.0
20.0
0.0
**
Oxacillin
Erythromycin
Tetracycline
Chloramphenicol
Antimicrobial
Pre-PCV7
Post-PCV7


Appendix C.9. Antimicrobial resistance comparison in invasive isolates, >2 years
Percent Resistant, Vaccine Type
100.0
80.0
60.0
40.0
20.0
0.0
Oxacillin
Erythromycin
Tetracycline
Chloramphenicol
Antimicrobial
Pre-PCV7
Post-PCV7

Appendix C.10. Clonal complex map of 208 invasive *S. pneumoniae* isolates, Peru



Appendix C.11. Clonal complex map of 521 carriage *S. pneumoniae* isolates, Peru