Short Communication

Macrolide Resistance Determinants in Erythromycin-Resistant
Streptococcus pneumoniae in Turkey

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SUMMARY: To determine the major molecular mechanisms of macrolide resistance among Streptococcus pneumoniae isolates in Turkey, we examined a total of 151 isolates collected from different regions of Turkey. Overall, 40 (26.4%) isolates were resistant to erythromycin. The most common mechanism (38/40) was target site modification due to erm (B) genes. Only two isolates harbored the mef (A)/(E) efflux gene. A clonal spread of resistant strains could not be demonstrated by BOX-polymerase chain reaction. The results from this study have shown that the erm (B) gene is predominant in Turkish S. pneumoniae isolates, as in isolates from the rest of the world, and a clonal dissemination is not responsible for this resistance profile.

Over the last 20 years, Streptococcus pneumoniae spp. resistant to penicillin and other antibiotics such as macrolides have become a global therapeutic problem (1-3). Macrolide resistance in S. pneumoniae is generally due to either target site modification or active efflux (4,5). These modifications have resulted in two major phenotypes, designated as MLSB and M. In the MLSB phenotype, methylation of 23 S rRNA causes resistance to macrolide, lincosamide and streptogramin B antibiotics. MLSB phenotype is usually constitutive or inducible. The M phenotype, on the other hand, is due to the presence of mef genes and causes resistance to macrolides only. Target modification by ribosomal gene mutations can also give rise to macrolide resistance, although it is a relatively rare mechanism (6). The aim of this study was to determine the molecular mechanisms of macrolide resistance of S. pneumoniae isolates collected from seven tertiary care hospitals located in different geographical locations in Turkey.

A total of 151 randomly selected S. pneumoniae (11 high level penicillin-resistant; 88 low level penicillin-resistant; 52 penicillin susceptible) isolates were collected from seven centers located in four cities (Ankara, Istanbul, Izmir, Kayseri) between 1998 and 2002. All were clinical isolates, recovered from sputum, blood, pus and conjunctivae. Pneumococcal Molecular Epidemiology Network (PMEN) clones (a kind gift from Dr. L. McGee) Finland6B (clone 12) (ATCC 700903), Spain23F (clone 1) (ATCC 700669) and Taiwan19F (clone 14) (ATCC 700905) were used as control strains in the susceptibility studies.

Minimal inhibitory concentrations (MICs) of erythromycin were determined by E Test methodology (AB Biodisk, Solna, Sweden). Penicillin susceptibility was screened by oxacillin disks (1 µg) (7). Penicillin MIC values were determined by E test for the isolates with inhibition zones of ≤19 mm for oxacillin. Susceptibility to penicillin was assessed by MIC values (≤0.06 µg/ml [S], 0.12 - 1 µg/ml [I or low-level penicillin-resistant], ≥2 µg/ml [R or high-level penicillin-resistant]) (8). S. pneumoniae ATCC 49619 was used as a control strain in the susceptibility studies.

Capsule serotypes of erythromycin-resistant isolates were determined by the quellung tests using specific antisera from the State Serum Institute, Denmark.

To determine the inducible MLSB resistance phenotype, we performed the erythromycin-clindamycin double disk (ECDD) test as described by Montanari et al. (9). After overnight incubation at 37°C, the absence of a zone of inhibition around the two disks indicated constitutive resistance, and blunting of the clindamycin zone of inhibition proximal to the erythromycin disk indicated inducible resistance. Susceptibility to clindamycin with no blunting of the inhibition zone indicated the M phenotype. Representative pictures of the phenotype are shown in Figure 1A-D.

