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Predominance of 23S rRNA Mutants among Non-Erm, Non-Mef Macrolide-Resistant Clinical Isolates of *Streptococcus pneumoniae* Collected in the United States in 1999-2000

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A total of 322 erythromycin-resistant pneumococci from TRUST 3 and TRUST 4 United States surveillance studies (1999–2000) were screened for 23S rRNA, L4, and L22 gene mutations. Nineteen isolates, two with *mefA*, had mutations at position 2058 or 2059 in 23S rRNA. Two had a $_{69}$GTG$_{71}$-to-TPS substitution in L4; one of these also contained *ermA*.

The most prevalent mechanisms of macrolide resistance in *Streptococcus pneumoniae* are mediated by *mefA*, a gene encoding an efflux pump specific for 14- and 15-membered macrolides, and *ermB*, a methylase that dimethylates A2058 in 23S rRNA, resulting in resistance to macrolides, lincosamides, and streptogramin B antibiotics. It has been shown that macrolide resistance in laboratory-generated mutants can be caused by mutations in 23S rRNA and ribosomal proteins L4 and L22 (1, 17). Recently, macrolide-resistant clinical isolates of *S. pneumoniae* that contain the same ribosomal mutations have been identified (3, 5, 8, 9, 12, 16).

In order to determine the specific ribosomal mutations (i.e., ribosomal protein or 23S rRNA) that exist in macrolide-resistant clinical isolates in the United States, isolates that were *mefA* and *ermB* negative were checked for mutations in the genes coding for ribosomal proteins L4 and L22 and 23S rRNA. Isolates were also checked for the presence of *ermA*. Specifically, from previous studies in our lab, we had in our possession 70 macrolide-resistant *S. pneumoniae* isolates collected in 1999 and 252 macrolide-resistant isolates collected in 2000, representing a partial collection of all the macrolide-resistant isolates from TRUST 3 and TRUST 4 surveillance studies, respectively. Most of the TRUST 3 isolates (53/70) were highly resistant to azithromycin (MIC $\geq$ 16 μg/ml; range, 4 to >128 μg/ml), while the 252 TRUST 4 isolates included strains with lower macrolide MICs (azithromycin MIC range, 1 to >128 μg/ml).

MICs were determined by broth microdilution using panels manufactured by Trek Diagnostic Systems (Westlake, OH) and using NCCLS methods (10). The *ermB* and *mefA* genes were detected by PCR as described by Sutcliffe et al. (14), and mutations in 23S rRNA, L4, and L22 genes were detected as described by Tait-Kamradt et al. (17). Detection of *ermA* by PCR was done as described by Syrogiannopoulos et al. (15). Genetic relatedness among macrolide-resistant isolates containing ribosomal mutations was determined by serotyping and pulsed-field gel electrophoresis (PFGE) as described previously (2).

The genotypes of the 70 TRUST 3 isolates and 252 TRUST 4 isolates are presented in Table 1. For the TRUST 3 isolates, *ermB* was the most prevalent resistance mechanism (Table 1), which can be attributed to the bias for very high levels of macrolide resistance in this isolate collection: 76% of isolates had azithromycin MICs of ≥16 μg/ml. However, *mefA* was the most common resistance mechanism in the TRUST 4 isolates (Table 1); these isolates had a broader range of azithromycin MICs and, hence, were more representative of the natural distribution of macrolide-resistant isolates (7). Mutations in the 23S rRNA genes were much more common than L4 mutations. Two isolates (5459 and 5486) had *mefA* but exhibited uncharacteristically high macrolide MICs (i.e., azithromycin MICs of >128 μg/ml) and resistance to clindamycin (Table 2). Upon further characterization, these two isolates were also found to contain 23S rRNA mutations (Table 2). In addition, one of these isolates (5486) had a mutation leading to a Glu77-to-Gly substitution in L22. While mutations in the gene encoding L22 have been shown to cause macrolide resistance in clinical isolates (5, 6, 8), this particular substitution has not been previously reported, and its role in resistance is uncertain.

