Macrolide- and Telithromycin-resistant Streptococcus pyogenes, Belgium, 1999–2003

Surbhi Malhotra-Kumar,* Christine Lammens,* Sabine Chapelle,* Monique Wijdooghe,* Jasper Piessens,* Koen Van Herck,* and Herman Goossens*

We found a 13% macrolide resistance in 3,866 Streptococcus pyogenes isolated from tonsillitis patients; 59% macrolide-resistant isolates were distributed in 5 clones, suggesting the importance of both resistance gene transfer and clonal dissemination in the spread of these organisms. We also report one of the largest collections of telithromycin-resistant isolates.

Streptococcus pyogenes causes several million cases of upper respiratory tract infection each year. The problem of these infections is growing as resistance increases among S. pyogenes to the macrolide group of antimicrobial drugs commonly used to treat such infections (1–4). S. pyogenes acquires resistance by 2 main mechanisms. The first is active drug efflux mediated by an ATP-binding cassette transporter wherein mef(A) encodes the transmembrane domains and msr(D) encodes the ATP-binding domains (5). This pattern of resistance is demonstrated by an M phenotype. In the second mechanism, gene products of ermA(B) or ermA(A) methylate the macrolide-binding site on 23S rRNA and stall bacterial protein synthesis. This pattern of resistance is demonstrated by either a constitutive (cMLS) or an inducible (iMLS) phenotype. A third, rare, mechanism is modification of the drug binding site on rRNA by mutation that is expressed as an M or a cMLS phenotype. The newest generation of macrolides, the ketolides, are also active against macrolide-resistant strains; however, few S. pyogenes strains of the cMLS phenotype have been found to be ketolide resistant (6).

In Belgium, the first ketolide to be used clinically, telithromycin, was approved in October 2002 to treat community-acquired respiratory infections in patients >12 years of age. We investigated the temporal trends in resistance and clonality among macrolide (including telithromycin)-resistant S. pyogenes recovered from patients with tonsillitis during surveillance studies conducted in Belgium.

The Study
During 1999–2003, a total of 4,031 nonduplicate, putative S. pyogenes isolates were collected from 10 Belgian provinces at the reference center with the date of isolation, specimen source, and patient’s age and residential address. By using a battery of tests, for example, β-hemolysis on blood agar, Gram stain, catalase production, pyrrolidonyl arylamidase, presence of Group A antigen, and bacitracin susceptibility, 3,866 isolates were confirmed to be S. pyogenes. The age of the patient was known in 3,654 cases. Population statistics are detailed in the first half of Table 1.

By using erythromycin (78 µg) and clindamycin (25 µg) double-disc diffusion (Neo-Sensitab discs; Rosco, Taastrup, Denmark), all 3,866 S. pyogenes isolates were further screened for a phenotypic expression of macrolide resistance, which was identified in 506 (13%) isolates. The proportion of macrolide-resistant isolates among the total S. pyogenes isolated from each of the 10 Belgian provinces fluctuated from 0% to 40% over the 5 years studied. The 3 known phenotypes, cMLS, iMLS, and M, were identified in 209 (41%), 18 (4%), and 279 (55%) isolates, respectively. Changes in prevalence of the 3 phenotypes among macrolide-resistant S. pyogenes over 5 years are presented in the second half of Table 1.

MICs of erythromycin, clindamycin, tetracycline (Sigma-Aldrich, St. Louis, MO, USA), clarithromycin (Abbott, Louvain-la-Neuve, Belgium), azithromycin (Pfizer, Groton, CT, USA), and telithromycin (Aventis, Romainville, France) were further determined by agar dilution (7). Susceptible and resistance breakpoints for telithromycin were ≤1 µg/mL and ≥4 µg/mL, respectively. Briefly, a 10^4 CFU/spot inoculum was incubated at 37°C for 18–24 h in ambient air. The MIC profiles of the 3 macrolide-resistant phenotypes to various antimicrobial drugs are presented in the online Appendix Table 1 (available at http://www.cdc.gov/ncidod/eid/vol11no06/04-1247_app1.htm). The yearly prevalence (1999–2003) of telithromycin resistance (MIC ≥4 µg/mL) among macrolide-resistant S. pyogenes was 2%, 7%, 11%, 13%, and 10%, respectively. Thus, the total telithromycin-resistant isolates (N = 50) identified here constitute the largest collection reported. Of the 50 telithromycin-resistant S. pyogenes, 49 belonged to the cMLS and 1 to the iMLS phenotype. These isolates exhibited erythromycin MICs of 128–512 µg/mL. Thirty (60%) telithromycin-resistant...

