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Impact of the heptavalent Pneumococcal Conjugate Vaccine (PCV7) on the molecular determinants of macrolide resistance among invasive isolates of *Streptococcus pneumoniae* in the United States

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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 Epidemiology

2011

ABSTRACT

Impact of the heptavalent Pneumococcal Conjugate Vaccine (PCV7) on the molecular determinants of macrolide resistance among invasive isolates of *Streptococcus pneumoniae* in the United States

By Paulina Ahues Hawkins

Background: Pneumococcal macrolide resistance is usually expressed as one of two phenotypes: the M phenotype, mediated by the *mef*A/E gene, or the MLS_B phenotype, mediated by the *erm*B gene. The predominant mechanism of resistance in the U.S. was until recently mediated by the *mef*A gene, but its prevalence has decreased since the introduction of the heptavalent pneumococcal vaccine (PCV7) in 2000. The main purpose of this study was to determine whether the *erm*B + *mef*A genotype has become the most prevalent mechanism. **Methods:** We determined serotype, phenotype and genotype for 4535 erythromycin-resistant isolates submitted to the ABCs program from participating sites pre (1999) and post (2002-2007) introduction of PCV7. Logistic regression models were fitted to determine risk factors associated with the MLS_B phenotype among these isolates. **Results:** The five most common serotypes associated with macrolide resistance were 19A, 14, 6A, and 15A; the prevalence of 14 and 6A was greatly reduced by PCV7, but 19A and 15A (non-vaccine serotypes) have increased significantly since 1999, along with macrolide resistance. The dual *erm*B + *mef*A/E genotype increased significantly among these isolates, while the *mef*A/E only genotype went from being the most predominant mechanism of erythromycin resistance in the U.S. in 1999 (80.2%) to accounting for only half of the resistant isolates in 2007. These changes were closely related to changes in serotype distribution. Isolates with a non-PCV7 serotype (especially 19A), that were collected in California, or that possessed multidrug resistance, were more likely to have an MLS_B phenotype. Fifteen isolates were found to have alternative resistance mechanisms (mutations in L4 ribosomal protein or presence of *erm*A (*erm*TR)). **Conclusions:** The *mef*A/E genotype, which was the predominant mechanism of erythromycin resistance among U.S. IPD isolates before the introduction of PCV7, is quickly being replaced by the dual *erm*B + *mef*A/E genotype, mostly due to increases in serotype 19A. Since the new 13-valent pneumococcal vaccine (PCV13) contains 19A, this trend will possibly be reversed in the next few years. Most significantly, alternative mechanisms that have rarely been reported were found among these isolates, including several previously unpublished mutations in the L4 ribosomal protein.

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ACKNOWLEDGEMENTS

I am sincerely grateful to my advisor, Prof. Keith Klugman for sharing his extensive knowledge, and providing guidance and support during the writing of this thesis. I also owe my deepest gratitude to my field advisor Dr. Lesley McGee, for being an amazing supervisor, mentor, and friend, in addition to a true inspiration in my life.

I am indebted to my colleague and friend Sopio Chochua for being an outstanding coworker, always so willing to lend a hand. I would also like to thank the members of the Klugman lab at Emory: Magderie, Allison, Jorge, and Herbert, for the help and support they provided, as well as all members of the Streptococcus lab at CDC. I am especially grateful to Dr. Bernard Bell for providing critical data and resources for this work.

I would not have made it through this journey without the unconditional love and support of my husband Allan, who acted as my emotional anchor and tireless cheerleader, and my family, who never doubted my ability to succeed. To them I dedicate this thesis.

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BACKGROUND

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, particularly in young children and elderly persons. The most important pneumococcal infections include meningitis, bacteremia/septicemia, and pneumococcal pneumonia, because of their high case-fatality rates and associated costs. In 2000, pneumococcal disease caused about 826,000 deaths (582,000-926,000) in children aged 1-59 months worldwide, of which 741,000 (542,000-805,000) were due to pneumonia and 60,500 (27,300-82,200) were due to meningitis (1). Otitis media, sinusitis and bronchitis are more common but less serious manifestations. It also causes osteomyelitis, septic arthritis, endocarditis, peritonitis, cellulitis and brain abscesses.

In 2007, pneumonia and influenza were the eighth leading cause of death in the United States, the sixth leading cause of death in those over age 65, and the number one cause of death from infectious diseases (2). In Europe and the United States, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia (CAP) in adults. Data from 2005 showed that there were 1.3 million hospitalizations for pneumonia in the United States, and about 60% of those who were hospitalized were over the age of 65; the cost of care for patients with community-acquired pneumonia was estimated to be over $40 billion, including both direct and indirect costs (3).

S. pneumoniae is a Gram-positive lancet shaped diplococcus, completely enveloped by a polysaccharide capsule. During invasion, this capsule is an essential determinant of virulence. Based on differences in the composition of this capsule, about 93 distinct

pneumococcal serotypes have been identified (4). The spectrum of prevalent capsular types varies with age, time and geographical region, although common IPD-causing serotypes have been identified throughout the world (5-6). Anti-pneumococcal vaccines are based on formulations of various capsular agents derived from these prevalent strains.

A heptavalent pneumococcal conjugate vaccine (PCV7; Prevnar) was licensed for use in children aged 2 to 23 months by the US FDA in 2000, and has since been registered in over 70 countries worldwide. The selected serotypes represented about 80% of invasive pneumococcal infections among young children in the United States: 4, 6B, 9V, 14, 18C, 19F, 23F. This vaccine has had a major effect on the incidence of pneumococcal disease, since PCV7 not only protects immunized children but also provides protection to non-immunized children and adults through herd immunity (7). The rate of IPD among US children under 2 had decreased by at least 60% by 2003 (8), and among adults ≥65 years old, the overall rate of IPD had decreased by 37% by 2007 (9). Vaccination also eliminated disparities in risk for PCV7-type IPD associated with race and group child care attendance (10).

Additionally, Kyaw et al. (11) observed that, since five of the seven serotypes in the vaccine (6B, 9V, 14, 19F, and 23F) were responsible for most antibiotic-resistant infections, the overall rate of penicillin-nonsusceptible disease decreased by 57% from 1999 to 2004. Similar decreases were found for disease caused by erythromycin-nonsusceptible strains (MIC >0.5μg/ml) and those resistant to multiple antibiotics.