Nineteen isolates had mutations in domain V of 23S rRNA at position 2058 or 2059 (*Escherichia coli* numbering), and two isolates had a three-amino-acid substitution in ribosomal protein L4 (Tables 1 and 2). All isolates with ribosomal mutations were resistant to azithromycin, clarithromycin, and erythromycin. Isolates with a A2058G mutation (three or four copies mutated) had the highest macrolide MICs, even higher than those of isolates with all four 23S rRNA gene copies with mutations at position 2059 (A to G or C) (Table 2). Seventeen of the 21 isolates with a ribosomal mutation were nonsusceptible to clindamycin (MIC, ≥0.5 μg/ml) (Table 2). While the isolate with *ermA* and an L4 mutation (5430) was susceptible to clindamycin by broth microdilution, a double-disk test with erythromycin and clindamycin showed this isolate to be inducibly clindamycin resistant.

A majority of isolates (14/21) with ribosomal mutations had low telithromycin MICs (≤0.06 μg/ml); however, four isolates with mutations at A2058/A2059 had elevated telithromycin

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TABLE 1. Genotypes of macrolide-resistant *S. pneumoniae* isolates

Resistance mechanism	No. (%) of isolates from indicated group with genotype[a]		
	TRUST 3 (yr 1999)	TRUST 4 (yr 2000)	TRUST 3 and TRUST 4 combined
mefA	20 (28.6)	159 (63.1)	179 (55.6)
ermB	34 (48.6)	53 (21.0)	87 (27.0)
mefA/*ermB*	6 (8.6)	29 (11.5)	35 (10.9)
23S rRNA[b]	9 (12.9)	8 (3.2)	17 (5.3)
mefA/23S rRNA[b]	0	2 (0.8)	2 (0.6)
L4[b]	1 (1.4)	0	1 (0.3)
ermA/L4[b]	0	1 (0.4)	1 (0.3)

[a] There were 70 isolates in the group from the TRUST 3 study and 252 in the group from the TRUST 4 study.
[b] Mutation in 23S rRNA or ribosomal protein L4.

MICs (0.25 to 1.0 μg/ml) (Table 2). A recent publication by Doern and Brown showed that in the PROTEKT 2000-2001 surveillance study, the telithromycin MIC_{90} among all isolates of *S. pneumoniae* was 0.5 μg/ml (4). However, among penicillin-susceptible pneumococci, the telithromycin MIC_{90} was 0.03 μg/ml. Three isolates with elevated telithromycin MICs in our study were penicillin-susceptible; thus, their telithromycin MICs were 8- to 32-fold higher than the reported MIC_{90} from the PROTEKT study (Table 2). This indicates that isolates with elevated telithromycin MICs were present before the introduction of telithromycin in the United States.

Other ribosomal-acting agents (tetracycline, linezolid, and quinupristin-dalfopristin) were tested and all isolates were susceptible except for isolate 5459, which was resistant to tetracycline (Table 2). Nine (43%) isolates were penicillin resistant (Table 2).

PFGE analyses showed that three isolates (5719, 5892, and 5948) with serotype 23F had almost identical SmaI patterns, but they did not all have the same resistance mechanisms (Table 2). Two isolates (5430 and 5501) with serotype 29 had the same PFGE pattern but different resistance mechanisms (Table 2). All other isolates had unique PFGE patterns.

The macrolide-resistant isolates with ribosomal mutations that were identified in this study had mutations primarily in domain V of 23S rRNA, and most were not clonally related. Non-Erm, non-Mef macrolide-resistant *S. pneumoniae* isolates from Finland were recently reported to contain mutations primarily in 23S rRNA. Some of these isolates were clonally related (12). In contrast, non-Erm, non-Mef macrolide-resistant *S. pneumoniae* isolates from Eastern Europe were reported to primarily have mutations in the ribosomal protein L4, and a majority of these isolates were clonally related (9, 16).

Macrolide/azalide usage continues to increase (7). Macrolide prescriptions increased 13% from 1993 to 1999, despite total antibiotic prescriptions decreasing 15% during the same time frame. The greatest increase in macrolide prescriptions was reported for children less than 5 years of age, for whom the number of prescriptions increased 320% from 1993 to 1999 (7). Twenty-nine percent (6/21) of the isolates containing ribo-

TABLE 2. MICs and genetic analysis of macrolide-resistant *S. pneumoniae* isolates containing ribosomal mutations