*University of Antwerp, Antwerp, Belgium

1A preliminary account of this work was presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, October 30–November 2, 2004, Washington DC, USA.
S. pyogenes were isolated from children, of which 28 (56%) were <12 years of age.

We further investigated clonality in all macrolide-resistant isolates and in a random selection of 331 macrolide-susceptible isolates by pulsed-field gel electrophoresis (PFGE) and *emm* typing on reverse line blotting as described previously (1). PFGE was performed by using *Sma*I; however, for most *mef*(A)-positive isolates that proved refractory to *Sma*I restriction, *Sfi*I restriction was utilized. PFGE patterns were analyzed by using GelCompar software 4.0 (Applied Maths, Kortrijk, Belgium). The 506 macrolide-resistant *S. pyogenes* were typed into 17 *emm* types and 76 PFGE types, of which 53 (70%) types were distributed among M phenotype isolates (Appendix Table 2 available at http://www.cdc.gov/ncidod/eid/vol11no06/04-1247_app2.htm). Ratios of PFGE types to number of *S. pyogenes* isolates were 0.18 and 0.09 for the M and cMLS phenotypes, respectively. Table 2 shows the temporal evolution over 5 years of the 5 major cMLS and M phenotype clones. Clones 1, 4, and 23 constituted 99%, 98%, and 100% of all the macrolide-resistant *emm*22, *emm*28, and *emm*11, respectively, isolated during the course of this study, while clones 1,001 and 1,002 constituted 97% and 39% of the *emm*1 and *emm*4 macrolide-resistant *S. pyogenes* serotypes, respectively. Serotypes *emm*1, *emm*4, *emm*11, *emm*22 and *emm*28 formed 70% of the total macrolide-resistant *S. pyogenes*. Among the 331 macrolide-susceptible *S. pyogenes* analyzed, the prevalence of clones 1, 4, 23, 1,001, and 1,002 was 5% (n = 18), 1% (n = 3), 0.3% (n = 1), 2% (n = 5), and 0.3% (n = 1), respectively (data not shown). Telithromycin resistance was distributed among 7 cMLS serotypes (*emm*22, *emm*28, *emm*11, *emm*12, *emm*77, *emm*6, and, *emm*2).

We next studied the genotype for the 3 macrolide-resistant phenotypes. Polymerase chain reaction was performed for *erm*(A), *erm*(B), and *mef*(A) (1,9,10). Isolates negative for all 3 genes were screened for ribosomal mutations in L4, L22, and portions of 23S RNA genes by using published primers (11). Amplimers were analyzed by direct double-strand sequencing (3730 DNA Analyzer, Applied Biosystems, Foster City, CA, USA) with the BigDye Terminator Version 3.1 Kit (Applied Biosystems). Nucleotide sequence alignment was done with SeqMan (DNASTAR Inc., Madison, WI, USA). Phenotypic and genotypic profiles of the macrolide-resistant *S. pyogenes* were generally consistent; however, 3% of the resistant iso-

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**Table 1. Yearly prevalence of *Streptococcus pyogenes* isolates screened and of macrolide-resistant *S. pyogenes* distributed by patient age group and phenotype, 1999–2003**

