Emergence of a *Streptococcus pneumoniae* Clinical Isolate Highly Resistant to Telithromycin and Fluoroquinolones

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*Streptococcus pneumoniae* is a major pathogen causing community-acquired pneumonia and acute bronchitis. Macrolides, fluoroquinolones (FQs), and, recently, telithromycin (TEL) constitute primary therapeutic options, and rare cases of resistance have been reported. In this report, we describe the emergence of an *S. pneumoniae* clinical isolate with high-level TEL resistance (MIC, 256 µg/ml) and simultaneous resistance to FQs. Ongoing studies are oriented to elucidate the precise mechanism of resistance to TEL.

Table 1. MICs and resistance determinants of *S. pneumoniae* clinical isolates M4256 and M4243

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result for isolate (sample date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>2</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.12</td>
</tr>
<tr>
<td>TMS</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>16</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.03</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
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<tr>
<td>Vancomycin</td>
<td>0.5</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>64</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>64</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>64</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>16</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>16</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>16</td>
</tr>
<tr>
<td>Clindamycin</td>
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</tr>
<tr>
<td>Azithromycin</td>
<td>32</td>
</tr>
<tr>
<td>Telithromycin</td>
<td></td>
</tr>
<tr>
<td>AD, air</td>
<td>0.12</td>
</tr>
<tr>
<td>AD, CO₂</td>
<td>0.25</td>
</tr>
<tr>
<td>BD, air</td>
<td>0.12</td>
</tr>
<tr>
<td>BD, CO₂</td>
<td>0.25</td>
</tr>
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</table>

Fluoroquinolone resistance mutation(s) in:
- gyrA
- gyrB
- parC
- parE

<table>
<thead>
<tr>
<th></th>
<th>gyrA</th>
<th>gyrB</th>
<th>parC</th>
<th>parE</th>
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<tr>
<td>S81F</td>
<td>E474K</td>
<td>S79F, K137N</td>
<td>I460V</td>
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<tr>
<td>S81F</td>
<td>E474K</td>
<td>S79F, K137N</td>
<td>I460V</td>
<td></td>
</tr>
</tbody>
</table>

Macrolide resistance gene carriage
- mfaA
- ermB
- ermA
- ermTR

SmaI-PFGE type
- A

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a TMS, trimethoprim-sulphametoxazole; AD, agar dilution; BD, broth dilution.

b +, PCR positive; −, PCR negative.
cies level by their susceptibilities to optochin and solubilities to bile salts. They were serotyped as 19F by the Quellung reaction (Quellung antisera; Staten Seruminstitut, Copenhagen). MICs by the agar dilution method were determined using Mueller-Hinton agar (Difco, BD Microbiology Systems, Sparks, Md.) supplemented with 5% sheep blood, 24 h of incubation at 35°C, and a 5% CO2 atmosphere. MICs were analyzed according to CLSI (formerly NCCLS) guidelines (17). The susceptibility profiles of M4256 and M4243 differed only in the MICs of TEL and macrolides (Table 1).

The prevalence of *S. pneumoniae* isolates that are resistant to FQs is still low worldwide (4), and the rate of resistance to levofloxacin in adults from Argentina was 2% in 2004, according to data from the National Surveillance Network, WHO-NET-Argentina (unpublished data). *S. pneumoniae* M4256 and M4243 showed high levels of resistance to FQs (Table 1). DNA sequence analysis of the quinolone resistance-determining regions of the *gyrA*, *gyrB*, *parC*, and *parE* genes was performed using conditions previously described (18). Both isolates showed identical mutations in the following four quinolone resistance-determining regions analyzed: Ser-81 → Phe, *gyrA*; Glu-474 → Lys, *gyrB*; Ser-79 → Asn, *parC*; and Ile-460 → Val, *parE* (Table 1). The same mutations were described by Nagai et al. in an *S. pneumoniae* mutant strain using gatifloxacin as selector (16), but this is the first time that such amino acid changes have been described in a clinical isolate. MICs of FQs determined in the presence and absence of reserpine have been successfully used by Morosini et al. to recognize resistance conferred by an efflux mechanism (15). Therefore, we determined MICs of ciprofloxacin with and without 64 μg/ml of reserpine. No reduction in the ciprofloxacin MICs was observed for either isolate with the addition of reserpine, suggesting the absence of an efflux pump contributing to the FQ resistance.

*S. pneumoniae* M4256 displayed phenotype M, resistance to erythromycin and azithromycin and susceptibility to clindamycin and TEL (MICs of 16, 32, 0.06, and 0.12 μg/ml, respectively). The subsequent isolate (M4243) showed a constitutive macrolide-lincosamide-streptogramin B (MLSB)-phenotype, with resistance to erythromycin, azithromycin, and clindamycin (MICs of 1,024, 1,024, and 8 μg/ml, respectively), but an alarmingly increased MIC of TEL (256 μg/ml). In order to detect mefA, ermB, *ermA*, or *ermTR* genes, PCR assays were performed (11, 21). Both isolates were positive for mefA and were repeatedly negative for *ermB*, *ermA*, and *ermTR* genes (Table 1). PCR-restriction fragment length polymorphism of the 348-bp mefA amplicon using BamHI was performed to discriminate between mefA and mefE alleles (14), and we found that both strains harbored the mefE allele (data not shown).