Isolate no.	Yr isolated	State	Patient age (yrs)	Source	Resistance mechanism[b]	Mut:wt[c]	MIC[a] (μg/ml)									
							ERY	CLR	AZM	TEL[d]	CLI	TET	LZD	Q-D	PEN	ST[e]
5296	1999	MI	67	Sputum	A2058G	2:2	8	64	128	0.015	1	0.12	1	0.5	≤0.03	7
5948	2000	TX	83	Sputum	A2058G	2:2	>128	>128	>128	0.12	4	0.5	2	1	16	23F
5304	1999	CA	4	Sputum	A2058G	3:1	>128	>128	>128	0.06	2	0.12	0.5	1	≤0.03	9V
5266	1999	IA	1	Ear	A2058G	3:1	>128	>128	>128	0.25	4	0.25	1	1	≤0.03	23F
5462	2000	MD	41	Sputum	A2058G	4:0	>128	>128	>128	0.12	16	0.25	1	1	≤0.03	19F
5501	2000	VA	51	Sputum	A2058G	4:0	>128	>128	>128	0.25	16	0.25	1	1	4	29
5259	1999	VA	54	Blood	A2058T	4:0	>128	64	128	1	2	0.25	1	1	≤0.03	22F
5301	1999	NY	57	Sputum	A2059C	4:0	>128	64	128	0.06	0.5	0.25	1	0.5	≤0.03	13
5250	1999	IN	43	Unknown	A2059G	2:2	8	2	128	0.004	0.03	0.12	1	0.12	≤0.03	9V
5853	2000	VT	29	Wound	A2059G	3:1	8	4	16	0.015	0.25	0.25	1	0.5	0.03	4
5892	2000	KY	29	Unknown	A2059G	3:1	32	8	>128	0.03	1	0.25	2	1	8	23F
5265	1999	IA	1	Ear	A2059G	4:0	128	16	>128	0.06	0.5	0.25	1	1	≤0.03	14
5437	2000	FL	81	Sputum	A2059G	4:0	64	32	>128	0.015	1	0.25	2	0.25	≤0.03	19F
5322	1999	NY	4	Trac asp	A2059G	4:0	128	16	>128	0.03	1	0.5	1	0.5	2	NT
5719	2000	CT	Un[f]	Unknown	A2059G	4:0	64	32	>128	0.03	1	0.5	2	0.5	16	23F
5252	1999	CA	1	BAL	A2059G	4:0	>128	16	128	0.03	1	0.25	1	0.5	≤0.03	16F
5431	2000	SC	53	Unknown	A2059G	4:0	64	16	>128	0.015	4	0.25	1	1	4	19F
5459	2000	NC	69	Sputum	A2059G/*mefA*	4:0	128	64	>128	0.03	1	>8	1	0.5	2	19F
5486	2000	VT	90	Sputum	A2059G/*mefA*[g]	4:0	>128	>128	>128	0.25	2	0.5	1	1	≤0.03	6A
5293	1999	LA	1	Eye	L4[h]	NA	>128	32	>128	0.12	0.06	0.25	1	0.5	4	23F
5430	2000	MI	41	Sputum	*ermA*/L4	NA	16	8	>128	0.06	0.06	0.5	2	1	2	29

[a] ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; TEL, telithromycin; CLI, clindamycin; TET, tetracycline; LZD, linezolid; Q-D, quinupristin-dalfopristin; PEN, penicillin.
[b] Mutation in domain V of 23S rRNA (*E. coli* numbering) except for isolates 5293 and 5430 that have mutations in ribosomal protein L4. Trac asp, trachial aspirate; BAL, bronchoalveolar lavage.
[c] Heterozygosity of the 23S rRNA genes. Mut:wt, number of mutant copies and number of wild-type copies; NA, not applicable.
[d] Telithromycin-susceptible breakpoint is 1.0 μg/ml (11).
[e] ST, serotype.
[f] Un, unknown.
[g] Also has a Glu77-to-Gly substitution in L22.
[h] L4 contains the substitutions $_{69}$GTG$_{71}$-TPS.

somal mutations in this study were isolated from children less than 5 years of age.

In summary, 21 of 322 macrolide-resistant *S. pneumoniae* isolates contained ribosomal mutations (23S rRNA or L4), with mutations in 23S rRNA being predominant. Two novel combinations of A2059G/*mefA* and L4 ($_{69}$GTG$_{71}$-TPS)/*ermA* were identified. The appearance of these mechanisms may in part be due to the continued widespread use of macrolide and azalide antibiotics. Recent TRUST 7 (2003) surveillance data showed that 27.5% of *S. pneumoniae* in the United States are macrolide resistant (13). How the introduction of telithromycin in the United States will affect macrolide resistance rates and the types of resistance mechanisms observed among pneumococci remains to be determined. This study provides a framework of the types of ribosomal mutations that existed among macrolide-resistant pneumococci prior to the use of telithromycin in the clinic.

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