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Mobile Elements and Chromosomal Changes Associated with MLS Resistance Phenotypes of Invasive Pneumococci Recovered in the United States

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Abstract

Pneumococcal macrolide resistance is usually expressed as one of two phenotypes: the M phenotype conferred by the *mef* gene or the MLS_B phenotype caused by modification of ribosomal targets, most commonly mediated by an *erm* methylase. Target-site modification leading to antibiotic resistance can also occur due to sequence mutations within the 23S rRNA or the L4 and L22 riboproteins. We screened 4,535 invasive isolates resistant to erythromycin and 18 invasive isolates nonsusceptible to quinupristin–dalfopristin (Q-D) to deduce the potential mechanisms involved. Of 4,535 erythromycin-resistant isolates, 66.2% were polymerase chain reaction (PCR)-positive for *mef* alone, 17.8% for *ermB* alone, and 15.1% for both *mef* and *ermB*. Thirty-seven isolates (0.9%) were PCR negative for both determinants. Of these, 3 were positive for *ermA* (subclass *ermTR*) and 25 had chromosomal mutations. No chromosomal mutations (in 23S rRNA, *rpD*, or *rpN*) nor any of the macrolides/lincosamides/streptogramin (MLS) resistance genes screened for (*ermT*, *ermA*, *cfr*, *lsaC*, and *vgaA*) were found in the remaining nine isolates. Of 18 Q-D nonsusceptible isolates, 14 had chromosomal mutations and one carried both *mef* and *ermB*; no chromosomal mutations or other resistance genes were found in 3 isolates. Overall, we found 28 mutations, 13 of which have not been previously described in *Streptococcus pneumoniae*. The role of these mutations remains to be confirmed by transformation assays.

Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, particularly in young children and elderly persons. The most important pneumococcal infections include pneumococcal pneumonia, meningitis, and bacteremia/septicemia, because of their high global disease burden, high case fatality rates, and associated costs.⁷ The introduction of the 7-valent vaccine in February of 2000 (PCV7, Prevnar; Wyeth

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Disclosure Statement

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Pharmaceuticals) had a major effect on the incidence of pneumococcal disease. By the end of 2001, the rate of invasive pneumococcal disease (IPD) among US children younger than two years had decreased by 69%.^{8,53} Additionally, since five of the seven serotypes in PCV7 (6B, 9V, 14, 19F, and 23F) were responsible for most antibiotic-resistant infections in the United States, the overall rate of disease caused by penicillin-nonsusceptible pneumococcal strains decreased by 57% from 1999 to 2004.³² However, the proportion of erythromycin-resistant invasive isolates increased from 20.5% during 1999 to 24.9% during 2009.¹

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, due to the *mef*-encoded efflux mechanism, and susceptible to clindamycin.⁴⁶ MLS_B phenotype isolates are resistant to macrolides (with generally higher minimum inhibitory concentrations [MICs]), lincosamides (clindamycin), and streptogramin B, as a result of the modification of ribosomal targets, most commonly mediated by an *erm* methylase⁵²; *ermB* is the predominant methylase found in *S. pneumoniae*, but *ermA* has also been reported.^{4,34,48} Both *erm* and *mef* genes are carried on mobile genetic elements.

While the *ermB* genotype is the most prevalent mechanism worldwide, occurring in 55% of all erythromycin-resistant *S. pneumoniae* isolates globally, the predominant mechanism of pneumococcal macrolide resistance in the United States was until recently mediated by the *mefA* gene. A study conducted in 1999, before the introduction of PCV7, found that the M phenotype accounted for 82% of erythromycin-resistant isolates. Most of these isolates were PCV7 types, which were most commonly associated with children younger than 5 years.²⁶ After the introduction of PCV7, the proportion of *mef* in the United States steadily decreased, while the prevalence of macrolide-resistant strains carrying both *ermB* and *mefA* genes has increased.^{15,18,19,20,21,27,28}

Macrolide resistance has also been frequently reported to occur due to substitutions in the 23S rRNA and in the L4 and L22 riboproteins.^{6,25,49,50,54,56} Efflux mechanisms encoded by other genes such as *vga* and *Isa* have also been associated with macrolides/lincosamides/streptogramin (MLS) resistance in other species, and the multidrug resistance gene *cfi*, encoding a 23S rRNA methyltransferase, was recently identified for the first time in streptococci.⁵¹

To determine the molecular mechanisms involved, we screened a large number of erythromycin and/or clindamycin-resistant isolates and all quinupristin–dalfopristin (Q-D) nonsusceptible isolates, submitted to the Active Bacterial Core Surveillance (ABCs) (part of the Centers for Disease Control and Prevention’s [CDC] Emerging Infections Program: <http://www.cdc.gov/abcs/index.html>), from participating sites pre- (1999) and post-PCV7 introduction (2002–2007). In this report, we describe previously unreported ribosomal changes associated with pneumococcal resistance to macrolides and related antibiotics.

