Activities of Newer Fluoroquinolones against *Streptococcus pneumoniae* Clinical Isolates Including Those with Mutations in the *gyrA*, *parC*, and *parE* Loci

J. H. JORGENSEN,1,* L. M. WEIGEL,2 M. J. FERRARO,3 J. M. SWENSON,2 AND F. C. TENOVER2

Department of Pathology, The University of Texas Health Science Center, San Antonio, Texas 78284; Hospital Infections Program, Centers for Disease Control and Prevention, Atlanta, Georgia 30333; and The Massachusetts General Hospital, Boston, Massachusetts 02114

Received 24 June 1998/Returned for modification 21 September 1998/Accepted 25 November 1998

Resistance to fluoroquinolone (FQ) antibiotics in *Streptococcus pneumoniae* has been attributed primarily to specific mutations in the genes for DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). Resistance to some FQs can result from a single mutation in one or more of the genes encoding these essential enzymes. A group of 160 clinical isolates of pneumococci was examined in this study, including 36 ofloxacin-resistant isolates (MICs, ≥8 μg/ml) recovered from patients in North America, France, and Belgium. The susceptibilities of all isolates to clinafloxacin, grepafloxacin, levofloxacin, sparfloxacin, and trovafloxacin were examined by the National Committee for Clinical Laboratory Standards reference broth microdilution and disk diffusion susceptibility testing methods. Among the ofloxacin-resistant strains, 32 of 36 were also categorized as resistant to levofloxacin, 35 were resistant to sparfloxacin, 29 were resistant to grepafloxacin, and 19 were resistant to trovafloxacin. In vitro susceptibility to clinafloxacin appeared to be least affected by resistance to the other FQs. Eight isolates with high- and low-level resistance to the newer FQs were selected for DNA sequence analysis of the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE*. The DNA and the inferred amino acid sequences of the resistant strains were compared with the analogous sequences of reference strain *S. pneumoniae* ATCC 49619 and FQ-susceptible laboratory strain R6. Reduced susceptibilities to grepafloxacin and sparfloxacin (MICs, 1 to 2 μg/ml) and trovafloxacin (MICs, 0.5 to 1 μg/ml) were associated with either a mutation in *parC* that led to a single amino acid substitution (Ser-79 to Phe or Tyr) or double mutations that involved the genes for both GyrA (Ser-81 to Phe) and ParE (Asp-435 to Asn). High-level resistance to all of the compounds except clinafloxacin was associated with two or more amino acid substitutions involving both GyrA (Ser-81 to Phe) and ParC (Ser-79 to Phe or Ser-80 to Pro and Asp-83 to Tyr). No mutations were observed in the *gyrB* sequences of resistant strains. These data indicate that mutations in pneumococcal *gyrA*, *parC*, and *parE* genes all contribute to decreased susceptibility to the newer FQs, and genetic analysis of the QRDR of a single gene, either GyrA or parC, is not predictive of pneumococcal resistance to these agents.

Increasing resistance to antimicrobial agents among contemporary clinical isolates of *Streptococcus pneumoniae* has been widely documented (3, 7, 8, 10, 27, 29). Resistance to penicillin, macrolides, trimethoprim-sulfamethoxazole, and extended-spectrum cephalosporins has complicated the therapy of both invasive and respiratory infections due to pneumococci (6, 9, 17, 18). However, resistance to fluoroquinolones with notable activity against gram-positive bacteria, such as levofloxacin and ofloxacin, has been rare (2, 11, 14, 29). Therefore, fluoroquinolones may represent an attractive choice for empiric therapy of common respiratory infections, such as community-acquired pneumonia. Although rare, clinical isolates of pneumococci with mutations in the quinolone resistance-determining regions (QRDRs) of the DNA gyrase and topoisomerase IV genes have been recognized (5, 11, 23, 24, 28) and have resulted in some therapeutic failures (5, 19, 23, 28).

