Prevalence and Molecular Genetics of Macrolide Resistance among 
Streptococcus pneumoniae Isolates Collected in Finland in 2002

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The prevalence and mechanisms of macrolide resistance among 1,007 clinical pneumococcal isolates collected in Finland were investigated. Of these, 217 (21.5%) were resistant to erythromycin and 11% to clindamycin. Among the erythromycin-resistant isolates, mef(E) was present in 95 isolates (44%), mef(A) was present in 12 isolates (6%), and erm(B) was present in 90 isolates (41%). A double mechanism, mef(E) and erm(B), was detected in five isolates (2%). Ribosomal mutation was detected in 14 (6%) macrolide-resistant isolates in which no other determinant was found. Based on the telithromycin MICs, two groups of isolates were formed: 83.3% of the isolates belonged to a major group for which the telithromycin MIC range was ≤0.008 to 0.063 µg/ml and 16.7% belonged to a minor group for which the telithromycin MIC range was 0.125 to 8 µg/ml. All except three isolates in the minor population carried a macrolide resistance gene.

Increasing resistance to macrolides among Streptococcus pneumoniae isolates is a worldwide problem. The proportion of resistant isolates ranges from 3 to 80% in different countries (2, 7, 20, 22, 23, 26, 33). Macrolide resistance is mediated by two main mechanisms in pneumococci: target site modification and drug efflux. The former is most often mediated by methylases encoded by the erm(B) gene, which is the most common methylase gene, or erm(A) [subclass erm(TR)], which is only infrequently found in pneumococci. Drug efflux is mediated by mef(A), which codes for an efflux pump (27). Two subtypes of mef efflux genes, mef(A) and mef(E), have been found in pneumococci (32, 40). These are variants of the same gene but are carried by different genetic elements (8, 36). An additional efflux mechanism, mediated by the msr(D) or the mel gene, has been found in genetic elements containing the mef gene (17, 38), but the significance of simultaneously carrying two efflux mechanisms is unknown. msr(D) and mel are homologues of the msr(A) gene found in staphylococci (38). Other possible mechanisms responsible for macrolide resistance in pneumococci include mutations in domain V or II of 23S rRNA or in genes coding for 50S ribosomal proteins L22 and L4 (27).

Telithromycin was the first ketolide introduced into clinical use. It is a semisynthetic derivative of erythromycin A composed of a 14-membered lactone ring, but the neutral sugar t-cladinose has been replaced by a keto group at position C-3. A C-11–C-12 carbamate side chain improves the affinity to ribosomes (1). According to present knowledge, telithromycin is effective against macrolide-resistant pneumococci, although some isolates may have elevated MICs to telithromycin (11, 12, 19, 24). Depending on the breakpoints and methods, the proportion of telithromycin nonsusceptibility has been reported to be 0.2% to 3.6% among macrolide-resistant pneumococci (11, 30).

The objectives of this study were to determine the prevalence of macrolide resistance in clinical isolates and the activity of telithromycin against clinical isolates and to investigate the molecular mechanisms of macrolide-resistant pneumococci.

(Preliminary results of this work have been presented at the 14th European Congress of Clinical Microbiology and Infectious Diseases, Prague, Czech Republic [P1475], and at the 4th International Symposium on Pneumococci and Pneumococcal Diseases, Helsinki, Finland [RES-40].)

MATERIALS AND METHODS

Pneumococcal isolates and susceptibility testing. Pneumococcal isolates (n = 1,007) were collected between May and December 2002 by a network of 24 Finnish Study Group for Antimicrobial Resistance (FiRe) laboratories, each of which was requested to send 50 consecutive pneumococcal isolates to the National Public Health Institute. Isolates were from both invasive sites (n = 129) and noninvasive sites (n = 878). The MICs for erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin were determined by an agar plate dilution technique in a 5% CO2 atmosphere (35). Telithromycin was kindly provided by Sanofi Aventis (Romainville, France), while the other antimicrobials were purchased from their respective manufacturers. S. pneumoniae ATCC 49619 and Staphylococcus aureus ATCC 29213 were used as quality controls. CLSI (formerly NCCLS) breakpoints were used (31) for all antimicrobials except azithromycin, for which, due to the effect of the CO2 atmosphere, we used the following breakpoints: susceptibility, ≤1 mg/liter; intermediate, 2 mg/liter; and resistant, ≥4 mg/liter. Both intermediate and resistant isolates were taken into account when resistance percentages were calculated.