The presence of erythromycin resistance genes was determined by polymerase chain reaction (PCR) using primer pairs specific for erm (B) (5’-GAA AAG GTA CTC AAC CAA ATA-3’ and 5’-AGT AAC GGT ACT TAA ATT GTT TGC-3’) and mec (A)/(E) (5’-ATC ATT ATATC CAC TAG TGC-3’ and 5’-TTC TTC TGG TAC TAA AAG TGG-3’) as reported by Sutcliffe et al. (10). BOX-PCR was performed using BOX AR1 primer (5’-CTA CGG CAA GCC GAC GCT GAC ATC-3’) (11).

To show the clonal relationship between macrolide-resistant isolates, we calculated the Jaccard coefficient (12) and constructed a dendrogram using unweighted pair-group method with arithmetic averages (UPGMA) analysis in Mega (v 2.1) software.
Isolates exhibiting identical banding patterns or patterns with only one band difference were classified as the same strain and “clonally related strains,” respectively (12).

The significance of the differences between the susceptibility rates to penicillin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole and ciprofloxacin among erythromycin-susceptible and -nonsusceptible isolates was determined by the chi-square test. The level of statistical significance was \( P < 0.05 \). Data were analyzed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, Ill., USA).

Forty of the 151 isolates were resistant to erythromycin, and 38 (95%) of these carried only the \( \text{erm} \) (B) determinant, which confers resistance by target modification (Table 1). Although capsule serotypes of erythromycin-resistant isolates were distributed over eight serotypes, most of the isolates were clustered in three serotypes, namely 6, 19 and 23, which were among the most common serotypes isolated from healthy carriers and invasive diseases in Turkey (13). Capsule serotype distribution and corresponding prevalences (%) among erythromycin-resistant isolates were as follows: 19 (including 19, 19A, 19F (32.5%), 6 (including 6, 6B) (27.5%), 23 (including 23, 23F) (15%), 14 (5%), 9V (7.5%), 11, 7, 15 B (2.5% each), nontypeable (NT) (5%). In accordance with the mentioned reports, the predominant serotype was serotype 19 in our study.

The results of this study suggest that the most common mechanism of resistance to macrolides in Turkey is the modification of the target site by 23S rRNA methylases (Fig. 1A, B), similar to those reported from previous studies that have included strains from a single center in Turkey (14,15). The \( \text{mef} \) gene was detected in only two isolates, of which one was in combination with an \( \text{erm} \) (B) gene (Fig. 1C). The pattern of the ECDD corresponded to the iMLS B phenotype for the isolate that carried both the \( \text{erm} \) (B) and \( \text{mef} \) determinants. Harboring both of the resistance determinants also has been reported in isolates from South Africa, North America and Europe (1,16,17). The capsule serotype of the isolate reported in the present study was 19 A, which was the most common serotype in our study. The isolate containing only the \( \text{mef} \) gene also had a high erythromycin MIC (32 mg/L), like that found by Reinert et al. in Germany (18), and is a rare isolate reported in the literature (Fig. 1D).

Susceptibility patterns of erythromycin-resistant isolates to other antibiotics are listed in Table 2. Thirty-four (85%) erythromycin-resistant isolates were not susceptible to penicillin (MIC ≥ 0.12 mg/L) whereas only 6 (15%) of \( \text{erm} \) (B)-positive isolates were penicillin susceptible (\( P = 0.003 \)).

![Fig. 1. Phenotypes of erythromycin-resistant pneumococci as determined by the ECDD test. The erythromycin disk (15 μg) is on the left and the clindamycin disk (2 μg) on the right in each panel. A, cMLS\(_\text{S}\) phenotype; B, iMLS\(_\text{S}\) phenotype; C, iMLS\(_\text{S}\) + M phenotype; D, M phenotype.](image)