Materials and Methods

Bacterial strains and antimicrobial susceptibility testing

Twenty-six thousand nine hundred seventy-four invasive *S. pneumoniae* isolates were submitted to ABCs from participating sites (www.cdc.gov/abcs/methodology/surv-pop.html) pre- (1999) and post- (2002–2007) introduction of PCV7. Isolates were serotyped by latex agglutination and the Quellung reaction. MICs to erythromycin, clindamycin, and Q-D were determined by the broth microdilution method. Antibiotic resistance was determined based on the Clinical and Laboratory Standards Institute (CLSI) breakpoints: for erythromycin and clindamycin, isolates with MIC ≥ 1 $\mu\text{g/ml}$ were classified as resistant, and for Q-D, isolates with MIC ≥ 2 $\mu\text{g/ml}$ were classified as nonsusceptible.^{9,11} Inducible clindamycin resistance was determined by the D-zone disk diffusion test^{10,11} and broth microdilution using an erythromycin–clindamycin (ERY-CLD) combination well. Four thousand five hundred thirty-five isolates (16.8%) were erythromycin resistant and available for testing. Eighteen additional Q-D-nonsusceptible isolates were identified through ABCs (submitted from 1996 to 2010) and included in the study. Q-D breakpoints were also determined using *E*-tests, following the manufacturer's instructions.

Polymerase chain reaction amplification and sequencing

Polymerase chain reaction (PCR) amplification of *ermT*, *ermA* (subclass *ermTR*), *ermB*, *mef*, *rpD* (L4), *rpN* (L22), and the 23S rRNA genes (domain II and V) was carried out using previously described protocols.^{45,47,48,57} Screening for genes *cfi*, *lsaC*, and *vgaA* was also carried out, as previously described.^{31,39,41} PCR products were purified using ExoSAP-IT (Affymetrix, Inc.) and cycle sequenced using the BigDye Terminator v3.1 chemistry (Life Technologies Corporation) according to the manufacturer's protocol.² Sequencing reactions were purified using a Sephadex-based method; briefly, sequencing reactions were transferred onto Sephadex G-50 columns prepacked into microtiter filter plates (Millipore) and then centrifuged at 750 *g* for 2 min onto clean 96-well plates. Purified reactions were analyzed on an ABI3130xl automated sequencer. The sequences obtained were compared to the TIGR4 (NC003028) genome, and then screened against all whole genome shotgun sequences available in the NCBI database.

Newly determined nucleotide sequence data have been submitted to GenBank under accession numbers KM098099-KM098108.

Results

Serotype and antimicrobial resistance

Forty-eight different serotypes were represented among the macrolide-resistant isolates included in this study. The five most common serotypes associated with macrolide resistance were 19A (26.2%), 14 (14.5%), 6A (9.1%), 15A (6.0%), and 6B (5.4%). During 1999, before the introduction of PCV7, the seven serotypes included in the vaccine accounted for 79.5% of macrolide-resistant invasive isolates; after PCV7 was introduced, this proportion decreased to 63.4% in 2002 ($p < 0.0001$) and to 8.3% in 2007 ($p < 0.0001$). Among non-PCV7 serotypes, the largest changes in proportion among macrolide-resistant invasive

isolates were observed in 19A (from 3.4% in 1999 to 42.3% in 2007, $p < 0.0001$) and 15A (from 0.3% to 12.5%, $p < 0.0001$), followed by 33F (from 0.3% to 7.3%, $p < 0.0001$) and 6C (from 0.7% to 5.9%, $p < 0.0001$).

Mechanisms of MLS resistance

The distribution of isolates positive for *ermB* and/or *mef* among PCV7 and non-PCV7 serotype isolates is shown in Table 1. The dual *ermB* + *mef* genotype increased significantly among these isolates from 1999 to 2007 ($p < 0.0001$), while the *mef*-only genotype went from being found in 80% of the resistant invasive isolates in 1999 to accounting for only half of the resistant isolates in 2007. No significant changes were observed in the proportion of *ermB*-only genotype.

