ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1999, p. 1177–1182 Vol. 43, No. 5
0066-4804/99/$04.00+0


# In Vitro Development of Resistance to Five Quinolones and Amoxicillin-Clavulanate in *Streptococcus pneumoniae*

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Received 17 September 1998/Returned for modification 4 December 1998/Accepted 11 February 1999

**The ability of 50 sequential subcultures in subinhibitory concentrations of ciprofloxacin, levofloxacin, grepafloxacin, sparfloxacin, trovafloxacin, and amoxicillin-clavulanate to select for resistance was studied for six penicillin-susceptible and four penicillin-intermediate pneumococci. Subculturing in ciprofloxacin, grepafloxacin, levofloxacin, and sparfloxacin led to selection of mutants requiring increased MICs for all 10 strains, with MICs rising from (i) 0.5 to 4.0 to (ii) 4.0 to 32.0 μg/ml after 7 to 12 passages for ciprofloxacin, from (i) 0.06 to 0.25 to (ii) 0.5 to 8.0 μg/ml after 5 to 23 passages for grepafloxacin, from (i) 0.5 to 1.0 to (ii) 4.0 to 64 μg/ml after 14 to 49 passages for levofloxacin, and from (i) 0.125 to 0.25 to (ii) 1.0 to 16.0 μg/ml after 8 to 26 passages for sparfloxacin. Subculturing in trovafloxacin led to increased MICs for eight strains, with MICs rising from (i) 0.06 to 0.125 to (ii) 0.5 to 8.0 μg/ml after 6 to 28 passages. Subculturing in amoxicillin-clavulanate led to raised MICs for only one strain, with the MIC rising from 0.015 to 0.125 μg/ml after 24 passages. Double mutations in both ParC and GyrA led to high-level quinolone resistance when ParC mutations were at S79. Trovafloxacin MICs were 1 to 2 μg/ml in double mutants with ParC mutations at positions other than S79 (e.g., D83). Mutations in ParE (at D435, R447, and E474) and GyrB (at S405, D406, and D435) were found in four and six mutants, respectively. In the presence of reserpine, 29 mutants had lower ciprofloxacin MICs (2 to 16 times lower), 8 mutants had lower levofloxacin MICs (2 times), and one mutant had a lower trovafloxacin MIC (2 times), suggesting the involvement of an efflux mechanism. In contrast to the case for quinolones, subculturing in the presence of amoxicillin-clavulanate did not select for resistance to this drug.**

Amoxicillin-clavulanate, a combination of a penicillin and a β-lactamase inhibitor, is the most active oral β-lactam agent overall against pneumococci (including those for which the penicillin MICs are increased), β-lactamase-positive and -negative *Haemophilus influenzae*, and *Moraxella catarrhalis*. In view of the increased incidence of community-acquired respiratory tract infections caused by penicillin-resistant pneumococci (1, 5), amoxicillin-clavulanate may be used as empiric therapy of sinusitis, acute exacerbations of chronic bronchitis, and other respiratory tract infections caused by these organisms, where chlamydiae, mycoplasmas, and legionellae are not involved. Levofloxacin, grepafloxacin, sparfloxacin, and trovafloxacin are quinolones with broader spectra than ciprofloxacin (2, 7, 20, 22), especially against pneumococci; this allows them to be used as alternate empiric therapy to amoxicillin-clavulanate for the same infections mentioned above.

Presently, the incidence of quinolone resistance in pneumococci is low. In a study by Felmingham in 1998 (6) examining 4,665 pneumococcal strains from 1992 to 1996, the incidences of resistance to ciprofloxacin (MIC $>$ 4 μg/ml) and ofloxacin (MIC $>$ 4 μg/ml) were 0.6 and 0.5%, respectively. In another study in France examining 4,804 strains from 1996, the incidence of resistance to ofloxacin was 0.79%. In a study presented by Jacobs et al. examining over 1,400 pneumococcal strains from the United States in 1997, there were none that were resistant to ciprofloxacin (9).

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The primary targets of fluoroquinolones are topoisomerase IV and DNA gyrase. Topoisomerase IV is composed of two C (ParC) and two E (ParE) subunits, which are encoded by *parC* and *parE*, respectively, and DNA gyrase is composed of two A (GyrA) and two B (GyrB) subunits, which are encoded by *gyrA* and *gyrB*, respectively. Resistance to fluoroquinolones in pneumococci usually occurs in a stepwise fashion, with low-level resistance caused by mutations in the quinolone resistance-determining region (QRDR) of *parC* and high-level resistance occurring after an additional mutation in the QRDR of *gyrA* (10, 15, 16, 21), except for sparfloxacin, which has been reported to target primarily GyrA (17). Mutations in the QRDRs of *parE* and *gyrB* are also believed to play a role in fluoroquinolone resistance (10, 16, 19).

The recent dramatic increase in the incidence of drug-resistant pneumococci may be due in part to abuse of oral drugs such as cephalosporins and macrolides (18). In a previous study (18), we found that sequential subcultures in subinhibitory concentrations of amoxicillin-clavulanate did not lead to increased pneumococcal MICs, in contrast to the case for azithromycin and, to a lesser extent, cefuroxime and cefaclor.

Fluoroquinolone resistance can be selected for in vitro in pneumococci (8, 10, 16, 21), and this must be considered during therapy. In order to shed further light on the capability of drugs potentially used for treatment of the same infections to select for raised MICs for pneumococci, we repeatedly exposed 10 strains of *Streptococcus pneumoniae* to subinhibitory concentrations of ciprofloxacin, grepafloxacin, levofloxacin, sparfloxacin, trovafloxacin, and amoxicillin-clavulanate to determine if resistance developed. The mutations in *parC*, *parE*, *gyrA*, and *gyrB* associated with quinolone resistance were also determined. In addition, many of the mutants were checked for

the presence of a quinolone efflux mechanism by comparing MICs in the presence and absence of reserpine (a known efflux pump inhibitor) (4).

## MATERIALS AND METHODS

**Bacteria and antimicrobial agents.** Ten strains of *S. pneumoniae* isolated within the past 5 years were randomly selected. Organisms were identified by optochin susceptibility and classified by serotyping. Six were susceptible to penicillin (MICs of ≤0.06 μg/ml), and four showed intermediate penicillin resistance (MICs of 0.125 to 0.25 μg/ml). Strains were stored at −70°C in double-strength skim milk (Difco Laboratories, Detroit, Mich.) before being tested. Antimicrobials were obtained as follows: amoxicillin-clavulanate from SmithKline Beecham Laboratories, Collegeville, Pa.; ciprofloxacin from Bayer, