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Mutations within the *rplD* Gene of Linezolid-Nonsusceptible *Streptococcus pneumoniae* Strains Isolated in the United States

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Three invasive *Streptococcus pneumoniae* strains nonsusceptible to linezolid were isolated in the United States between 2001 and 2012 from the CDC's Active Bacterial Core surveillance. Linezolid binds ribosomal proteins where structural changes within its target site may confer resistance. Our study identified mutations and deletions near the linezolid binding pocket of two of these strains within the *rplD* gene, which encodes ribosomal protein L4. Mutations in the 23S rRNA alleles or the *rplV* gene were not detected.

Linezolid was the first oxazolidinone to be licensed in the United States (in 2000) and marketed worldwide (1–3). Linezolid is approved by the U.S. Food and Drug Administration (FDA) for the treatment of complicated skin infections, meningitis, nosocomial pneumonia, endocarditis, sepsis, osteomyelitis, concurrent bacteremia, and bacteremia associated with community-acquired pneumonia (1, 2).

Linezolid blocks the assembly of a functional initiation complex for protein synthesis, thereby preventing mRNA translation. Other antibiotics that prevent mRNA translation include chloramphenicol, tetracycline, macrolides, and lincosamides. They allow the formation of an initiation complex but inhibit subsequent peptide elongation (3, 4).

The LEADER (Linezolid Experience and Accurate Determination of Resistance) program, which monitors linezolid-resistant clinical isolates, reports that, in the United States, linezolid-sensitive *Streptococcus pneumoniae* isolates have an MIC_{90} of 1 μg/ml (5–9). Therefore, *S. pneumoniae* clinical strains with linezolid MICs of >1 μg/ml should be monitored and investigated for potential mechanisms of resistance. This is consistent with the Clinical and Laboratory Standards Institute (CLSI) breakpoint of 2 μg/ml (10).

The mechanisms of resistance to linezolid that have been described to date include target modification and use of a mobile *cfr* element (2, 8, 11). The linezolid target (the 50S subunit) is composed of 5S and 23S rRNAs and 36 riboproteins (L1 through L36). Linezolid-resistant strains present mutations in one or more alleles of the 23S rRNA gene, decreasing the affinity of ribosomes for the drug (12). A clear correlation between the number of 23S rRNA alleles mutated and increased linezolid resistance has been demonstrated (13, 14). The most frequently reported mutation in linezolid-resistant clinical isolates of staphylococci and enterococci occur by G-to-U substitution in the peptidyl transferase center of 23S rRNA at position 2576 (2, 8). Additional mutations within the same 23S rRNA gene have also been described (e.g., A2059G, C2190T, and G2447T) (15–17).

The *cfr* mobile element includes the *cfr* gene, which encodes a methyltransferase that methylates the 23S rRNA at position A2503. This affects binding of linezolid to the 50S subunit (11, 18, 19). While carried by *Staphylococcus aureus* strains (20, 21) and recently described in *Streptococcus suis* (22), this mobile element has not been described in *S. pneumoniae*.

Only a few *S. pneumoniae* strains with reduced susceptibilities to linezolid have been isolated from disease cases (16, 23). For these strains, it was suggested that mutations in 23S rRNA genes and those encoding ribosomal proteins L4 and L22 confer linezolid resistance (16). However, direct evidence demonstrating deletions within the *rplD* gene of *S. pneumoniae* strain TN33388, encoding ribosomal protein L4, which is linked to reduced susceptibility to linezolid, was published by Wolter et al. (23). Strain TN33388 was identified through the Active Bacterial Core surveillance (ABCs), part of the Centers for Disease Control and Prevention's (CDC's) Emerging Infections Program.

In this study, the CDC *Streptococcus* laboratory identified two other additional *S. pneumoniae* strains (7828-04 and 2008227074) with reduced susceptibilities to linezolid. Overall, 3 of 45,099 pneumococci tested (<1%) were isolated from invasive disease in the United States between 2001 and 2012 through the ABCs, and they showed reduced susceptibilities to linezolid (Table 1). Mutations within demonstrated linezolid targets were investigated in these two isolates.