It was hoped that the vaccine would also provide cross protection against vaccine related serotypes such as 6A, 23A and 19A. Indeed, Pilishvili et al. (9) showed that IPD caused by PCV7-related serotypes in the United States declined significantly among children aged <5 years after the introduction of the vaccine, mostly due to declines in rates of serotype 6A IPD. However, they also observed that the rate of IPD caused by serotypes 23A and 19A increased significantly from 1998 to 2007.

Similarly, Moore et al. (12) found that among children aged <5 years, the incidence of IPD due to serotype 19A had increased by >2.5 fold by 2005. Of some concern is the fact that >60% of invasive 19A isolates in that study were nonsusceptible to penicillin (MIC >2ug/ml), a fact that was echoed in other studies (11,13), where serotype 19A was found to account for 35% of all penicillin-nonsusceptible strains among children < 2 years of age in 2004 (compared to only 2% in 1999), while non-susceptibility to TMP-SMZ (56%) and erythromycin (38%) among serotype 19A isolates was also high. Gertz et al. (14) also found increased penicillin nonsusceptibility among non-PCV7 serotypes 15A, 23A, 35B and 6C.

Furthermore, according to data from year 6 of the PROTEKT US study (2005-2006) macrolide resistance among *S. pneumoniae* isolates collected from community-acquired respiratory tract infections (CA-RTI) increased significantly from about 30% in years 3-5 to 35.3% in year 6 (15). As for invasive isolates, the rate of erythromycin resistance increased from 20.5% in 1999 to 24.9% in 2009 in the United Sates (16).

Pneumococcal macrolide resistance is usually expressed as one of two phenotypes: the M phenotype or the MLS_B phenotype. M phenotype isolates are moderately resistant to 14- and 15-membered macrolides and almost always susceptible to clindamycin, as a result of a drug efflux pump encoded by the *mef* gene (17). MLS_B phenotype isolates are resistant to macrolide, lincomsamide (clindamycin), and streptogramin B antibiotics, as a result of the modification of ribosomal targets, most commonly mediated by an *erm* methylase (18); *erm*B is the predominant methylase found in *S. pneumoniae*, but *erm*A (*erm*TR) has also been reported (19-20). Target-site modification has also been shown to occur by substitutions in the 23S rRNA and in the L4 and L22 riboproteins (21-22).

While the *erm*B genotype is the most prevalent mechanism, occurring in 55% of all erythromycin-resistant *S. pneumoniae* isolates globally and in more than 88% of isolates in some European countries in 2004 (23), the predominant mechanism of pneumococcal macrolide resistance in the U.S. was until recently mediated by the *mef*A gene. A study conducted in 1999, before the introduction of PCV7, found that the M phenotype accounted for 82% of erythromycin-resistant isolates, and was most closely associated with children younger than 5 years. Most of these isolates were PCV7-types (24).

However, the prevalence of *mef*A in the United States has steadily decreased since the introduction of PCV7, while the prevalence of macrolide-resistant strains carrying both *erm*B and *mef*A genes has increased. This was first reported by the PROTEKT study among North American CA-RTI isolates in 2002 (25) and has subsequently been confirmed in several other PROTEKT reports (15, 23, 26- 29). The proportion of *S.*

pneumoniae CA-RTI isolates positive for both *erm*B and *mef*A genes increased from 9.3% in year 1 (2001-2001) to 24.1% in year 6 (2005-2006), while isolates positives for *mef*A only decreased over the same time from 69.0% to 53.8%. Most of the dual genotype strains were serotype 19A (69.2%) and penicillin nonsusceptible (90%). In addition, *mef*A only isolates showed an increased level of macrolide resistance (MIC >16μg/ml) than previously reported (15).

To our knowledge, no similar study on the distribution of macrolide resistant genotypes among IPD isolates in the United States has been conducted. Hence, the aims of this study were to determine if the *erm*B + *mef*A genotype has increased among macrolide resistant invasive *S. pneumoniae* isolates in the United States, whether this genotype has become more prevalent than the *mef*A only genotype, and whether there are particular risks factors associated with the MLS_B phenotype.

METHODS

Surveillance

The Active Bacterial Core surveillance (ABCs) is a core component of CDC's Emerging Infections Programs Network (EIP). ABCs is an active laboratory- and population-based surveillance system for invasive bacterial pathogens of public health importance. For each case of invasive disease in the surveillance population, a case report with basic demographic information is completed and bacterial isolates are sent to CDC and other reference laboratories for additional laboratory evaluation. The surveillance areas in 1999 included: California (San Francisco County); Connecticut; Georgia (20 county Atlanta area); Maryland (6 county Baltimore area); Minnesota (7 county Twin Cities area); New York (7 county Rochester area and 8 county Albany area); Oregon (3 county Portland area); Tennessee (5 urban counties), with a total population of over 18 million people. Colorado (5 Denver counties) was added in 2001 and New Mexico in 2004. The combined population under surveillance by 2007 was over 28 million individuals. (www.cdc.gov/abcs/reports-findings/surv-reports.html). Invasive *S. pneumoniae* isolates submitted to ABCs pre (1999) and post (2002-2007) introduction of the PCV7, and determined to be erythromycin-resistant (MIC≥1μg/ml) were included in this study. The Emory IRB determined this study to be exempt from review (see Appendix).

Serotyping and antimicrobial susceptibility testing

Isolates were serotyped by latex agglutination and the Quellung reaction using typing antisera prepared in the CDC Streptococcus Laboratory. Minimum inhibitory concentrations (MIC) were determined by the broth microdilution method and resistance

was determined based on the CLSI breakpoints (30). An Etest was performed on all 1999 strains with MIC ≥16μg/ml and all 2007 strains with MIC ≥32μg/ml, following the manufacturer's instructions (31). Additionally, disk diffusion testing was carried out on 118 strains with an M phenotype that were positive for *erm*B, in order to determine inducible clindamycin resistance (32); all isolates showing positive induction results (a "D-shaped" zone) were tested by broth microdilution.

DNA extraction

Genomic DNA was extracted using a Chelex-based method (33), briefly: using a 1ul inoculating loop, overnight cultures of *S. pneumoniae* were resuspended in a 5% Chelex solution containing 200ug/ml of proteinase k and incubated at 56^{o}C for 1 hr, then pulse vortexed for 10s, incubated at 95^{o}C for 10 min, and finally vortexed again for 10s then centrifuged at full speed for 2 min. The resulting DNA was then diluted in sterile water (1 in 5) before using in a PCR reaction.