Detection of macrolide resistance mechanisms. All erythromycin-resistant isolates (n = 217), 4 clindamycin-resistant isolates, and 41 randomly selected macrolide-susceptible isolates were investigated for the presence of the macrolide resistance genes mef(A/E), erm(B), and erm(TR) by a multiplex PCR method (16) with the primers described previously (16, 34, 39). Separate PCRs were run to differentiate efflux gene subclasses mef(A) and mef(E) in all mef-positive isolates, as well as to detect the presence of msr(D). The primers used for the detection of mef(A) and mef(E) have been described previously (5, 8). A modified primed pair was used for the detection of msr(D): 5'-CAGTTGGACGAA-3' (forward primer) and 5'-CTTTACGTCTCCTCTTTCC-3' (5). Testing for the detection of msr(D) was performed with 53 randomly selected isolates: 30 isolates with mef(E), 12 isolates with mef(A), 6 isolates with

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erm(B), and 5 susceptible isolates. The PCR run for **\text{mef}(A)\**, **\text{mef}(E)\**, and **\text{msr}(D)\** included initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for **\text{mef}(A)\** and **\text{msr}(D)\** or 58°C for **\text{mef}(E)\**, and elongation at 72°C for 1 min. The magnesium concentration was 1.5 mM. All PCRs were run with a Whatman Biometra thermocycler (Biometra, Goettingen, Germany). Positive and negative controls were included in every run. Ribosomal mutations at positions 2058-2059 and 2611 of domain V of 23S rRNA (Escherichia coli numbering) and mutations in genes coding for 50S ribosomal proteins L4 and L22 were sought if the isolate was nonsusceptible to any of the antimicrobials tested and no known resistance gene was present. In addition, mutations were investigated in 13 randomly selected isolates: 7 with **\text{erm}(B)\**, 2 with **\text{mef}(E)\**, 2 with both **\text{erm}(B)\** and **\text{mef}(E)\**, and 2 that showed a macrolide-susceptible phenotype. Mutations at positions 2058-2059 and 2611 of domain V of 23S rRNA were detected by a pyrosequencing technique (18, 37), and mutations in L4- and L22-coding genes were detected by sequencing (25, 42) with known primers (42). Primers for the detection of mutations at positions 2058 and 2059 have been described previously (18). The following primers were used for the detection of mutations at position 2611: for PCR, primers 5'-TGG GTCAGAAGCTGCGTGAAGA-3' (forward primer) and 5'-GCGTGTAAGTCC ACTCTGGTC-3' (reverse primer), and for pyrosequencing, primer 5'-GCAAGTTCGCTGTC-3' (EMBL accession number AE0088386).

**RESULTS**

The prevalences of erythromycin, azithromycin, and clindamycin resistance were 21.5%, 22.3%, and 11.0%, respectively. The proportion of telithromycin-nonsusceptible isolates was not determined because of the lack of breakpoints for the method used here. Based on the telithromycin MICs, two groups of isolates were formed: a major group (83.3% of isolates) with an MIC range of ≤0.008 to 0.063 μg/ml and a minor group (16.7% of isolates) with an MIC in the range 0.125 to 8 μg/ml. All except three isolates in the minor group carried a macrolide resistance gene (Fig. 1). Of the 217 erythromycin-resistant isolates, 95 (44%) had **\text{mef}(E)\**, 12 (6%) had **\text{mef}(A)\**, and 90 (41%) had **\text{erm}(B)\**. Only one isolate had **\text{erm}(TR)\**. Five (2%) isolates carried both the **\text{erm}(B)\** and the **\text{mef}(E)\** genes.

