In Vitro Activities of Six Quinolones and Mechanisms of Resistance in *Staphylococcus aureus* and Coagulase-Negative Staphylococci

HANS-JÖRG LINDE,* MARIO SCHMIDT, EMMI FUCHS, UDO REISCHL, HANS-HELMUT NILLER, AND NORBERT LEHN

Institute for Medical Microbiology and Hygiene, University of Regensburg, Regensburg, Germany

Received 31 October 2000/Returned for modification 18 December 2000/Accepted 30 January 2001

Of 94 clinical isolates of *Staphylococcus aureus* (n = 51) and coagulase-negative staphylococci (CNS) (n = 43), mutations in the quinolone resistance-determining region of topoisomerases GrlA, GrlB, GyrA, and GyrB together with MICs of six quinolones were analyzed. Amino acid substitutions at identical residues (GrlA residues 80 and 84; GyrA residues 84 and 88) were found in *S. aureus* and CNS. Active efflux, as suggested by blocking by reserpine, contributed substantially to the resistance phenotype in some strains. Among ciprofloxacin, clinafloxacin, levofloxacin, nalidixic acid, trovafloxacin, and sparfloxacin, a 0.5-μg/ml concentration of sparfloxacin discriminated best between strains with two or three mutations and those with no mutations.

Considerable information about the mechanisms of quinolone resistance is available for *Staphylococcus aureus* (2, 5, 7, 12, 16, 17, 20, 23); however, less is known for *Staphylococcus epidermidis* (9, 19) and other coagulase-negative staphylococci (2, 5, 7, 12, 16, 17, 20, 23). In the present study, we analyzed 94 unique clinical isolates with regard to the MICs of various quinolones and to the combinations of ciprofloxacin (CIP)-reserpine (RES) and trovafloxacin (TV-A)-RES for these isolates. The MICs were correlated with mutations in the quinolone-resistance determining region (QRDR) of the grlA and gyrA genes (all strains) and grlB and gyrB genes (*S. aureus* and *S. epidermidis*).

All strains (except methicillin-resistant [Met']) *S. aureus* were consecutive isolates collected from individual patients at the Institute for Medical Microbiology, University of Regensburg, between 1995 and 1998 and included 27 methicillin-susceptible (Mets) *S. aureus* isolates, 24 (Mets) *S. aureus* isolates (each with a unique pattern in pulsed-field gel electrophoresis [24]), 12 Met' *S. epidermidis* isolates, 19 Met' *S. epidermidis* isolates, 8 Met' CNS (1 *Staphylococcus haemolyticus* isolate, 5 *Staphylococcus hominis* isolates, and 2 *Staphylococcus capitis* isolates), and 4 Met' CNS (3 *S. haemolyticus* isolates and 1 *Staphylococcus simulans* isolate). CNS were isolated from normally sterile sites. Isolates were identified by a latex agglutination test (Slidex Staph-Kit; bioMérieux sa, Marcy-l’Étoile, France) and by biochemical reactions (ID 32 STAPH; bioMérieux sa). Antimicrobial agents were provided by the manufacturers: CIP (Bayer AG, Leverkusen, Germany), clinafloxacin (Parke-Davis Pharmaceutical Research, Freiburg, Germany), levofloxacin (Hoechst Marion Roussel, Frankfurt, Germany), sparfloxacin (SPX) (Rhone-Poulenc-Rorer, Köln, Germany), and TV-A (Pfizer, Karlsruhe, Germany). Nalidixic acid was purchased from Sigma (Deisenhofen, Germany) (catalog no. N8878). MICs were determined by the agar dilution method on Mueller-Hinton agar (Oxoid, Wesel, Germany) according to NCCLS guidelines (13). In two determinations the effect of RES (catalog no. R0875; Sigma) on MICs of TVA and CIP was evaluated on Mueller-Hinton agar plates with and without RES (20 μg/ml) and Etest strips (AB BIODISK, Solna, Sweden) and was expressed as the change of dilution steps. If the MIC exceeded the maximum concentration on the strip, double the concentration was arbitrarily used for further calculations. Protocols for the amplification of grlA, grlB, gyrA, and gyrB of *S. aureus* and grlA of CNS as published previously were used (3, 21, 26). Primers and PCR conditions for amplification of grlB, gyrA, and gyrB genes of CNS are listed in Table 1.

### Table 1. Primers and PCR conditions used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide (5′→3′)</th>
<th>Position</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>MgCl₂ (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>grlA (S. epidermidis, CNS)</td>
<td>GCTTTAAACCCAGATACACG</td>
<td>2141−2159</td>
<td>49</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>CAGCAATGTGACTGGATTC</td>
<td>2184−2190</td>
<td></td>
<td></td>
</tr>
<tr>
<td>grlB (S. epidermidis)</td>
<td>GTGACCAAAAAAGCCAACCG</td>
<td>1531−1550</td>
<td>53</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CCGGTTAGATGCAAGACG</td>
<td>1780−1761</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCGATTCCGTCACAAATGG</td>
<td>1526−1549</td>
<td>65</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>TTAAGTCGGTACCAACACCAGCACCAA</td>
<td>1850−1823</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbering according to *S. aureus* sequence.

* Corresponding author. Mailing address: Institut für Medizinische Mikrobiologie und Hygiene, Universität Regensburg, Franz-Josef-Strauss-Allee 11, D-93049 Regensburg, Germany. Phone: 49-941-944-6461. Fax: 49-941-944-6402. E-mail: hans-joerg.linde@klinik.uni-regensburg.de.