Polymerase Chain Reaction (PCR) amplification and Sequencing

PCR amplification of *erm*A (*erm*TR), *erm*B, and *mef*A/E was carried out using primers previously described (34); 5ul of DNA extract was used in a 25ul reaction containing 1X buffer, 200uM dNTP, 600uM of each primer, and 2U of Taq polymerase (New England Biolabs, Ipswich, MA). The resulting PCR products were analyzed by agarose gel electrophoresis. Amplification of the genes encoding L4 (*rpl*D) and L22 (*rpl*V) riboproteins was carried out as previously described (21). PCR products were then purified using ExoSAP-IT (Affymetrix, Inc., Santa Clara, CA) and cycle sequenced using

the BigDye Terminator v3.1 chemistry (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer's protocol (35). Sequencing reactions were purified using a Sephadex-based method, briefly: sequencing reactions were transferred onto Sephadex G-50 columns pre-packed into microtiter filter plates (Millipore, Billerica, MA) and then centrifuged at 750xg for 2 min onto clean 96-well plates. Purified reactions were analyzed on an ABI3130xl automated sequencer. The *rpl*D and *rpl*V sequences obtained were run through the Basic Local Alignment Search Tool (BLAST) at NCBI (www.ncbi.nlm.nih.gov) and then manually compared to the TIGR4 (NC003028) genome, in order to identify possible changes in the riboproteins.

Statistical Analyses

Statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, NC) statistical software. Simple logistic regression models were used to determine crude odds ratios using phenotype as the dependent (outcome) variable and serotype, location, age group, and resistance to multiple drugs (two or more antibiotics other than clindamycin and erythromycin), as the independent variables. Multivariable logistic models were then fitted to calculate adjusted odds ratios for each independent variable, adjusting for the other variables. The variable serotype was coded in two different ways, first as PCV7 vs non-PCV7 (excluding 19A) and then as serotype 19A vs other, while the variable location was coded first as one category for each state and then as California vs other. When used as covariates to calculate adjusted ORs, these two variables were coded as 19A vs other, and California vs other. P values were calculated using the χ^2 test; a P value <0.05 was considered statistically significant.

RESULTS

Isolates

Twenty six thousand nine hundred and seventy four (26,974) invasive *S. pneumoniae* isolates were submitted to ABCs from participating sites pre (1999) and post (2002-2007) introduction of the PCV7. Four thousand five hundred and thirty five (4535) of these isolates (16.8%) were erythromycin-resistant (defined as having an erythromycin MIC≥1μg/ml) and available for testing. Table 1 summarizes the general characteristics of these isolates by year of submission. The age distribution shows that the proportion of these isolates cultured from children <2 years old decreased significantly from 1999 to 2002 (from 38.7% to 12.6%, P<0.0001) and then remained stable for the following years (at 13-14%). Conversely, the proportion of these isolates obtained from the 45-64 and >65 age groups increased significantly during the same period (from 16.2% to 31.6% P<0.0001, and from 26.1% to 33.3% P=0.002, respectively). Isolates were evenly distributed among genders, and most of them were cultured from blood or CSF.

Invasive serotypes

Figure 1 illustrates the changes in serotype distribution among erythromycin-resistant invasive isolates from pre (1999) to post (2002-2007) introduction of PCV7. The seven serotypes included in the vaccine declined rapidly from 79.5% in 1999 to 63.4% in 2002 (P<0.0001) to a low 8.3% in 2007 (P<0.0001). Among the non-PCV7 serotypes, the largest changes from 1999 to 2007 were observed in 19A and 15A (from 3.4% to 42.3% P<0.0001, and from 0.3% to 12.5% P<0.0001, respectively) followed by 33F and 6C (from 0.3% to 7.3% P<0.0001, and from 0.7% to 5.9% P<0.0001, respectively).

Conversely, non-PCV7 serotype 6A decreased from 9.9% in 1999 to 4.2% in 2007 ($P<0.0001$).

Serotype and antimicrobial resistance

Forty eight different serotypes were represented among the study isolates. The five most common serotypes were 19A (26.2%), 14 (14.5%), 6A (9.1%), 15A (6.0%) and 6B (5.4%). The serotypes with the largest proportion of isolates resistant to at least 5 antibiotics (in addition to erythromycin) were 23F (53.7%), 19F (46.7%), 19A (43.5%), 14 (38.6%), 9A (21.9%) and 6B (20.0%).

Mechanisms of erythromycin resistance

The dual *erm*B + *mef*A/E genotype increased significantly among these isolates from 1999 to 2007, while the *mef*A/E only genotype went from being the most predominant mechanism of erythromycin resistance in the U.S. in 1999 to accounting for only half of the resistant isolates in 2007 (Fig. 2). No significant changes were observed in the proportion of *erm*B only genotype. Twenty four isolates were negative for both *erm*B and *mef*A/E mediated mechanisms, and were therefore tested for the presence of *erm*A (*erm*TR), and mutations in the ribosomal proteins L4 and L22. None of these *erm*B-/ *mef*A/E- isolates were positive for *erm*A (*erm*TR) on PCR, but ten of them were found to have mutations in the L4 ribosomal protein (Table 2): all of them had S20N mutations, one had an additional S112L mutation, and two had another 8 mutations, including a 3-amino-acid substitution ($_{69}$GTG$_{71}$ to $_{69}$TPR$_{71}$) in a highly conserved region of L4 ($_{63}$KPWRQKGTGRAR$_{74}$). The *rpl*D (L4) sequence of these two isolates (3802-99 and

6275-99) had only a 91% identity with the *rplD* sequence of TIGR4. There was no particular serotype associated with these isolates, but 9 out of 10 showed moderate- to high-level resistance to erythromycin, and 7 of those were also resistant to clindamycin; the two isolates with 10 mutations were susceptible to clindamycin, as was the one strain with low-level resistance to erythromycin. An additional five strains were found to have mutations in the *rplV* gene encoding riboprotein L22, but none of these resulted in amino acid changes. The remaining isolates are still under investigation.