Decreased susceptibility to TEL is associated with (i) mutations in the L4 and L22 riboproteins, domain II or V of the 23S rRNA, (ii) the presence of the *ermB* gene, or (iii) a combination of these mechanisms (19, 22, 24). In vitro selection of TEL-resistant *S. pneumoniae* carrying the mefA gene has been
described, but these strains displayed lower levels of resistance (MIC, 8 \mu g/ml) than those of M4243 (24). Sequence analysis of domains II and V of four individual rrl (23S rRNA) genes from M4243 strains revealed an A→T point mutation at position 2058 which was absent in parental strain M4256 (7). In addition, we identified a three amino acid deletion located at the C-terminal portion of the protein in the rplV (L22 riboprotein) gene of the S. pneumoniae M4243 strain by using standard conditions for PCR and L22-F (5′-CAT GGT AGG CCA CAA ACT TGG T-3′) and L22-R (5′-CAC GCA TAC CAA TTG GAT GT-3′) primers (Fig. 1). Interestingly, this deletion has not been previously described. Analysis results of rplD (L4 riboprotein), using L4-F (5′-CCT TAT CAA AGG TAA CGT ACC A-3′) and L4-R2 (5′-GAT CAA AAG TTT GTG TGC ACG-3′) primers, were identical between the S. pneumoniae M4243 and S. pneumoniae M4256 strains; however, they differed in a point mutation resulting in a Ser→Asn amino-acid change at position 20 (S20N) relative to the wild-type S. pneumoniae R6 strain. The implication of each ribosomal and riboproteins mutation, as well as the combination of different mechanisms of resistance, is under investigation.

Discrepancies in TEL susceptibility due to variations in the MIC methodology employed have been reported for S. pneumoniae (6). The atmosphere of incubation, air versus 5% CO₂ (the latter is associated with a “pH effect” due to CO₂ on the media), and the method used are the most significant variables (2, 6, 25). Therefore, MICs of TEL were determined both by agar dilution, as described above, and by broth dilution (Mueller-Hinton broth plus 5% lysed horse blood and 24 h of incubation at 35°C), in both the presence and the absence of 5% CO₂ (Table 1). For S. pneumoniae M4256, the MICs of TEL determined in ambient air were 0.12 \mu g/ml both by agar and by broth dilution, and this value increased to 0.25 \mu g/ml when the strain was incubated with 5% CO₂ (Table 1). For S. pneumoniae M4243, the MICs in air were 256 and 512 \mu g/ml by agar and broth dilution, respectively, and increased to 1,024 \mu g/ml when the strain was incubated in CO₂ (Table 1). In summary, there were no differences between TEL MICs determined by agar or broth dilution methods, but differences of 1- or 2-log₂ dilutions were detected when the strains were incubated in a CO₂-enriched atmosphere.

Pulsed-field gel electrophoresis (PFGE) was performed using Sma-I enzyme and previously described conditions (5). Both isolates showed the same PFGE profile (Fig. 2), which was not related to any of the international clones already described (13). We conclude that the high level of resistance to TEL displayed in S. pneumoniae M4243 could probably be due to the selection of an intratreatment mutant.

TEL has demonstrated good clinical efficacy in patients with mild to moderate community-acquired pneumonia (9, 23). Moreover, Farrel and Felmingham analyzed 13,874 S. pneumoniae clinical isolates and reported a low rate of TEL resistance (0.2%), with 8 \mu g/ml being the highest MIC found in that survey (8). To the best of our knowledge, there has been only one previous report of clinical selection of TEL resistance (16 \mu g/ml) in S. pneumoniae (19). In laboratory-generated mutants of S. pneumoniae carrying the ermB gene, observed TEL MICs were 64 \mu g/ml (24). Current information thus suggests that the selection of isolates showing resistance to TEL is a possible but infrequent phenomenon. However, there are insufficient data to date to evaluate the role of prior therapy with azithromycin in the selection of the M4243 TEL-resistant isolate.

TEL has been available for clinical use for the past few years, and resistance to this drug is extremely rare at present (20). However, the emergence of the clinical isolate of S. pneumoniae with very high-level TEL resistance (MIC 256 \mu g/ml) described in this report and simultaneous resistance to FQs constitutes a public health concern that requires worldwide attention. Continuous antimicrobial resistance surveillance of this pathogen and conscientious use of macrolides, FQs, and
ketolides are urgently needed to preserve the scarce therapeu-
tic alternatives.

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