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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 MIC₉₀ 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 occurs 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).
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% CO₂. Chromosomal DNA was then extracted by using the QIAamp DNA minikit (Qiagen, Inc., Valencia, CA) and the primers L4F (AAATCAGCAGTTAAAGCTGG) and L4R (GAGCTTTCA

titation kit (Qiagen, Inc., Valencia, CA). Genes were sequenced at Eurofins MWG Operon (Huntsville, AL). Genes from strain R6 (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.

### Table 1: Phenotypic findings of S. pneumoniae strains with reduced susceptibilities to linezolid

<table>
<thead>
<tr>
<th>Strain (yr of isolation, state)</th>
<th>Serotype</th>
<th>L4 phenotype</th>
<th>MIC (μg/ml)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>0566-02 (2001, GA)</td>
<td>19A</td>
<td>S20N</td>
<td>0.25</td>
<td>This study</td>
</tr>
<tr>
<td>3084-03 (2002, GA)</td>
<td>19A</td>
<td>S20N</td>
<td>1</td>
<td>This study</td>
</tr>
<tr>
<td>7828-04 (2004, CT)</td>
<td>014</td>
<td>ΔW65R66b</td>
<td>4</td>
<td>This study</td>
</tr>
<tr>
<td>2008227074 (2007, NM)</td>
<td>09N</td>
<td>Q67R,R72G</td>
<td>4</td>
<td>This study</td>
</tr>
<tr>
<td>TN33388 (2003, TN)</td>
<td>33F</td>
<td>ΔK68G69</td>
<td>4</td>
<td>This study</td>
</tr>
</tbody>
</table>

* L4D, linezolid; VAN, vancomycin; PEN, penicillin; AMO, amoxicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline. Current CLSI breakpoints: L4D 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; CLI S, ≤4 μg/ml, and CLI R, ≥8 μg/ml; CLT S, ≤0.25 μg/ml, and CLT R, ≥1 μg/ml; and TET S, ≤1 μg/ml, and TET R, ≥4 μg/ml (10). 

b Δ, Deletion.

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**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.
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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