Genotype and serotype

It was also observed that the changes in prevalence among the mechanisms of erythromycin resistance were closely related to changes in serotype distribution. Figure 3(a) shows that the distribution of genotypes remained stable among PCV7-serotype isolates across the years in the study, with *mef*A/E being the predominant mechanism and the *erm*B + *mef*A/E genotype remaining as a minimal proportion. Meanwhile, among non-PCV7-serotype isolates, the proportion of *mef*A/E only isolates declined from 91.3% in 1999 to 48.5% in 2007 ($P<0.0001$), while the proportion of *erm*B + *mef*A/E isolates increased from 0.7% in 1999 to 31.3% in 2007 ($P<0.0001$). The proportion of *erm*B only isolates also increased among non-PCV7 serotypes, but not significantly. Among the non-PCV7 serotypes, 19A (85.5%) and 15A (5.88%) were the predominant serotypes among *erm*B + *mef*A/E positive isolates in 2007, as well as among the *erm*B positive isolates (16.5% and 54.6%, respectively); this is clearly illustrated in Figure 3(b) where serotypes are broken down into PCV13 (which includes 19A) and non-PCV13.

Genotype and macrolide resistance levels

Previous studies have reported that the *mef*A/E encoded efflux pump confers low level resistance to macrolides (36-38), and that this resistance level has increased over time (24, 27). We indeed found that the erythromycin MIC_{50} and MIC_{90} of *mef*A/E only isolates (8μg/ml and 16μg/ml, respectively) were significantly lower than those of isolates containing the *erm*B gene (512-1024μg/ml and 1024μg/ml, respectively), but we failed to find a significant difference in MIC ranges. We also failed to find an increase in erythromycin MICs from 1999 to 2007 among any of the genotypes (Table 3).

Genotype and MIC phenotype

MIC phenotype can generally be predicted from genotype, and vice versa, but we found several isolates with a discordant genotype/MIC phenotype (Table 4). Thirty six isolates that had an MLS_B phenotype and were positive for the *mef*A/E gene only were retested to confirm phenotype and genotype, five of them were determined to be susceptible to clindamycin by disk diffusion testing, while an additional twenty five isolates were found to carry the *erm*B gene when PCR was repeated. Six of these *mef*A/E only/MLS_B isolates remained unresolved after retesting; of these, two were found to carry the *erm*A (*erm*TR) gene, two carried the *erm*A (*erm*TR) gene and had a mutation in the L4 riboprotein (S20N), and one had a mutation in the L4 riboprotein (S20N) only (Table 5). In addition, one hundred and eighteen isolates with an M phenotype and positive for *erm*B were tested for inducible clindamycin resistance using a "D-zone" test. Forty of these isolates were determined to have inducible clinamycin resistance (confirmed by broth microdilution), and another thirty six were found to have constitutive resistance

(indicating an MIC error). PCR was repeated on the remaining forty two isolates and twenty eight were found to carry the *mef*A/E gene only, while fourteen isolates remained unresolved. These are in addition to the previously mentioned twenty four isolates that were neither *mef*A/E- nor *erm*B-positive, of which 6 were M phenotype and 18 were MLS_B phenotype (Table 4).

Factors associated with MLS_B phenotype

Regression analyses were carried out in order to determine the factors associated with an MLS_B phenotype. Logistic regression models were fitted to examine the association between serotype, location, age group, and multidrug resistance (MDR). Results are summarized in Table 6. Having a non-PCV7 serotype, being serotype 19A, having been collected in California, and possessing multidrug resistance, were all found to be associated with an MLS_B phenotype when using crude odds ratios. The same factors, plus the age group the host belonged to, were found to be associated with an MLS_B phenotype when using adjusted odds ratios.

DISCUSSION

The rate of erythromycin resistance among invasive U.S. isolates of *S. pneumoniae* increased from 20.5% in 1999 to 22.7% in 2007, in spite of substantial reductions in the prevalence of IPD after the introduction of PCV7. This increase was primarily due to the emergence and expansion of resistant clones of serotype 19A; the proportion of 19A isolates increased from 3.39% in 1999 to 42.3% in 2007. As it has been previously shown (39-40) most of the 19A isolates (72.0%) submitted to ABCs in 1999 belonged to the moderately antimicrobial-resistant clonal complex CC199, while the 2007 ones were distributed among complexes CC199 (40.3%), CC320/271^{19A} (32.9%) and CC695^{19A} (13.6%); 98% of CC320/271^{19A} isolates collected during 2005-2007 were penicillin-resistant, the majority of which (91-96%) were also resistant to macrolides (40).

The *mef*A/E genotype, which was the predominant mechanism of erythromycin resistance among U.S. IPD isolates before the introduction of PCV7, is quickly being replaced by the dual *erm*B + *mef*A/E genotype. The *mef*A/E genotype decreased from 80.2% in 1999 to 50.1% in 2007, while the dual *erm*B + *mef*A/E genotype increased from 2.6% to 29.7% from 1999 to 2007. Among the dual genotype isolates, the prevalence of 19A serotype increased from 5.0% in 1999 to 85.5% in 2007; 15A was the next most common serotype among these isolates in 2007 (5.88%). These results confirm the genotype shift that has been previously reported among respiratory tract isolates (15, 23, 25- 29). The higher-level resistance to macrolides conferred by this dual genotype is also of concern: dual genotype isolates were found to have a MIC_{50} 128-fold higher than that of mefA/E only isolates (8 vs. 1024μg/ml). Since the new 13-valent pneumococcal

vaccine (PCV13, Prevnar 13) approved by the FDA in 2010 contains serotype 19A, the increase in prevalence of this dual genotype (and its associated high-level resistance) may be reversed in the next few years as the new vaccine comes into use. However, the importance of continued surveillance is clear, in order to quickly identify new serotypes, such as 15A (not included in PCV13), that might emerge to fill this niche; 15A already represents the second most common serotype among isolates with dual genotype and its prevalence has increased steadily since 1999.

One of the most notable findings of this study was the fifteen isolates with alternative Erythromycin resistance mechanisms. We found twenty four isolates that were negative for both *erm*B and *mef*A/E mediated mechanisms, ten of them were found to have nine different mutations in the *rpl*D gene that encodes for the L4 ribosomal protein, and fourteen remain unresolved. An S20N substitution which has been previously reported to confer high-level resistance to erythromycin (41) but rarely resistance to clindamycin was found in all ten; indeed, 9 of these 10 isolates had and erythromycin MIC >16 μg/ml, but 7 of them were resistant to clindamycin. The remaining eight mutations found in the *rpl*D gene of two isolates have never reported in *S. pneumoniae*; a $_{69}$GTG$_{71}$ to $_{69}$TPS$_{71}$ substitution has been previously reported (22, 42) but we found a $_{69}$GTG$_{71}$ to $_{69}$TPR$_{71}$ substitution instead. Both isolates carrying these eight mutations showed high-level resistance to erythromycin. In addition, five isolates with MLS_B phenotype, and positive only for *mef*A/E, had the S20N substitution in L4 and/or carried the *erm*A (*erm*TR) gene.