**TABLE 1. Isolates with ribosomal mutations (including three isolates whose resistance mechanism remained unresolved) and their respective MICs to erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin**

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Type and position</th>
<th>No. of mutated alleles total no.</th>
<th>Mutation in 23S rRNA gene, Domain V</th>
<th>Mutation in 50S ribosomal proteins</th>
<th>Resistance gene</th>
<th>MIC (μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L4</td>
<td>L22</td>
<td></td>
<td>ERY AZM SPI TEL CLI</td>
</tr>
<tr>
<td>45</td>
<td>A2059G</td>
<td>1/4</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>560</td>
<td>A2059G</td>
<td>1/4</td>
<td>E\text{\textsubscript{2611}}→K</td>
<td>Wild</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>561</td>
<td>A2059G</td>
<td>1/4</td>
<td>E\text{\textsubscript{2611}}→K</td>
<td>Wild</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>588</td>
<td>A2059G</td>
<td>2/4</td>
<td>T\text{\textsubscript{2058}}→N</td>
<td>Wild</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>904</td>
<td>A2059G</td>
<td>3/4</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>1166</td>
<td>A2059G</td>
<td>4/4</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>&gt;128</td>
</tr>
<tr>
<td>5</td>
<td>C2611T</td>
<td>4/4</td>
<td>V\text{\textsubscript{2058}}→G</td>
<td>A\text{\textsubscript{2059}}→P</td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>152</td>
<td>C2611T</td>
<td>4/4</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>522</td>
<td>C2611T</td>
<td>4/4</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td>48</td>
<td>Wild</td>
<td>6\text{\textsubscript{2058}}↓</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>1</td>
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<tr>
<td>438</td>
<td>Wild</td>
<td>6\text{\textsubscript{2058}}↓</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>156</td>
<td>Wild</td>
<td>S\text{\textsubscript{2611}}→N</td>
<td>B\text{\textsubscript{2622}}→C</td>
<td>Wild</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>551</td>
<td>Wild</td>
<td>S\text{\textsubscript{2611}}→N</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>2</td>
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<tr>
<td>545</td>
<td>Wild</td>
<td>S\text{\textsubscript{2611}}→N</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>837</td>
<td>Wild</td>
<td>S\text{\textsubscript{2611}}→N</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>&gt;128</td>
</tr>
<tr>
<td>843</td>
<td>Wild</td>
<td>E\text{\textsubscript{2611}}→K</td>
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<td>Wild</td>
<td></td>
<td>&gt;128</td>
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<tr>
<td>354</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>&gt;128</td>
</tr>
<tr>
<td>695</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>&gt;128</td>
</tr>
<tr>
<td>965</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td>Wild</td>
<td></td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

* ERY, erythromycin; AZM, azithromycin; SPI, spiramycin; TEL, telithromycin; CLI, clindamycin.
Table 2. Macrolide resistance mechanisms and MIC data for erythromycin, azithromycin, spiramycin, telithromycin, and clindamycin

<table>
<thead>
<tr>
<th>Mechanism (no. of isolates)</th>
<th>MIC ( \mu g/ml )</th>
<th>Erythromycin</th>
<th>Azithromycin</th>
<th>Spiramycin</th>
<th>Telithromycin</th>
<th>Clindamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>50%</td>
<td>90%</td>
<td>Range</td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>(E)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mef (12)</td>
<td>0.03–0.25</td>
<td>0.125</td>
<td>0.25</td>
<td>&gt;128</td>
<td>0.063–2</td>
<td>&gt;128</td>
</tr>
<tr>
<td>(A)</td>
<td>0.063–2</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>(B)</td>
<td>0.063–2</td>
<td>0.25</td>
<td>0.25</td>
<td>&gt;128</td>
<td>0.063–2</td>
<td>&gt;128</td>
</tr>
<tr>
<td>Mutation only (14)</td>
<td>0.016–0.25</td>
<td>0.031</td>
<td>0.125</td>
<td>&gt;128</td>
<td>0.016–0.25</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

The erythromycin MICs in the mef(A)-positive isolates were higher than those in the mef(E) isolates \( (P = 0.002, \text{Mann-Whitney U test}) \). msr(D) was present in all mef-positive isolates tested but not in those with erm(B) or in susceptible isolates. Fourteen isolates (6%) in which no other mechanism was found had a mutation in domain V of 23S rRNA or in ribosomal protein L4 or L22 (Table 1). No macrolide resistance mechanisms were detected in susceptible isolates. The resistance mechanism remained unresolved in three isolates (Table 1). MIC data for isolates with different resistance mechanisms are summarized in Table 2.