Strain TN33388 from the CDC (for whom its mechanism of resistance to linezolid had been investigated), two serotype 19A linezolid-susceptible strains, and the reference *S. pneumoniae* strain R6 were utilized as controls (23). The MICs for linezolid, vancomycin, penicillin, amoxicillin, erythromycin, chloramphenicol, clindamycin, and tetracycline were determined using the broth microdilution methodology according to the CLSI (24). The linezolid-susceptible strains shown in Table 1 had linezolid MICs of 0.25 or 1 μg/ml, whereas linezolid-nonsusceptible strains had MICs of 4 μg/ml. The strains were susceptible to penicillin, vancomycin, amoxicillin, and tetracycline. Except for one strain (3084-03), they were also susceptible to clindamycin. Linezolid-nonsusceptible strains were resistant to chloramphenicol and erythromycin (Table 1).

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TABLE 1 Phenotypic findings of *S. pneumoniae* strains with reduced susceptibilities to linezolid

Strain (yr of isolation, state)	Serotype	L4 phenotype	MIC (μg/ml)[a]								Reference or source
			LZD	VAN	PEN	AMO	ERY	CHL	CLI	TET	
0566-02 (2001, GA)	19A	S20N	0.25	0.25	0.06	0.06	0.12	≤2	0.12	≤2	This study
3084-03 (2002, GA)	19A	S20N	1	0.25	0.12	0.06	0.25	≤2	0.5	≤2	This study
7828-04 (2004, CT)	014	ΔW65R66[b]	4	0.5	2	2	2	8	0.12	≤2	This study
2008227074 (2007, NM)	09N	Q67R, R72G	4	0.25	≤0.03	≤0.03	1	>8	0.06	≤2	This study
TN33388 (2003, TN)	33F	ΔK68G69	4	0.25	≤0.03	≤0.03	1	8	0.25	≤2	22

[a] LZD, linezolid; VAN, vancomycin; PEN, penicillin; AMO, amoxicillin; ERY, erythromycin; CHL, chloramphenicol; CLI, clindamycin; TET, tetracycline. Current CLSI breakpoints: LZD susceptible (S), ≤2 μg/ml; VAN S, ≤1 μg/ml; PEN S, ≤2 μg/ml, PEN resistance (R), ≥8 μg/ml; AMO S, ≤2 μg/ml, and AMO R, ≥8 μg/ml; ERY S, ≤0.25 μg/ml, and ERY R, ≥1 μg/ml; CHL S, ≤4 μg/ml, and CHL R, ≥8 μg/ml; CLI S, ≤0.25 μg/ml, and CLI R, ≥1 μg/ml; and TET S, ≤1 μg/ml, and TET R, ≥4 μg/ml (10).
[b] Δ, Deletion.

To investigate the molecular mechanism of reduced susceptibility to linezolid, we amplified, purified, and sequenced the *rplD* gene (encoding the ribosomal protein L4), the *rplV* gene (encoding the ribosomal protein L22), and all four 23S rRNA alleles. The presence of the *cfr* gene in these linezolid-nonsusceptible strains was also sought.

For DNA extraction, *S. pneumoniae* strains were cultured on Trypticase soy agar (TSA) supplemented with 5% sheep blood and incubated overnight at 37°C in 5% CO_2. Chromosomal DNA was then extracted by using the QIAamp DNA minikit (Qiagen, Inc., Valencia, CA). An aliquot (100 ng) was used as the template in PCR mixtures containing 1× Platinum *Taq* DNA polymerase high fidelity (Life Technologies, Carlsbad, CA) and the primers L4F (AAATCAGCAGTTAAAGCTGG) and L4R (GAGCTTTCA GTGATGACAGG) for the *rplD* gene and primers L22F (GCAGA CGACAAGAAAACACG) and L22R (ATTGGATGTACTTTTTG ACC) to amplify *rplV* (23). Each of the four copies of the 23S rRNA alleles carried by the pneumococcus was amplified by using a previously published method (25). Briefly, the genes were initially amplified as overlapping contigs. Then, nested PCRs utilizing unique primers downstream of each 23S rRNA allowed the amplification of the peptidyl transferase region from each allele. The presence of the *cfr* gene was investigated with primers and conditions described previously (26–28).