Changes in the prevalence of these mechanisms of erythromycin resistance among invasive isolates were closely related to changes in serotype distribution (Fig. 1). The *mef*-only genotype decreased slightly in prevalence, but still remained the predominant mechanism among PCV7 serotype isolates across the years in the study. The *ermB* + *mef* genotype increased somewhat among PCV7 serotype isolates due to the relative number of 19F isolates (data not shown), but remained as a minor proportion. In contrast, among non-PCV7 serotype isolates in our study, the proportion of *mef*-only isolates declined from 91.3% in 1999 to 48.5% in 2007 ($p < 0.0001$), while the proportion of *ermB* + *mef* isolates increased as a function of 19A emergence from 0.7% in 1999 to 31.3% in 2007 ($p < 0.0001$). Most isolates positive for *ermB* + *mef* from 2007 were serotype 19A (85.5%). The proportion of *ermB*-only isolates also increased among non-PCV7 serotypes, but not significantly.

Thirty-seven macrolide-resistant isolates were negative for both *ermB*- and *mef*-mediated mechanisms and were therefore screened for the presence of *ermA* (subclass *ermTR*), *cfi*, *IsaC*, and *vgaA* genes, as well as changes (relative to TIGR4) in genes encoding L4 (*rpD*) and L22 (*rpN*), and in the 23S rRNA. Three isolates were positive for *ermA* (subclass *ermTR*) and 25 had changes in the 23S rRNA or the ribosomal proteins (L4 and L22). No changes were found in nine of the isolates. None of the isolates contained any of the other MLS resistance genes tested, and clindamycin resistance was not inducible in any of the clindamycin-susceptible strains (Table 2).

Of the 25 isolates with ribosomal changes, 18 isolates had an A to G substitution at position 2061 (A2059 in *Escherichia coli*) in the 23S rRNA, 7 had substitutions or insertions in L4, 3 of those with multiple changes, and 3 isolates had insertions in L22.

Since all 3 isolates with insertions in L22 were also resistant to Q-D, we screened an additional 18 Q-D nonsusceptible isolates from ABCs (submitted from 1996 to 2010). Six of them had insertions similar to those previously observed and one had a substitution in L22. Three isolates had insertions, and two isolates had substitutions in L4. Lastly, one isolate had a C-to-G change at position 2613 (C2611 in *E. coli*) and another had an A-to-C change at position 2060 (A2058 in *E. coli*) in the 23S rRNA. Incidentally, one of the isolates was positive for both *ermB* and *mef*, and one was positive for *mef* only. In three of these isolates, the potential mechanisms were not apparent (Table 3).

Overall, we found a total of 28 changes in the 23S rRNA and the ribosomal proteins L4 and L22 (relative to TIGR4), 11 of which have never been reported (Table 4). In addition, we found four changes relative to TIGR4 that correspond to the natural variants of L4, L22, and 23S rRNA and do not correlate with changes in macrolide susceptibility: an S20N substitution in L4, an I39T substitution in L22, plus a ⁶⁸³AA₆₈₄ to CC substitution, and a U676C substitution in domain II of the 23S rRNA.

We also identified three isolates that were clindamycin susceptible in spite of carrying the *ermB* gene. They were checked by the D-zone disk diffusion test and broth microdilution, and resistance was not found to be inducible. The *ermB* structural gene was sequenced and no changes were found. This was also the case for one of the isolates carrying *ermA* (4336-05), where clindamycin resistance was not inducible, and no changes were found in the *ermA* structural gene sequence, suggesting a defect in gene expression.

Discussion

PCV7 implementation had a marked impact on the distributions of macrolide resistance determinants among invasive isolates in the United States, due to decreases in vaccine-targeted strains and increases in certain nonvaccine serotype strains. Before the introduction of PCV7, the majority of *mef*-only isolates among the macrolide-resistant isolates in the study were associated with serotype 14 (64.4%), which was the most common serotype among all macrolide-resistant isolates in the study (44.5%). After the introduction of PCV7, the proportion of serotype 14 among the study isolates declined rapidly to 21.7% in 2002 and to 2.2% by 2007, contributing to the decrease of the *mef*-only genotype among macrolide-resistant pneumococci from 80.2% in 1999 to only 51.3% in 2007. In contrast, the proportion of *ermB* + *mef* isolates increased from 0.7% in 1999 to 31.3% in 2007, mainly driven by the emergence of macrolide-resistant serotype 19A in the post-PCV7 period. Serotype 19A accounted for 3.4% and 42.3% of pneumococcal macrolide resistance in 1999 and 2007, respectively. During 2007, 85.5% isolates positive for *ermB* + *mef* were of serotype 19A.