<table>
<thead>
<tr>
<th></th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total <em>S. pyogenes</em> screened</td>
<td>508</td>
<td>336</td>
<td>633</td>
<td>1,226</td>
<td>1,073</td>
</tr>
<tr>
<td>Isolated from adults (mean age, 34.7 y; SD, 11.1 y; range, 17 to 91 y)</td>
<td>220 (36.7%)</td>
<td>144 (43.1%)</td>
<td>245 (38.7%)</td>
<td>469 (38.2%)</td>
<td>453 (42.0%)</td>
</tr>
<tr>
<td>Isolated from children (mean age, 7.2 y; SD, 3.5 y; range, 3 mo to 16.9 y)</td>
<td>357 (59.6%)</td>
<td>172 (51.2%)</td>
<td>367 (58.0%)</td>
<td>675 (55.0%)</td>
<td>552 (51.0%)</td>
</tr>
<tr>
<td>Macrolide-resistant <em>S. pyogenes</em></td>
<td>81 (14%)</td>
<td>41 (12%)</td>
<td>73 (12%)</td>
<td>215* (18%)</td>
<td>96* (9%)</td>
</tr>
<tr>
<td>Isolated from adults</td>
<td>23 (4%)</td>
<td>16 (5%)</td>
<td>29 (5%)</td>
<td>82 (7%)</td>
<td>38* (4%)</td>
</tr>
<tr>
<td>Isolated from children</td>
<td>56 (9%)</td>
<td>22 (7%)</td>
<td>44 (7%)</td>
<td>126 (10%)</td>
<td>50* (5%)</td>
</tr>
<tr>
<td>Constitutive phenotype</td>
<td>49/81 (8%)</td>
<td>10/41§ (3%)</td>
<td>20/73 (4%)</td>
<td>68/215 (6%)</td>
<td>54/96 (5%)</td>
</tr>
<tr>
<td>M phenotype</td>
<td>32/81 (5%)</td>
<td>29/41 (9%)</td>
<td>39/73 (6%)</td>
<td>141/215 (12%)</td>
<td>36/96§ (4%)</td>
</tr>
<tr>
<td>Inducible phenotype</td>
<td>–</td>
<td>2/41 (1%)</td>
<td>6/73 (1%)</td>
<td>7/215 (1%)</td>
<td>4/96 (0.4%)</td>
</tr>
</tbody>
</table>

*Increase and decrease in macrolide resistance from 2001 to 2002 and from 2002 to 2003, respectively, was significant (p<0.001).
†Prevalence of macrolide-resistant *S. pyogenes* decreased significantly among both children and adults from 2002 to 2003 (p<0.0001).
‡Prevalence of macrolide-resistant *S. pyogenes* increased significantly among children from 2001 to 2002 (p = 0.005).
§Decrease in prevalence of cMLS isolates from 1999 to 2000 (p = 0.005) and of M phenotype isolates from 2002 to 2003 (p<0.0001) was significant.

*P*earson's *χ*²-test with Bonferroni post-hoc adjustments was used for all multiple comparisons. *p*<0.05 (2-sided) was significant.

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**Table 2. Temporal changes in the distribution of major pulsed-field gel electrophoresis and *emm* types among the 3 macrolide-resistant *Streptococcus pyogenes* phenotypes**

<table>
<thead>
<tr>
<th>Macrolide-resistant phenotype</th>
<th>Pulsed-field gel electrophoresis cluster (<em>emm</em> type)</th>
<th>Frequency (n = 506)</th>
<th>1999 (n = 81)</th>
<th>2000 (n = 41)</th>
<th>2001 (n = 73)</th>
<th>2002 (n = 215)</th>
<th>2003 (n = 96)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive</td>
<td>1 (<em>emm</em>22)</td>
<td>70</td>
<td>45 (56%)</td>
<td>7 (17%)†</td>
<td>9 (12%)</td>
<td>7 (3%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td></td>
<td>4 (<em>emm</em>28)</td>
<td>45</td>
<td>–</td>
<td>–</td>
<td>4 (5%)</td>
<td>15 (7%)</td>
<td>26 (27%)</td>
</tr>
<tr>
<td></td>
<td>23 (<em>emm</em>11)</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>1 (1%)</td>
<td>6 (3%)</td>
<td>21 (22%)</td>
</tr>
<tr>
<td>M</td>
<td>1001 (<em>emm</em>1)</td>
<td>128</td>
<td>7 (9%)</td>
<td>12 (29%)</td>
<td>23 (32%)</td>
<td>80 (37%)†‡</td>
<td>6 (6%)‡‡</td>
</tr>
<tr>
<td></td>
<td>1002 (<em>emm</em>4)</td>
<td>26</td>
<td>2 (2.5%)</td>
<td>2 (5%)</td>
<td>7 (10%)</td>
<td>7 (3%)</td>
<td>10 (10%)</td>
</tr>
</tbody>
</table>