MIC phenotype can generally be predicted from genotype, but we found several isolates with a discordant genotype/MIC phenotype. One hundred and eighteen isolates that were *erm*B+/M phenotype were found, but after repeating PCR and MIC testing only six remained unresolved; forty of these isolates had inducible clindamycin resistance, something that is not commonly reported for *S. pneumoniae,* but that must be taken into account in clinical settings when considering long-term clindamycin monotherapy. An additional thirty six isolates were *mef*A+/*erm*B-/MLS_B phenotype, but only fourteen remained unresolved after retesting. These results illustrate the limitations of determining phenotype from genotype, which might impact clinical decisions when it comes to choosing an antimicrobial for treatment. We found that the MLS_B phenotype was more common among isolates with a non-PCV7 serotype (especially 19A), isolates collected in California, and isolates that were also resistant to multiple drugs.

This study had a few limitations. First, because of the self-reporting nature of the surveillance program some cases may go unreported, this is minimized however by performing routine audits of reporting laboratories. Second, the PCR determination of resistance mechanisms is susceptible to false-positive and false-negative errors, because of variations in template and the risk of carryover contamination, but this was addressed by using phenotypic data to identify unexpected combinations. Finally, the exclusion of clinical data limited the identification of factors associated with specific resistant mechanisms, but this was beyond the scope of this particular study.

This study also had its strengths. The very large sample size allowed for significant patterns to be determined, and the fact that this in an ongoing surveillance program will allow for changes in these patterns to be observed over time, which will be highly relevant now that the new pneumococcal vaccine has been introduced. In addition, this was the first study of erythromycin resistance mechanisms on invasive isolates collected from a broad geographical area of the U.S., and spanning seven years pre and post introductions of PCV7. Previous publications focused mainly on respiratory tract isolates or in a specific region of the country.

Possible future directions for this study include further investigating isolates that were negative for both *erm*B and *mef*A/E mediated mechanisms and isolates of discrepant genotype/phenotype, updating the data to include years 2008-2010 in order to obtain a complete picture of the distribution of mechanisms pre-introduction of PCV13, and determining the clonal composition of invasive isolates other than 19A.

TABLES AND FIGURES

Table 1. General Characteristics of Erythromycin-resistant *S. pneumoniae* Isolates Submitted to ABCs pre (1999) and post (2002-2007) introduction of PCV7.

	All isolates (%) N=4535	**1999 (%)** N=767	**2002 (%)** N=515	**2003 (%)** N=538	**2004 (%)** N=524	**2005 (%)** N=679	**2006 (%)** N=742	**2007 (%)** N=770
Age group								
Under 2	17.8	38.7	12.6	13.0	14.1	12.8	13.9	14.6
3-14	5.4	5.0	5.8	4.8	4.8	5.6	5.7	5.8
15-44	16.5	14.1	20.8	19.7	17.0	14.9	16.9	14.8
45-64	27.2	16.2	28.4	25.5	28.2	31.4	29.8	31.6
Over 65	33.1	26.1	32.4	37.0	35.9	35.4	33.8	33.3
Gender								
Female	49.5	49.4	45.2	49.3	50.9	48.4	51.9	50.5
Male	50.5	50.6	54.8	50.7	49.1	51.6	48.1	49.5
State								
CA	3.5	6.1	3.8	3.2	2.7	4.1	2.7	2.0
CO	4.1		6.6	5.6	3.2	4.6	5.5	4.0
CT	12.3	12.1	15.7	11.3	14.1	10.9	9.9	13.1
GA	24.2	37.6	20.9	27.1	22.1	19.3	23.1	17.5
MD	10.7	9.0	9.1	11.3	10.9	10.3	10.1	13.9
MN	16.6	15.3	17.9	16.5	16.8	18.2	15.6	16.2
NM	3.6				2.7	6.2	6.7	7.5
NY	7.7	5.3	11.5	6.3	9.9	6.8	7.6	8.2
OR	1.6		2.3	2.2	1.7	2.4	2.8	0.7
TN	15.6	14.6	12.0	16.4	15.8	17.2	15.9	16.8
Source								
Blood	94.1	94.9	92.6	93.7	94.5	94.1	94.3	94.3
CSF	3.3	3.3	4.5	3.7	3.1	3.8	2.7	2.3
Pleural fluid	0.6	0.4	0.6	0.7	0.4	0.6	0.7	0.7
Joint	0.2	0.5	0.2	0.4	0.0	0.2	0.0	0.4
Other	1.8	0.9	2.1	1.5	2.1	1.3	2.3	2.3

Table 2. Erythromycin Resistant *S. pneumoniae* Isolates with Susbtitutions in the L4 Ribosomal Protein and Corresponding Erythromycin and Clindamycin MIC.

Isolate	L4 mutation	Serotype	ery MIC(µg/ml)	cld MIC(µg/ml)
3494-00	S20N	19A	16	1
4056-00	S20N	6B	>32	2
4066-00	S20N	14	16	1
6306-99	S20N	24F	16	2
7853-05	S20N	6A	>32	2
4882-07	S20N	7F	4	0.06
7509-06	S20N	15C	>32	>2
5473-07	S20N S112L	22F	>32	2
3802-99	S20N E13Q L19F E30Q $_{69}$GTG$_{71}$/$_{69}$TPR$_{71}$ V88I G98A A128S S130E	19A	>32	0.5
6275-99	S20N E13Q L19F E30Q $_{69}$GTG$_{71}$/$_{69}$TPR$_{71}$ V88I G98A A128S S130E	19A	>32	0.12

Table 3. MIC_{50}, MIC_{90}, and MIC Ranges for Each Mechanism of Macrolide Resistance in 1999 and 2007.

.