Population-based surveillance in the United States revealed that 82% of penicillin-resistant invasive serotype 19A isolates recovered during 2007 belonged to clonal complex CC320/271^{19A}, and the majority of these were also resistant to macrolides (96%) and lincosamides (79%).³ It should be noted that CC320/271 includes the Taiwan^{19F}-14 clone, which is the likely ancestral strain of this complex and also carries *ermB* and *mef*. Serotype 19A and its associated high-level resistance has already decreased dramatically within ABCs surveillance areas following the introduction of the 13-valent pneumococcal vaccine (PCV13, Prevnar13; Wyeth Pharmaceuticals) in February of 2010 (unreported data), and is likely to continue to decrease, as also evidenced by early reports of decreased carriage of 19A and decreased rates of IPD caused by 19A.^{12,30,44} MLS_B-type resistance within non-PCV13 serotype 15A, seen here associated with *ermB*, modestly increased in the post-PCV7 period due to the increase in multilocus sequence-type ST63.²⁴ In the post-PCV13 period, these and other nonvaccine serotype-resistant strains could potentially continue to emerge, possibly benefitting from the removal of PCV13 serotypes from the carriage reservoir.

Aside from the changes in serotype and the *ermB*- and/or *mef*-mediated mechanisms, we also identified potentially important resistance-associated changes within the ribosomal proteins L4 and L22 and the 23S rRNA. Eleven of the 28 changes identified have not been previously described and an additional 2 have been reported in other bacterial species, but not in *S. pneumoniae*.

Seven different changes were found in a highly conserved region of L4 (Table 4), ${}_{63}\text{KPWRQKGTGRAR}_{74}$, including three previously unreported insertions (ranging from 3 to 12 bp) associated with macrolide and/or Q-D resistance, and one insertion (KG after position 69) previously reported to confer resistance to azithromycin, but not erythromycin nor Q-D, in *Streptococcus pyogenes*.³⁷ Similar insertions have been reported in *S. pneumoniae* and other gram-positive species in association with resistance to macrolides and streptogramins.²³ We also found three substitutions, including a ${}_{69}\text{GTG}_{71}$ to TPS substitution that has been previously shown to confer resistance to macrolides, and streptogramin B,^{17,50,54} and to telithromycin as well when combined with the presence of *ermB*⁵⁵; plus two substitutions, Q67R and R72G in a Q-D, which were described in a previous report.¹⁴ Although previous linezolid nonsusceptible clinical *S. pneumoniae* isolates have only been associated with deletions in L4,^{17,55} similar substitutions have been reported in laboratory-derived mutants of pneumococcal strains²² and clinical isolates of other species.³⁶ In addition, a Ser-to-Leu substitution was found at position 112, but only in combination with other changes.

In L22, we found a total of 10 changes in conserved regions of the C-terminal sequence (Table 4), including 2 substitutions and 8 insertions (ranging from 15 to 27 bp), associated with macrolide and Q-D resistance. Five of the insertions were in the region ${}_{102}\text{KRTAHITVA}_{110}$; three have been previously reported,^{6,17,29,40} and two newly observed insertions are very similar as well (KRTAHIT and VRTAHIT). The three remaining insertions were in a region where an insertion has been previously reported in macrolide-resistant *S. pneumoniae* isolates (${}_{85}\text{PMKRFRPR}_{92}$).⁴³ Deletions have also been reported in this region of the *Staphylococcus aureus* L22 protein in association with Q-D resistance.³⁸ The two substitutions T85P and R103C have not been previously reported.