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA), and the concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Genes were sequenced at Eurofins MWG Operon (Huntsville, AL). Genes from strain R6 (29) were also sequenced and analyzed for comparison. Sequences were analyzed utilizing the software DNASTAR Lasergene 10 and aligned against the nucleotide sequence of a linezolid-susceptible *S. pneumoniae* R6 reference strain (GenBank accession number AE007317) using BLAST (23, 29).

Our sequence analysis found that, when compared to those genes carried by wild-type strain R6, linezolid-nonsusceptible strains had mutations and deletions within only the *rplD* gene (Table 1 and Fig. 1). Strain 2008227074 contained two mutations leading to the amino acid substitutions Q67R and R72G. These two mutations had not been described before in linezolid-nonsusceptible *S. pneumoniae* strains. Strain 7828-04 presented a 6-bp deletion (ΔW65R66) that was similar, but not identical, to that previously identified in strain TN33388 (23). The two linezolid-susceptible strains had a substitution (S20N) which was caused by a single-nucleotide change in position 59 (G59A) of the nucleotide sequence. S20N is apparently out of the linezolid binding pocket within L4 and has been reported in fully susceptible pneumococcal strains and in isolates resistant to macrolides (29).

The sequences of the *rplV* gene of the linezolid-nonsusceptible and linezolid-susceptible strains were identical to that of strain R6. In contrast to the mechanism of linezolid resistance commonly found in staphylococcal isolates, these *S. pneumoniae* strains did not have mutations in any of the four copies of the 23S rRNA alleles. Moreover, the *cfr* gene could not be identified in any of these *S. pneumoniae* strains.

In conclusion, the 2 clinical isolates of *S. pneumoniae* with reduced susceptibilities to linezolid described in this study over a 12-year period have mutations only in the *rplD* gene, leading to changes in the amino acid sequence of the L4 protein. Part of ribosomal protein L4 is placed relatively close to the linezolid binding site on the ribosomes, suggesting that the mechanism for reduced susceptibility may include structural perturbation of the linezolid binding site.

Recently, mutations in 23S rRNA genes have been described in an *in vitro*-generated linezolid-resistant *S. pneumoniae* strain with an MIC of 32 μg/ml (30), which suggests another potential mechanism for resistance. However, these mutations have not been detected to date in clinical pneumococcal strains. Prudent use of linezolid in the United States may account for the low mutation rates in its target and therefore the continued activity against *S.*

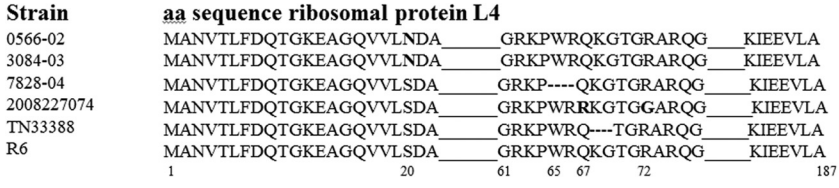

FIG 1 Alignment of ribosomal protein L4 of linezolid-nonsusceptible isolates of *S. pneumoniae* and linezolid-susceptible strains. Mutations are shown in bold type. Dashes represent deletions, and identical sequences, in comparison to strain R6, are indicated by a straight line. Numbers underneath the specific amino acids (aa) represent the position in the R6 sequence.

pneumoniae strains. Similarly, a global study that utilized strains ($n = 636$) isolated in 22 different countries showed susceptibility to linezolid in all *S. pneumoniae* strains (31). Despite many years of exposure to the drug, the very low rate of linezolid resistance in pneumococci suggests that the fitness cost of resistance (32) may be suppressing the successful dissemination of these strains in the pneumococcus.

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