	***erm*B**	***erm*B+*mef*A/E**	***mef*A/E**
1999			
MIC50 (μg/ml)	512	1024	8
MIC90 (μg/ml)	1024	1024	16
Range	1-1024	16-1024	1-512
2007			
MIC50 (μg/ml)	512	1024	8
MIC90 (μg/ml)	1024	1024	32
Range	1-1024	2-1024	1-512

Table 4. Number of *S. pneumoniae* Isolates From all 7 Years (1999, 2002-2007) with Discordant Erythromycin resistance genotype and MIC phenotype.

Genotype	**Expected phenotype**	**Observed phenotype**	**Nbr of isolates**
ermB+	MLS_B	M	12
*erm*B+/*mef*A/E+	MLS_B	M	2
*erm*B-/*mef*A/E-	Susceptible	M	6
*mef*A/E+	M	MLS_B	6
*erm*B-/*mef*A/E-	Susceptible	MLS_B	18
Total			44 (0.97%)

Table 5. Summary of Alternative Erythromycin Resistance Mechanisms Found Among *S. pneumoniae* Isolates From all 7 Years (1999, 2002-2007).

Isolate	Phenotype	Mechanism
3494-00	MLS_B	L4 mutation
4056-00	MLS_B	L4 mutation
4066-00	MLS_B	L4 mutation
6306-99	MLS_B	L4 mutation
7853-05	MLS_B	L4 mutation
5473-07	MLS_B	L4 mutation
7509-06	MLS_B	L4 mutation
4882-07	M	L4 mutation
3802-99	M	L4 mutation
6275-99	M	L4 mutation
3662-06	M	*erm*A (*erm*TR)
2008008613	M	*erm*A (*erm*TR)
7541-06	M	*erm*A (*erm*TR) and L4 mutation
8043-06	M	*ermA (erm*TR) and L4 mutation
7582-06	M	L4 mutation

Table 6. Factors Associated with and MLS_B Phenotype Among Invasive Erythromycin-resistant *S. pneumoniae* Isolates.

		M Phenotype (n=3020)	MLS Phenotype (n=1515)				
	No of Isolates	**%**	**%**	**cOR (95% CI)**	**P value**	**aOR (95% CI)**	**P value**
Serotype							
Non PCV7 [1]	1708	49.7	54.8	1.23 (1.05-1.44)	0.0085	2.31 (1.92-2.79)	<0.0001
PCV7	1637	50.3	45.2	Referent		Referent	
19A	1190	18.6	41.5	3.11 (2.71-3.56)	<0.0001	3.38 (2.90-3.93)	<0.0001
Other	3345	81.4	58.5	Referent		Referent	
State							
Colorado	184	4.5	3.1	0.39 (0.28-0.55)	<0.0001	0.28 (0.19-0.41)	<0.0001
Connecticut	558	10.3	16.2	0.63 (0.44-0.89)	0.0089	0.47 (0.32-0.69)	<0.0001
Georgia	1095	26.8	18.9	0.87 (0.61-1.24)	0.4379	0.66 (0.44-0.97)	0.0335
Maryland	486	8.8	14.5	0.91 (0.64-1.30)	0.6167	0.70 (0.47-1.04)	0.0792
Minnesota	751	17.7	14.3	0.39 (0.27-0.58)	<0.0001	0.40 (0.26-0.61)	<0.0001
New Mexico	164	3.8	3.3	0.45 (0.32-0.63)	<0.0001	0.42 (0.29-0.62)	<0.0001
New York	351	8.6	6.1	0.38 (0.24-0.60)	<0.0001	0.32 (0.20-0.53)	<0.0001
Oregon	75	1.8	1.4	0.48 (0.31-0.76)	0.0017	0.45 (0.28-0.75)	0.0021
Tennessee	709	14.9	17.0	0.43 (0.24-0.77)	0.0050	0.39 (0.21-0.74)	0.0039
California	162	2.7	5.2	Referent		Referent	
California	162	2.8	5.1	1.85 (1.35-2.53)	0.0001	2.32 (1.64-3.28)	<0.0001
Other	4373	97.2	94.9	Referent		Referent	
Age group							
Under 2	808	18.4	16.6	Referent		Referent	
3-14	244	5.1	6.0	1.32 (0.98-1.78)	0.0691	1.42 (1.02-1.97)	0.0397
15-44	750	16.7	16.2	1.08 (0.87-1.33)	0.4976	1.60 (1.26-2.02)	0.0001
45-64	1232	26.4	28.8	1.22 (1.01-1.47)	0.0433	1.86 (1.51-2.30)	<0.0001
Over 65	1501	33.4	32.5	1.08 (0.90-1.30)	0.4005	1.66 (1.36-2.04)	<0.0001
MDR[2]							
Yes	2501	43.0	79.3	5.09 (4.41-5.87)	<0.0001	5.35 (4.60-6.22)	<0.0001
No	2034	57.0	20.7	Referent		Referent	

1. Excluding serotype 19A
2. Resistance to more than two antibiotics, other than erythromycin or clindamycin

Figure 1. Changes in Serotype Distribution among Invasive Erythromycin-resistant *S. pneumoniae* Isolates from pre (1999) to post (2002-2007) introduction of PCV7

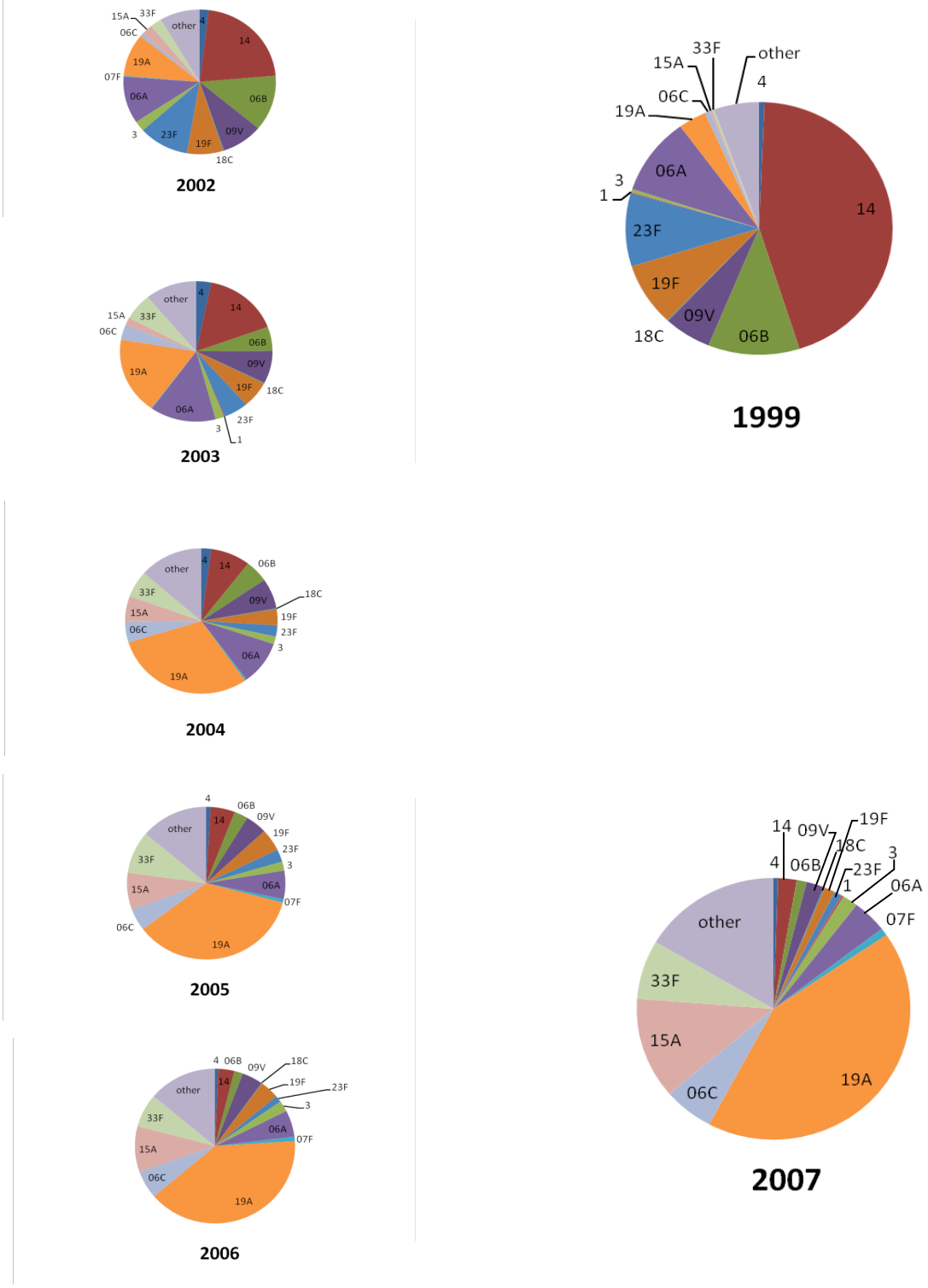


Figure 2. Distribution of Macrolide Resistance Mechanisms by Year.

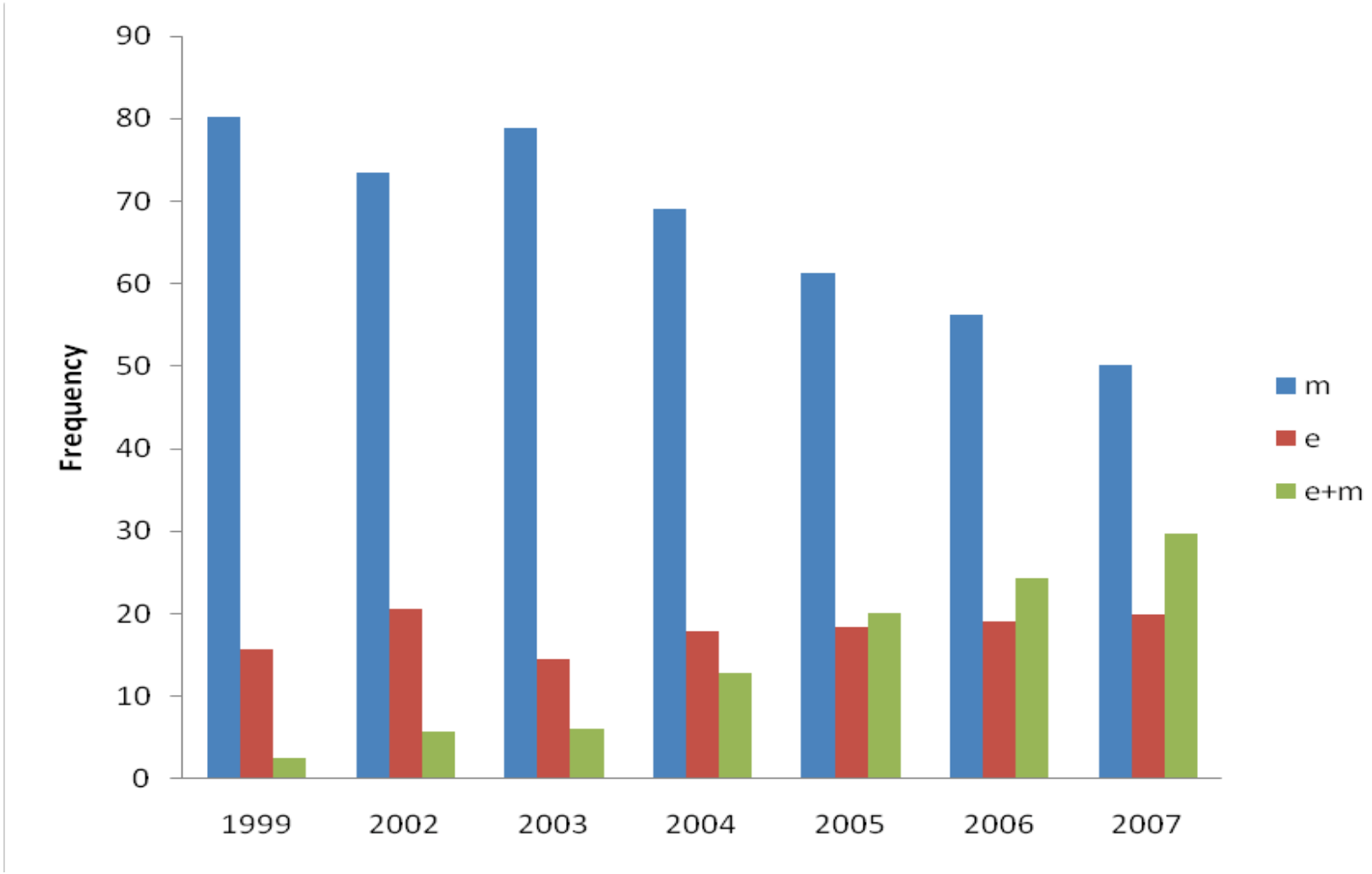
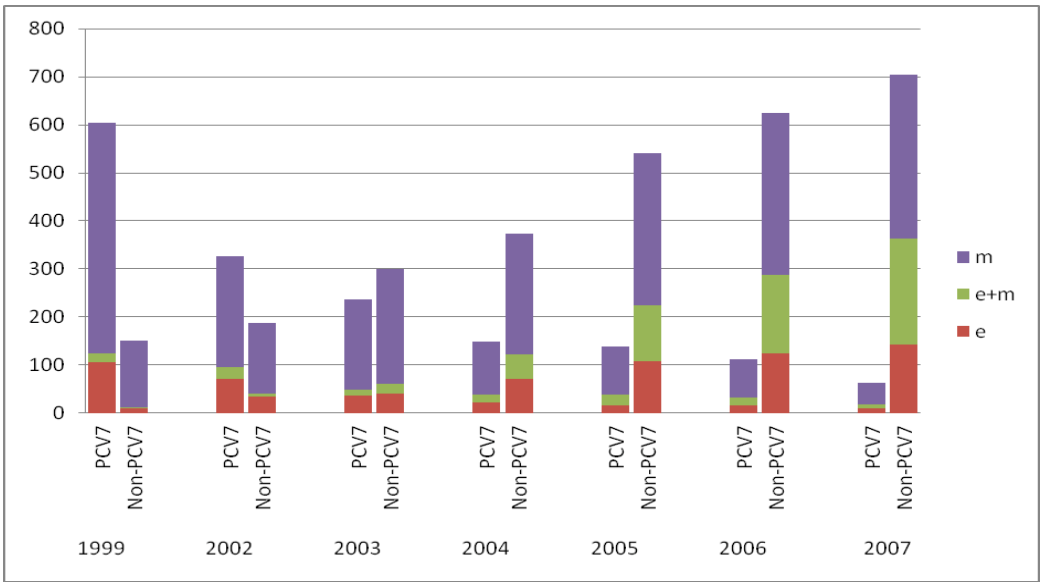
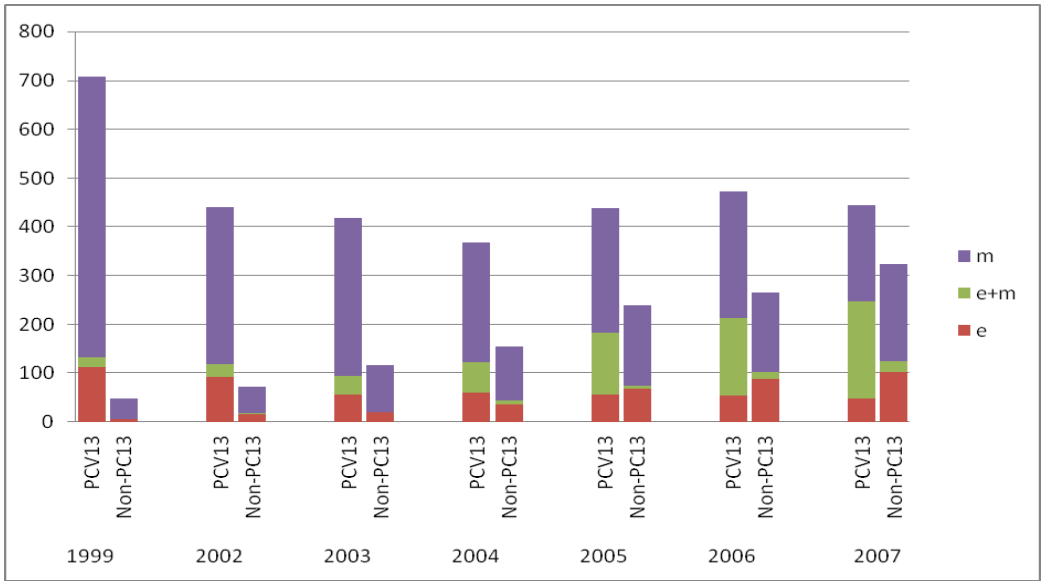


Figure 3. (A) Distribution of Macrolide Resistance Mechanisms by Year and PCV7 Serotype. (B) Distribution of Macrolide Resistance Mechanisms by Year and PCV13 serotype.

A



B



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APPENDIX

EMORY UNIVERSITY

Institutional Review Board

TO: Paulina Hawkins
Principal Investigator

DATE: October 7, 2009

RE: **Notification of Submission Determination: No IRB Review Required**
IRB00029216
Impact of the heptavalent Pneumococcal Conjugate Vaccine (PCV7) on the molecular determinants of Macrolide resistance among invasive isolates of Streptococcus pneumonia in the United States

The above-referenced study has been vetted by the Institutional Review Board (IRB), and it was 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" under applicable federal regulations. Accordingly, IRB review is not required. This study is a secondary data analysis of a de-identified dataset obtained from the Active Bacterial Core Surveillance (ABCs) program of the CDC.

45 CFR Section 46.102(f)(2) defines "Research involving Human Subjects" as follows:

> Human subject means a living individual about whom an investigator (whether professional or student) conducting research obtains:
>
> (1) data through intervention or interaction with the individual, or
> (2) identifiable private information
>
> Intervention includes both physical procedures by which data are gathered (for example, venipuncture) and manipulations of the subject or the subject's environment that are performed for research purposes. Interaction includes communication or interpersonal contact between investigator and subject. Private information includes information about behavior that occurs in a context in which an individual can reasonably expect that no observation or recording is taking place, and information which has been provided for specific purposes by an individual and which the individual can reasonably expect will not be made public (for example, a medical record). Private information must be individually identifiable (i.e., the identity of the subject is or may be ascertained by the investigator or associated with the information) in order for obtaining the information to constitute research involving human subjects.

In addition, the IRB has determined that the study is not a "Clinical Investigation" under applicable Food & Drug Administration regulations because it does not involve a test article and does not otherwise meet the requirements of the definition of "Clinical Investigation" as set forth in 21 CFR Section 50.3(c).

Please note that any changes to the protocol could conceivably alter the status of this research under the federal regulations cited above. Accordingly, any substantive changes in the protocol should be presented to the IRB for consideration prior to their implementation in the research.