Mutations in primary rRNA sequence have been found to play an important role in antibiotic resistance and there is growing evidence pointing to their role in MLS resistance.^{13,52} In this study, three potentially resistance-conferring mutations were found in the peptidyltransferase loop of the 23S rRNA of 20 invasive isolates. Position 2611 (*E. coli* numbering) is important in maintaining the stem preceding the single-stranded portion of the peptidyltransferase loop containing positions A2058 and A2059,^{5,35} and a C-to-G substitution at this position has been previously reported to confer resistance to macrolides and Q-D¹⁶; this substitution was observed in one of our isolates with the same phenotype (7799-07). Another isolate in our study carried an A2060C (A2058 in *E. coli*) substitution that has not been previously reported in *S. pneumoniae*, but has been reported in *Mycobacterium intracellulare*,⁵² *Mycoplasma pneumoniae*,⁴² and *C. jejuni*,³³ in association with an MLS_B phenotype. The isolate in our study was found to be resistant to erythromycin and clindamycin and nonsusceptible to Q-D; the elevated Q-D MIC (2 µg/ml) is likely explained by resistance to the streptogramin B unit (quinupristin) of Q-D. In addition, 18 isolates carried an A2061G

(A2059 in *E. coli*) substitution that has been frequently reported in association with an ML, MLS_B, or MS_B phenotype,^{16,49,50} which are the same phenotypes we observed. It has been previously suggested that the different phenotypes and levels of resistance associated with this substitution depend on the number of 23S rRNA alleles that are mutated.¹⁶ However, another study on a large population of isolates argued against this and instead suggested that differential expression due to environmental pressure may be the explanation for these phenomena.¹⁷ Since we did not individually amplify the four alleles present in *S. pneumoniae* nor measured their expression, we are unable to draw conclusions either way, but believe that both possibilities merit further investigation.

Future plans include performing transformation assays to determine the role of the 13 changes found in this study, as well as further characterization by whole genome sequencing of the isolates where the mechanism of resistance remains unexplained.

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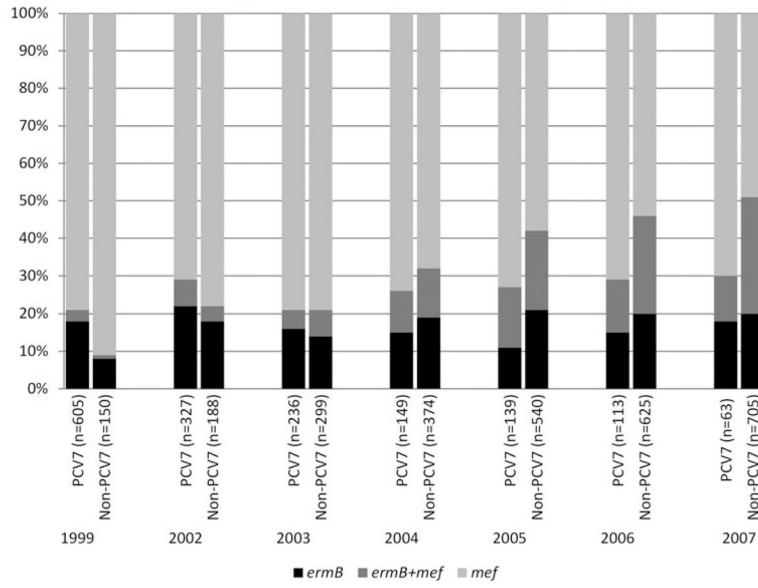


FIG. 1. Changes in distribution of the two major macrolide resistance determinants among PCV7 versus non-PCV7 serotypes.

Table 1
Distribution of *ermB* and *mef* Among Macrolide-Resistant Isolates Pre- and Post-PCV7 Introduction

Year	<i>ermB</i> + (%)	<i>ermB</i> + / <i>mef</i> + (%)	<i>mef</i> + (%)	<i>ermB</i> - / <i>mef</i> - (%)	Total
1999	120 (15.6)	20 (2.6)	615 (80.2)	12 (1.6)	767
2002	106 (20.6)	29 (5.6)	376 (73.0)	4 (0.8)	515
2003	76 (14.1)	37 (6.9)	421 (78.3)	4 (0.7)	538
2004	95 (18.1)	71 (13.5)	356 (67.9)	2 (0.4)	524
2005	123 (18.1)	133 (19.6)	420 (61.7)	3 (0.6)	679
2006	139 (18.7)	173 (23.3)	422 (56.9)	8 (1.1)	742
2007	150 (19.5)	221 (28.7)	395 (51.3)	4 (0.5)	770

Table 2
 Chromosomal Changes Associated with MLS Resistance Among Isolates Not Containing *ermB* and/or *mef* ($n = 37$)