This study examined the activities of ofloxacin, four recently marketed fluoroquinolones, and one investigational fluoroquinolone by the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution and disk diffusion susceptibility testing procedures (20, 21) against a group of clinical pneumococcal isolates from North America and Europe that included 36 ofloxacin-resistant strains. To understand better the effect of DNA gyrase and topoisomerase IV mutations on the activities of the newer members of this class of antimicrobial agents, selected strains with high- and low-level fluoroquinolone resistance were characterized by genetic analysis. Mutations in the QRDRs of these genes were correlated with the susceptibility profiles of eight fluoroquinolone-resistant clinical isolates.

MATERIALS AND METHODS

**Participating laboratories.** This collaborative study was conducted in microbiology laboratories at three separate institutions: the Centers for Disease Control and Prevention (CDC), The Massachusetts General Hospital (MGH), and The University of Texas Health Science Center at San Antonio (UTHSC). The testing protocol, the quality control strains, the two microdilution antibiotic panels, and the lots of antibiotic disks were the same for the three laboratories, but the Mueller-Hinton sheep blood agar plates were from different sources (see below).

**Antimicrobial agents.** Reagent powder of each antimicrobial agent was kindly provided for this study by the manufacturers. The agents (and their manufacturers) included clinafloxacin (Parke-Davis, Ann Arbor, Mich.), grepafloxacin (Glaxo-Wellcome, Research Triangle Park, N.C.), levofloxacin and ofloxacin (Ortho-McNeil Pharmaceutical, Raritan, N.J.), sparfloxacin (Rhône-Poulenc...
grown on sheep blood agar plates that had been incubated at 35°C for 20 to 24 h.

Test inocula were prepared from pneumococcal colonies. Broth microdilution panels were prepared to include each antimicrobial agent diluted in Mueller-Hinton broth supplemented with 5% sheep blood. Each laboratory used Mueller-Hinton medium from two commercial sources, Becton-Dickinson and Difco Laboratories.

This included use of cation-adjusted Mueller-Hinton broth supplemented with 5% sheep blood. Each laboratory used Mueller-Hinton basal medium used for testing (data not shown).

Preparation of chromosomal DNA. Genetic analysis of eight selected fluoroquinolone-resistant strains and fluoroquinolone-susceptible control strains was conducted at CDC. S. pneumoniae cells were grown to the late exponential phase in 10 ml of Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (Difco) and were harvested by centrifugation. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0)–10 mM EDTA containing 0.5% deoxycholate and 0.1 mg of RNase per ml, and the mixture was incubated for 30 min at 37°C. Proteinase K and Buffer AL (QIAamp Tissue Kit; QIAGEN, Chatsworth, Calif.) were added, and the mixture was incubated at 70°C for 30 min. The lysates were applied to QIAamp spin columns, and the genomic DNA was eluted according to the manufacturer’s protocol.

PCR and DNA sequencing. Oligonucleotide primers PNC6 and PNC7 or PNC10 and PNC11 were used to amplify a 329-bp or a 512-bp gene fragment (excluding primers) of gyrA and parC, respectively (13), from the chromosomal DNA of each of the eight clinical isolates, isolate R6, and reference strain ATCC 49619. A 321-bp gene fragment of parE (excluding primers) was amplified with oligonucleotide primers SPPARE7 and SPPARE9 as described by Perichon et al. (26). Primers H4025 and H4026, described by Pan et al. (23), were used to amplify a 422-bp gene fragment of gyrB.

Amplification products were purified with the QIAquick PCR purification kit (Qiagen). DNA sequencing was performed by ABI Prism dye terminator cycle sequencing (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) with the ABI 377 automated sequencer (Perkin-Elmer, Applied Biosystems). DNA sequences were determined for both strands by using the products of independent PCRs. The GCG (Genetics Computer Group, Madison, Wis.) genetic analysis programs were used for alignment of DNA sequences and deduced amino acid sequences.

Nucleotide sequence accession numbers. The DNA sequence data obtained in this study for gene fragments from S. pneumoniae ATCC 49619 were assigned the following GenBank accession nos.: gyrA, AF065152; parC, AF065151; and parE, AF065153. The partial DNA sequence of parE from S. pneumoniae R6 was assigned GenBank accession no. AF058920.

RESULTS

The MICs of the six fluoroquinolones examined in this study did not differ significantly on the basis of the source of Mueller-Hinton basal medium used for testing (data not shown). Because MIC endpoints were somewhat better defined with the Difco medium, only those values will be detailed in the following paragraphs.

The investigational agent clinafloxacin was the most active

<table>
<thead>
<tr>
<th>Strain and quinolone</th>
<th>No. of strains for which MICs (µg/ml) were as follows:</th>
<th>% Resistancea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.12</td>
<td>≤0.25</td>
</tr>
<tr>
<td>Ofloxacin-resistant strains (n = 36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Clinafloxacin</td>
<td>6</td>
<td>28</td>
</tr>
</tbody>
</table>

a Based on approved NCCLS resistance breakpoints.
fluoroquinolone examined in this in vitro study against both the ofloxacin-susceptible and -resistant strains (Tables 1 and 2). The next most active agent was trovafloxacin, followed by grepafloxacin, sparfloxacin, and levofloxacin. The MICs of the agents for the high-level ofloxacin-resistant strains increased from 16- to 64-fold, whereas the clinafloxacin MICs for the resistant isolates appeared to increase less. Table 2 indicates the MICs recorded for the study isolates and the percentage of strains resistant to each agent.

Graphs that relate MICs to disk diffusion zone diameters are presented in Fig. 1A to F. Approved NCCLS MIC and zone diameter breakpoints for each agent with the exception of clinafloxacin (F).

FIG. 1. Scatterplots of quinolone MICs and disk diffusion zone diameters derived from the testing of 160 pneumococcal isolates. NCCLS MIC and disk diffusion interpretive breakpoints are indicated by double horizontal and vertical lines, respectively, on each graph (except for clinafloxacin, which currently lacks approved breakpoints). The fluoroquinolone agents tested in this study included ofloxacin (A), levofloxacin (B), sparfloxacin (C), grepafloxacin (D), trovafloxacin (E), and clinafloxacin (F). Asterisks indicate ofloxacin-resistant strains.
clinafloxacin are indicated on each graph; the MIC and zone diameter breakpoints for clinafloxacin have not yet been established. The source of Mueller-Hinton agar used to prepare the agar disk diffusion plates did not appear to affect the fluoroquinolone zone diameters appreciably (data not shown).

With grepafloxacin, levofloxacin, sparfloxacin, and trovafloxacin, the MICs were increased and the zone diameters were reduced for the ofloxacin-resistant strains. A comparison of the error rates generated with the NCCLS MIC and disk diffusion tests is shown in Table 3. Application of the approved NCCLS breakpoints resulted in only a few minor errors for all drugs but three very major errors with sparfloxacin. Test strains segregated into three groups according to the MICs: (i) those highly susceptible to all agents, (ii) those highly resistant to grepafloxacin, sparfloxacin, and trovafloxacin (i.e., MICs ≥ 4 μg/ml), and (iii) isolates for which MICs were moderately elevated (MICs of 1 to 2 μg/ml for sparfloxacin and grepafloxacin and 0.5 to 1 μg/ml for trovafloxacin). Eight isolates from the latter two categories were selected for genetic characterization (Table 4).

Oligonucleotide primers for gyrA, gyrB, parC, and parE were used to amplify gene fragments that included the QRDRs from the eight selected strains, isolate R6, and reference strain ATCC 49619. Amplification with oligonucleotide primers PNC6 and PNC7 produced the expected 232-bp gyrA gene fragment from each of the pneumococcal strains. Direct sequencing of the amplified gyrA gene fragments and alignment of the DNA sequences revealed a C-to-T mutation at the second position of the codon, resulting in an amino acid change of Ser-81 to Phe in five of the isolates, as shown in Table 4 (position numbers are based on the gyrA sequence of *S. pneumoniae* [1]). No mutations were detected in the gyrA gene fragment from strains TN3659-6, TN4201, or F30078, and no changes in gyrB were noted for any of the eight isolates.

The DNA sequences of the 329-bp parC gene fragments were aligned with the corresponding parC sequence from ATCC 49619. Five of the strains exhibited mutations in the Ser-79 codon, leading to substitutions with Phe (4 strains) or Tyr (1 strain) (Table 4; amino acid positions are based on those of the *S. pneumoniae* parC sequence [24]). Two mutations in the QRDR of the highly fluoroquinolone-resistant strain J3810 resulted in alterations of amino acids Ser-80 to Pro and Asp-83 to Tyr. No mutations were detected in the parC QRDR of the low-level-resistant strains F30084 or F31324.

Alignment of the 323-bp gene fragments of parE revealed mutations in pneumococcal isolates F30084 and F31324, resulting in the substitution of Asn for Asp-435 (position numbers are based on those of the *S. pneumoniae* parE sequence [24]). In both strains, the parE mutations were associated with Ser-81-to-Phe alterations of GyrA. The ParE sequences of the five remaining isolates were identical to those of R6 and the susceptible reference strain.

The strains highly resistant to ofloxacin, levofloxacin, sparfloxacin, grepafloxacin, and trovafloxacin expressed mutations in both the GyrA and ParC genes. For strains that possessed a single amino acid alteration in the ParC QRDR, the MICs were elevated but the isolates were not highly resistant to all of the newer fluoroquinolones. Double mutations involving GyrA and ParE resulted in higher MICs of ofloxacin and levofloxacin compared with those for the strains with a single amino acid change in ParC. Mutations in the gyrase A and topoisomerase IV genes did not have a pronounced effect on the susceptibilities of the study isolates to clinafloxacin (Fig. 1F).

**DISCUSSION**

This multicenter study has examined the in vitro activities of six contemporary fluoroquinolones against a collection of pneumococcal isolates, including 36 strains resistant to ofloxacin, that were recovered during recent active surveillance studies in North America, Belgium, and France. Our study was not designed to assess the incidence of such strains. However, resistance to ofloxacin occurred in 0.2% of isolates in the initial phase of the CDC North American surveillance study conducted from 1994 to 1996 (14), and resistance to levofloxacin was reported for 0.6% of isolates in a U.S. surveillance study conducted in 1996 and 1997 (29). Thus, while uncommon at

---

**TABLE 3. Determination of interpretive error rates associated with NCCLS MIC and zone diameter breakpoints with isolates included in this study**

<table>
<thead>
<tr>
<th>Quinolone</th>
<th>MIC breakpoints (μg/ml)</th>
<th>Zone breakpoints (mm)</th>
<th>Error rates (no. [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Ofl oxacin</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>≤0.5</td>
<td>1</td>
<td>≥2</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>≤0.5</td>
<td>1</td>
<td>≥2</td>
</tr>
<tr>
<td>Trovafloxacin</td>
<td>≤1</td>
<td>2</td>
<td>≥4</td>
</tr>
</tbody>
</table>

*Abbreviations: S, susceptible; I, intermediate; R, resistant; VM, very major error; M, major error; Minor, minor error.*

**TABLE 4. Alterations in GyrA, ParC, and ParE genes observed in isolates of *S. pneumoniae* demonstrating reduced susceptibility to fluoroquinolones**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation(s)¹ in QRDR of:</th>
<th>MIC (μg/ml)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GyrA</td>
<td>ParC</td>
</tr>
<tr>
<td>ATCC 49619</td>
<td>Ser-81</td>
<td>Ser-79</td>
</tr>
<tr>
<td>R6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TN4201</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>TN3659-6</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>F30078</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>F31324</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>J3810</td>
<td>&gt;16</td>
<td>16</td>
</tr>
<tr>
<td>MD3904</td>
<td>&gt;16</td>
<td>16</td>
</tr>
<tr>
<td>CT5-5821</td>
<td>&gt;16</td>
<td>16</td>
</tr>
</tbody>
</table>

*GyrA, ParC, and ParE amino acid positions are based on the respective gene sequences for *S. pneumoniae* (1, 24). —, no difference from *S. pneumoniae* 49619.

OFLX, ofloxacin; LEVO, levofloxacin; GREP, grepafloxacin; SPAR, sparfloxacin; TROV, trovafloxacin; CLFX, clinafloxacin.
the present time, the possibility exists that fluoroquinolone-resistant pneumococci will increase in frequency as these agents are used for treatment of community-acquired respiratory infections.