Copyright © 2001, American Society for Microbiology. All Rights Reserved.
1. Three units of Expand High Fidelity Taq polymerase (pre-cast solution; Roche Molecular Biochemicals, Mannheim, Germany) was used for all amplifications. The PCR products were purified with a PCR purification kit (QIAQuick; Qiagen, Hilden, Germany). Complementary strands were sequenced on a 310 DNA sequencer (Perkin-Elmer, Foster City, Calif.) using PCR primers (6 μmol). Sequences were compared with published wild-type sequences of *S. aureus* (gyrA and gyrB: GenBank accession number M86227; grlA and grlB: GenBank accession number D67075). SPSS 10.0 for Windows was used for calculation of the chi-square and Mann-Whitney U test results. The partial sequences of the *grlA* gene of *S. haemolyticus*, *S.
TABLE 2. Degree of similarity of nucleotide and amino acid sequences in the QRDRs of GyrA, GyrB, GrlA, and GrlB of staphylococci.

<table>
<thead>
<tr>
<th>Species</th>
<th>% Similarity to <em>S. epidermidis</em> sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GyrA nt</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>85.8</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>86.7</td>
</tr>
<tr>
<td><em>S. capitis</em></td>
<td>90.5</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>87.5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>83.3</td>
</tr>
</tbody>
</table>

*nt*, nucleotide.  
*aa*, amino acid.  
—, not done.

The degree of similarity in nucleotide and amino acid sequences of the QRDRs of GyrA, GyrB, GrlA, and GrlB of staphylococci was determined using the GenBank nucleotide sequence database and compared to the sequence of *S. epidermidis* as a reference. The degree of similarity was calculated using the BLAST algorithm.

**TABLE 3.** Changes in MICs of CIP and TVA for *S. aureus* and CNS on Mueller-Hinton agar with RES (20 μg/ml)

<table>
<thead>
<tr>
<th>Strains</th>
<th>No.</th>
<th>CIP</th>
<th>TVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>All</td>
<td>1.4</td>
<td>1</td>
<td>4–6</td>
</tr>
<tr>
<td>CIP-S</td>
<td>1.9</td>
<td>2</td>
<td>2–4.4</td>
</tr>
<tr>
<td>CIP-R</td>
<td>1.1</td>
<td>1</td>
<td>1–4.4</td>
</tr>
<tr>
<td>CNS</td>
<td>0.8</td>
<td>1</td>
<td>4–6</td>
</tr>
</tbody>
</table>

* CIP, ciprofloxacin; TVA, trovafloxacin; RES, resistant strain.

---

*hominis, S. capitis, and S. simulans* and of the gyrA gene of *S. hominis, S. capitis, and S. simulans* appear in the GenBank nucleotide sequence database under accession numbers AF159150, AF159151, AF159152, AF159153, AF159154, AF159155, and AF159156, respectively. Partial sequences of the grlB and gyrB genes of *S. epidermidis* are listed under accession numbers AF314403 and AF314404, respectively.

The MICs at which 50 and 90% of *S. aureus* and CNS strains tested were inhibited were comparable to those found in previous studies (1, 4, 8, 18). The distribution of the MICs for the different groups of staphylococci is shown in Fig. 1. In both *S. aureus* and CNS, resistance to methicillin and to quinolones was highly correlated (*P* < 0.0001 [chi-square test]).

In *S. aureus*, resistance to quinolones was correlated with the number of point mutations in *grlA* and *gyrA*, leading to amino acid changes in residues Ser80 and/or Glu84 of GrlA and Ser84 and/or Glu88 of GyrA. The association of the GrlB432 alteration with resistance is unclear (seen in two strains; MICs of SPX for these strains were 0.06 to 0.25 μg/ml, indicating no apparent effect of the Glu88Asp change in GyrA of *S. haemolyticus* or *S. hominis*.

Identical to *S. aureus*, elevated MICs for CNS were found in strains with amino acid changes in residues Ser80 and Asp84 of GrlA and residues Ser84 and Glu88 of GyrA. In *S. epidermidis* only Ser80Phe or Ser80Tyr changes were found by Li et al. (9) and in the present study, while *S. hominis* had Ser80Val or Ser80Leu amino acid changes (21). No mutations in gyrB or grlB in any strain of *S. epidermidis* were found. In CNS other than *S. epidermidis*, only the grlA and gyrA genes were analyzed. Different primers designed for amplification of the grlB and gyrB genes of *S. aureus* or *S. epidermidis*, tested under various nonstringent conditions, did not yield any product in *S. capitis, S. hominis, S. haemolyticus*, and *S. simulans*.

In strains with identical amino acid changes but different MICs, additional resistance mechanisms may be active. We investigated whether inhibition of efflux pump systems (presumably NorA [11, 14; F. J. Schmitz, B. Hertel, B. Hoffmann, S. Scheuring, J. Verhoef, A. C. Fluit, H. P. Heinz, K. Kohrer, and M. E. Jones, Letter, J. Antimicrob. Chemother., 42:561–563, 1998] and/or NorB [15, 16]) might be involved.

**Table 2** shows the degree of similarity of nucleotide and amino acid sequences in the QRDRs of GyrA, GyrB, GrlA, and GrlB of staphylococci, compared to *S. epidermidis* as a reference. The degree of similarity was calculated using the BLAST algorithm.

**Table 3** shows the changes in MICs of CIP and TVA for *S. aureus* and CNS on Mueller-Hinton agar with RES (20 μg/ml). The MICs of CIP and TVA were determined for *S. aureus* and CNS strains. The changes in MICs were compared to the previous MICs, and the correlation coefficients were calculated. The data show that the changes in MICs were comparable to those found in previous studies (1, 4, 8, 18).
The results of typing Met’ S. aureus by pulsed-field gel electrophoresis were kindly provided by Michaela Metz. We thank Markus Bollwein and Christine Irtenkauf for excellent technical assistance. Nucleotide sequence determination was performed by Holger Melzl and Josef Köstler.

REFERENCES