Year	Strain ID	Serotype	ERY	CLD ^a	Q-T ^b	Changes in L4 ^{c,d}	Changes in L22 ^{c,d}	Changes in 23S rRNA ^c	Other ^e
1999	3802-99	19A	> 32	0.12	1.5	⁶⁹ GTG ₇₁ to TPS, E13Q, E30Q, V88I, G98A, A128S, S130E			
1999	6275-99	19A	> 32	0.06	0.5	⁶⁹ GTG ₇₁ to TPS, E13Q, E30Q, V88I, G98A, A128S, S130E			
2002	5087-03	19A	> 32	0.12	1	⁶⁹ GTG ₇₁ to TPS, E13Q, E30Q, V88I, G98A, A128S, S130E			
2002	5486-02	23F	2	< 0.03	1	E30K ⁶³ KPWRQKIGTGRA ₇₃			
2002	5001-02	22F	2	0.12	8	S112L	¹⁰² KRTAHTTRFAHIVA ₁₁₀		
2005	5309-05	22F	4	0.06	24	S112L	¹⁰² KRTAHTKRTAHITVA ₁₁₀		
2007	6466-07	19A	16	0.25	> 32		⁸⁵ PMKRFRPPMKRFRPR ₉₂ T85P		
1999	2009219579	35F	2	0.12	0.5			A2061G	
1999	3494-00	19A	16	0.5	1			A2061G	
1999	4397-99	004	32	1	1			A2061G	
1999	1361-00	06B	> 32	1	1			A2061G	
1999	4056-00	06B	> 32	2	1.5			A2061G	
1999	7783-99	09N	> 32	2	1			A2061G	
1999	5667-99	06B	> 32	> 2	2			A2061G	
2003	6752-04	10A	> 32	0.25	1			A2061G	
2003	8297-04	35B	> 32	2	1			A2061G	
2004	9764-04	034	32	1	1			A2061G	
2005	3662-06	06C	> 32	> 2	1.5			A2061G	
2005	7853-05	06A	> 32	2	2			A2061G	
2006	5473-07	22F	> 32	2	2.0	S112L		A2061G	
2006	6549-07	17F	32	1	0.5			A2061G	
2006	7509-06	15C	> 32	> 2	2			A2061G	
2006	8043-06	23A	> 32	2	1.5			A2061G	
2007	2010200843	09N	32	1	2			A2061G	
2007	7948-07	034	> 32	> 2	1.5			A2061G	
2007	2008008613	19A	32	1	1			A2061G	<i>ermA</i> +
2006	7541-06	003	> 32	2	1			A2061G	<i>ermA</i> +
2004	4336-05	038	2	0.12	0.5			A2061G	<i>ermA</i> +

Year	Strain ID	Serotype	ERY	CLD ^a	Q-D ^b	Changes in L4 ^{c,d}	Changes in L22 ^{c,d}	Changes in 23S rRNA ^c	Other ^e
1999	6306-99	24F	16	1	0.5				None
2006	7582-06	23A	32	2	1				None
2003	7995-03	07F	16	0.12	0.5				None
1999	4066-00	014	16	1	1				None
2006	4882-07	07F	4	0.12	0.5				None
2006	7701-07	35F	> 32	2	1.5				None
2003	2003000312	10A	> 32	2	2				None
2002	2009214597	06A	> 32	1	0.5				None
1999	5272-03	001	8	0.25	0.5				None

^a Clindamycin resistance was not inducible in any of the clindamycin-susceptible strains.

^b Determined by Etest.

^c Relative to TIGR4.

^d Insertions are underlined.

^e *ermT*, *ermA* (subclass *ermTR*), *cfi*, *IsxC*, and *vgaA*.

CLD, clindamycin; ERY, erythromycin; MLS, macrolides/lincosamides/streptogramin; Q-D, quinupristin-dalfopristin (Synercid).