The newer fluoroquinolones included in the study showed improved activity over that observed with ofloxacin. As expected, levofloxacin, the active I isomer of ofloxacin, was approximately twofold more active than ofloxacin, although most strains that were resistant to ofloxacin were also resistant to levofloxacin (Fig. 1B). The rank order of activity of the newer agents on a weight basis against the ofloxacin-resistant strains was clinafloxacin (greatest) followed by trovafloxacin, grepafloxacin, and then sparfoxcin. Two populations of strains were apparent when the activities of the latter three compounds against the strains were tested, i.e., those most resistant (MICs \( \geq 4 \mu g/ml \)) to grepafloxacin, sparfoxcin, and trovafloxacin and those with lower-level resistance characterized by MICs two- to eightfold higher than those for the normal, susceptible population (Fig. 1C, D, and E). When representative strains with lower levels of resistance were characterized genetically, they exhibited mutations in either \( \text{parC} \) alone or \( \text{gyrA} \) and \( \text{parE} \) but not in \( \text{gyrA} \) or \( \text{parC} \). In contrast, selected representatives of the highly fluoroquinolone-resistant strains proved to have mutations in both \( \text{gyrA} \) and \( \text{parC} \) (Table 4). These data are consistent with the descriptions of the first clinical isolates of \( \text{S. pneumoniae} \) which demonstrated low-level resistance to ciprofloxacin as a result of mutations in the \( \text{parC} \) gene (19). Additional isolates showing high-level resistance to ciprofloxacin had mutations in \( \text{gyrA} \) and \( \text{parC} \) (19). Further studies indicated that \( \text{ParC} \) was the preferential target of ciprofloxacin and that mutations in \( \text{parC} \) preceded mutations in \( \text{gyrA} \) (12, 13, 19, 23, 24, 26). In our study, the ofloxacin-resistant isolates showed \( \text{ParC} \) changes of Ser-79 to Phe or Tyr, analogous to the changes described for ciprofloxacin-resistant strains (12, 13, 19, 23, 24). The high-level ofloxacin-resistant isolates also showed \( \text{GyrA} \) alterations of Ser-80 to Pro and Asp-83 to Tyr. However, the NCCLS breakpoints categorizing the \( \text{parC} \) single mutants and the \( \text{gyrA} \) and \( \text{parE} \) double mutants as susceptible to trovafloxacin, even though the MICs of that agent were elevated in a manner similar to those for grepafloxacin and sparfoxcin (Fig. 1E). With the exception of some very major errors with sparfoxcin disk tests, the interpretative category error rates recorded in this study with the NCCLS breakpoints are similar to those observed with other agents (15) and indicate that testing of pneumococci can be performed reliably by either the NCCLS MIC or the disk diffusion procedure. It should be noted that the NCCLS breakpoints were established with several data sets in addition to the data presented here. If only our data were considered, it would seem reasonable to increase the sparfloxacin zone diameter breakpoints by 1 mm.

In summary, this study has demonstrated that clinical pneumococcal isolates with mutations in the QRDRs of both \( \text{gyrA} \) and \( \text{parC} \) are classified by the current NCCLS breakpoints as resistant to the newer fluoroquinolones included in this study. Isolates with only \( \text{parC} \) or \( \text{gyrA} \) and \( \text{parE} \) mutations are associated with borderline resistance (sparfoxcin and grepafloxacin) or elevated MICs (trovafloxacin) and diminished zone diameters that are proximate to the current interpretative category breakpoints. Clinical studies will be required to determine the significance of the reduced susceptibilities of the latter strains to the newer fluoroquinolones. It will also be important to monitor the susceptibilities of contemporary pneumococci to these agents as their use for the treatment of respiratory infections increases.

ACKNOWLEDGMENTS

This study was supported in part by Glaxo-Wellcome, Ortho-McNeil, Parke-Davis, Pfizer, Remel, and Rhône-Poulenc Rorer. The quinolone-resistant strains tested by MGH were graciously provided by the Centre National de Reference des Pneumocoques, Créteil, France, and André Bryskier, of Hoehst Marion Roussel, Inc., Romainville, France.

We thank Jean Spargo (MGH), Leticia McElmeel (UTHSC), and Sharon Crawford (UTHSC) for excellent technical assistance.

REFERENCES