### Table 1. Erythromycin and clindamycin resistance phenotypes according to macrolide resistance determinants

<table>
<thead>
<tr>
<th>No. of strains with the following gene(^{1)}) (no.)</th>
<th>Erythromycin (\text{MIC (mg/L)})(^2)</th>
<th>Clindamycin susceptibility(^3)</th>
<th>Phenotype of macrolide resistance(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{erm} ) (B) (37)</td>
<td>&gt;256</td>
<td>256</td>
<td>R</td>
</tr>
<tr>
<td>(\text{erm} ) (B) (1)</td>
<td>2</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>(\text{mef} ) (A)/(E) (1)</td>
<td>32</td>
<td>ND</td>
<td>S</td>
</tr>
<tr>
<td>(\text{erm} ) (B) and (\text{mef} ) (A)/(E) (1)</td>
<td>4</td>
<td>ND</td>
<td>S</td>
</tr>
</tbody>
</table>

\(^{1)}\) \(\text{erm} \) (B) and \(\text{mef} \) (A)/(E) genes were determined by PCR.
\(^{2)}\) MICs of erythromycin were determined by E test.
\(^{3)}\) Clindamycin susceptibility was determined by disk diffusion.
\(^{4)}\) Macrolide resistant phenotypes were determined according to the susceptibility pattern, ECDD test and PCR.
ND, Not determined; R, Resistant; S, Sensitive.

iMLS\(_\text{B}\), phenotype with constitutive coreistance to macrolide, lincosamide and streptogramin B; cMLS\(_\text{B}\), phenotype with inducible coreistance to macrolide, lincosamide and streptogramin B; M, phenotype with resistance to macrolides only.

### Table 2. Susceptibility patterns of the study isolates to other antibiotics

<table>
<thead>
<tr>
<th>Erythromycin resistant (n = 40)</th>
<th>Penicillin (^{5)})</th>
<th>Tetracyclin (^{5)})</th>
<th>Chloramphenicol (^{5)})</th>
<th>TMP-SMX (^{5)})</th>
<th>Ciprofloxacin (^{5)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 (15)</td>
<td>15 (37.5)</td>
<td>20 (50.0)</td>
<td>1 (2.5)</td>
<td>40 (100)</td>
<td></td>
</tr>
<tr>
<td>Erythromycin susceptible (n = 111)</td>
<td>46 (41.4)</td>
<td>96 (86.5)</td>
<td>106 (95.5)</td>
<td>45 (40.5)</td>
<td>111 (100)</td>
</tr>
</tbody>
</table>

\(^{5)}\) \( P = 0.003, P \leq 0.001, P \leq 0.001, P \leq 0.001, P > 0.05. 

[Table source: Data were analyzed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, Ill., USA).]
finding is consistent with the previous data obtained from some European countries suggesting an obvious relationship between erythromycin and penicillin resistance (2,19). Tetracycline resistance has also been reported to be associated with macrolide resistance, as both resistance determinants are carried by a common transposon (17). In the present study, 62.5% of erythromycin-resistant isolates were also more resistant to tetracycline, with a clear statistical relationship ($P < 0.001$). Erythromycin-resistant isolates were also more resistant to other selected antimicrobials, such as chloramphenicol ($P < 0.001$) and trimethoprim-sulfamethoxazole ($P < 0.001$). On the other hand, all of the isolates (macrolide susceptible and resistant) were susceptible to ciprofloxacin.

Dissemination of resistant clones has been reported to be responsible for the predominance of a macrolide-resistance phenotype in several countries (3,16). In our study, 30 BOX-PCR patterns were detected among erythromycin-resistant isolates (Figs. 2, 3). Although three clusters of clonally related isolates, one of seven isolates and two others of three isolates each, were identified, when the level of relatedness was taken as $>0.8$, clonal spreading of resistant strains could not be demonstrated by BOX-PCR, a technique especially useful for local epidemiological studies.

In conclusion, although there are a few previous reports on erythromycin resistance of $S$. pneumoniae isolates from individual centers, this investigation is the first to explore the molecular mechanisms of erythromycin resistance of $S$. pneumoniae isolates collected from various geographical locations in Turkey. The results from this study suggest that although the ermA (B) gene is predominantly found in macrolide-resistant isolates, a clonal dissemination is not responsible for this resistance profile.

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