Table 3

Changes Associated with Quinupristin–Dalfopristin Nonsusceptibility

Year	Strain ID	Serotype	ERY	CLD	Q-D ^d	Changes in L4 ^{c,d}	Changes in L22 ^{c,d}	Changes in 23S rRNA ^c	Other ^e
1997	3179-97	09V	4	0.12	2.0				
1997	1095-99	11A	> 8	4	2.0			A2060C	e + m
2007	2008236056	19A	> 32	> 2	2.0				
2007	2008227074 ^b	09N	1	0.06	3.0	Q67R R72G			
2000	4339-00	19F	0.5	0.06	4.0	<u>63</u> KPW/RQK <u>KG</u> GTGRAR ₇₄			
2001	5663-01	19F	32	0.06	4.0	<u>63</u> KPW/RQK <u>GSOK</u> GTGRAR ₇₄			m
2003	6766-04	12F	0.25	0.03	4.0	<u>63</u> KPW/RP <u>WRQK</u> GTGRAR ₇₄			
2008	2008237895	07F	0.5	0.06	4.0		<u>78</u> AFANEGPTIAFANEGPTM ₈₆		
2000	7697-03	23F	1	0.12	8.0	E30K			
2001	2009216547	014	2	0.06	8.0		<u>102</u> KRTAHITRTAHITVA ₁₁₀		
2001	6434-01	06A	2	0.06	8.0		<u>102</u> KRTAHITKRTAHIVA ₁₁₀		
1996	0571-97	004	1	0.06	12.0		<u>102</u> KRTAHITKRTAHITVA ₁₁₀	C2613G	
2007	7799-07	11A	> 32	1	12.0				
2010	2010223420	19A	4	0.06	12.0		<u>102</u> KRTAHITVTRTAHITVA ₁₁₀		
2008	2009208197	16F	1	0.06	16.0		<u>85</u> TMKRRFRPRA <u>SFR</u> PRAK ₉₄		
2000	6356-00	06A	4	0.06	32.0		R103C		None
1995	3262-95	06A	2	0.03	12.0				None
2001	5951-01	004	1	0.06	8.0				None

^aDetermined by Etest.

^bThis strain is nonsusceptible to linezolid (minimum inhibitory concentration = 4 µg/ml).

^cRelative to TIGR4.

^dInsertions are underlined.

^e*ermT*, *ermA* (subclass *ermTR*), *ermB* (e), *mef* (m), *ctr*, *lsaC*, and *vgxA*.

Table 4

Summary of Alterations Found in L4, L22, and 23s rRNA, Among ERY-Resistant (*erm* - /*mef*⁻, *n* = 37) and Q/D Nonsusceptible Isolates (*n* = 18)

Gene	Mutation ^a	Observed phenotype ^b	Reported phenotype ^b	Reference
<i>rpD/L4</i>	E13Q, E30Q, V88I, G98A, A128S, S130E	See notes c and d	No resistance	56
	E30K	Q-D	M	54
	S112L	See note d		This study
	Q67R + R72G	Q-D LZD	LZD	14
	69GTG71 to TPS	MS _B	ML, M, MS _B	17, 50, 56
	63KPWRQKTGTGRAR74	M		This study
	63KPWRQKGGTGRAR74	Q-D	ERY-I AZI-R	37 ^e
	63KPWRQKGSQKGTGRAR74	M Q-D		This study
	63KPWRPWRQKGTGRAR74	Q-D		This study
<i>rpN/L22</i>	R103C	M Q-D		This study
	102KRTAHIT <u>RTAH</u> IVA110	M Q-D	M Q-D	29
	102KRTAHIT <u>RTAH</u> ITVA110	M Q-D	ML, M, M Q-D	17, 40
	102KRTAHIT <u>KRTAH</u> IVA110	M Q-D	M Q-D	6
	102KRTAHIT <u>KRTAH</u> ITVA110	M Q-D		This study
	102KRTAHIT <u>VRTAH</u> ITVA110	M Q-D		This study
	T85P	See note 4		This study
	85PMKRFRPPMKRFRPRAK94	M Q-D		This study
	85TMKRFRPRASFRPRAK94	M Q-D		This study
23s d. V	A2061G (2059 in <i>Escherichia coli</i>)	M, MLS _B	ML, MLS _B , MS _B	16, 49, 50, 54
	C2613G (2611 in <i>E. coli</i>)	M Q-D	M, M Q-D	16, 49, 52
	A2060C (2058 in <i>E. coli</i>)	MLS _B	ML, M	33, 42, 52 ^e

^aInsertions are underlined.

^b“Observed phenotype” refers to this study, while “Reported phenotype” refers to phenotypes observed in previous reports.

^cThese changes were found in combination with the 69GTG71 to TPS substitution.

^dOnly found in combination with other changes, its role in resistance is unclear.

^eThese reports pertain to other bacterial species. Our study is the first to report these changes in *Streptococcus pneumoniae*.

AZI, azithromycin; ERY, erythromycin; LZD, linezolid; L, lincosamides; M, macrolides; S_B, streptogramin B; Q-D, quinupristin–dalfopristin (Synercid).