**DISCUSSION**

The prevalence of macrolide resistance among pneumococci in Finland doubled between 1999 and 2002 (www.ktl.fi/extras/fire). Currently, there are no signs that this worrying trend is slowing, despite recommendations to avoid the overuse of macrolides, and more effective measures such as encouraging the use of vaccines should be considered.

In this study we used a 5% CO₂ supplement to confirm the proper growth of resistant isolates (15). The CO₂ supplement may elevate macrolide, ketolide, and clindamycin MICs (6, 15). Despite the CO₂ supplement, the results of this study can be considered reliable since 99% of the isolates with erythromycin MICs \( \geq 0.05 \mu g/ml \) harbored a macrolide resistance determinant or had a mutation, thus reflecting the resistance category and genotype well. Moreover, none of the susceptible isolates carried macrolide resistance genes or mutations.

Two groups of pneumococci were formed on the basis of telithromycin MICs: a highly susceptible major group and a minor group of isolates in which the presence of macrolide resistance genes was associated with elevated telithromycin MICs. Nevertheless, the MICs were not constant among isolates with the same macrolide resistance determinant. This was especially true for erm(B)-positive isolates. It is not yet clear why some isolates carrying the same macrolide resistance determinant are fully susceptible to telithromycin but others are not. It may also be possible that true telithromycin resistance in pneumococci evolves in macrolide-resistant isolates that have a moderately elevated MIC to telithromycin.

The proportions of different macrolide resistance determinants recorded here were similar to those from a previous Finnish study on invasive pneumococci (33) and resemble those in North America and Scotland, where the efflux mechanism is the most prevalent (2, 13, 21). This is in contrast to the situation in Europe, where erm(B) dominates (7, 21, 29, 30). There have recently been reports of pneumococci carrying a double mechanism, both erm(B) and mef(E) (3, 14, 28). The spread of similar strains is considered of great concern, since they are often multiresistant and are clonally related (14, 28).

In a recent report on the global situation, the prevalence of isolates having both the erm(B) and the mef(E) genes was 7% (14). In our study, only 2% of isolates carried a double mechanism; and in those isolates, the mef(E) subtype was always present together with erm(B).

mef(A) and mef(E) have 90% similarity at the nucleotide level and are considered variants of the same gene, mef(A) (36); but because they are carried in different genetic elements in pneumococci, they should be differentiated (8). In addition,
there are epidemiological and phenotypic differences between these subtypes (2, 8, 17). For instance, it has been reported that mef(A) isolates have higher MICs to erythromycin than mef(E) isolates (2). A similar observation was recorded in this study. mef(E) is the prevailing efflux gene subtype in the United States, Asia, and South Africa (5) and, according to this study, also in Finland. mef(A) has been more frequently reported in other parts of Europe (2, 5, 29, 32).

The proportion of isolates with mutations in this study was relatively high (6%) compared to that indicated in a recent report on the global prevalence (10). The most frequent mutation in our study was an A2059G change in domain V of 23S rRNA, which has been reported to be one of the most common mutations in clinical isolates. Modification at the erythromycin binding site, which causes resistance to 14-, 15-, and 16-membered macrolides and elevated MICs to clindamycin but not to azithromycin or clindamycin. The MICs to these MICs to clindamycin but not to azithromycin or clindamycin. The MICs to these isolates (2). A similar observation was recorded in this study. Although telithromycin has good activity against pneumococci, activated MIC to telithromycin should be further investigated. The significance of macrolide-resistant isolates having an elevated MIC to telithromycin should be further investigated.

In conclusion, the level of erythromycin resistance is increasing in Finland. The dominant macrolide resistance mechanism is an efflux mechanism caused by either mef(E) or mef(A). Although telithromycin has good activity against pneumococci, the efficacy of macrolides in the treatment of respiratory infections in vitro should be further investigated.

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