*A ≤6-band difference was employed to assign isolates to a clone according to Tenover et al. (8). PFGE clusters up to 100 designate restriction with *Sma*I and clusters >1,000 designate restriction with *Sfi*I.
†Decrease in prevalence of the 1/1emm22 clone from 1999 to 2000 was highly significant (p<0.001).
‡Both the increase and decrease in prevalence of the 1001/1emm1 clone from 2001 to 2002 and from 2002 to 2003, respectively, were significant (p<0.01).
lates carried 2 resistance genes. Of the 209 cMLS isolates, 199 carried erm(B), 9 carried erm(B)+mef(A), and 1 carried erm(B)+erm(A). Of the 279 M phenotype isolates, 273 carried mef(A), 1 carried erm(B)+mef(A), and 4 carried mef(A)+erm(A). The 1 isolate that was negative for all 3 genes carried a single A2059G (Escherichia coli numbering system) mutation in the 23S rRNA gene. The A2059G mutation, although quite frequent in S. pneumoniae, has been rarely observed in S. pyogenes. Finally, of the 18 iMLS isolates, 5 carried erm(B) and 13 carried erm(A).

Ten percent of the macrolide-resistant strains harboring erm(B) alone or with mef(A) were also telithromycin-resistant, and telithromycin has additional binding sites on 23S rRNA. Therefore, we hypothesized that either mutations in the erm gene promoter region have upregulated methylase expression or that mutations in the coding region have changed the methylase specificity to include the additional binding sites of telithromycin. Alternatively, mutations at the additional binding sites on the 23S rRNA genes might also disable the binding arm; however, a recent study described only a low level of telithromycin resistance in the presence of these mutations (12). Utilizing 3 pairs of overlapping primers (primer sequences available on request), DNASTAR software, and sequence data of Tn1545 (National Center for Biotechnology Information, Rockville, MD, USA, accession no. X52632), the entire erm(B) gene, including the promoter and control peptides, were sequenced from 10 telithromycin-resistant isolates. In addition, L4, L22, and portions of 23S rRNA genes were also amplified as above. Analysis of the sequencing data showed a single H118R (A677G) substitution in the erm(B) coding region of all 10 telithromycin-resistant isolates. While our study was ongoing, the H118R substitution in erm(B) was also confirmed independently for 2 telithromycin-resistant isolates (6).

Conclusions

We demonstrated in this study that overall macrolide resistance in Belgium is driven by an epidemic spread of a few major clones as well as by resistance gene transfer among genetically diverse S. pyogenes. On average, we demonstrated a 2-fold (13%) increase in macrolide resistance in Belgium from 1999 to 2003, compared to that observed from 1995 to 1997 (6.5%) (1). Although, a general increase in macrolide-resistance in Europe has been observed during the last few years, resistance levels tend to differ considerably between countries. For instance, while resistance rates in Germany (6) and Poland (4) were similar to those observed in Belgium, considerably higher resistance levels were observed in Spain and Portugal (2), as well as in Italy (3). Provincial variations in macrolide-resistance observed in Belgium have also been reported in other countries (3); however, the precise causes underlying such variations within or between countries are not fully understood. Macrolide consumption might be one factor that explains the regional variations in macrolide-resistant S. pyogenes in Spain and Finland (13,14), especially when consumption surpasses a critical threshold (15). However, in France, one of the highest macrolide consumption within Europe is not paralleled by an equally high resistance in S. pyogenes (16,17). The identification of telithromycin-resistant S. pyogenes in our study, many of which were already present in the community before the introduction of telithromycin in Belgium, also suggest that antimicrobial drug use and development of resistance might be dissociated to some extent. Clearly, other factors like natural fluctuations in prevalence of clones (18), patient compliance with antimicrobial drug regimens, fitness costs of drug resistance, or even tetracycline consumption (tetracycline and macrolide-resistance genes cosegregate) (19) might be important determinants for the development and spread of macrolide-resistant S. pyogenes. Thus, any direct link between macrolide use and resistance in S. pyogenes should be interpreted cautiously.

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Ms. Malhotra-Kumar holds a double master’s degree in medical microbiology and molecular biology and is a final-year PhD student at the University of Antwerp. Her main research focuses on the epidemiology and molecular genetics of antimicrobial resistance in oral streptococci.

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Address for correspondence: Surbhi Malhotra-Kumar, Department of Medical Microbiology, Campus Drie Eiken, University of Antwerp, S3, Universiteitsplein 1, B-2610 Wilrijk, Antwerp, Belgium; fax: 32-3-820-2663; email: surbhi.malhotra@ua.ac.be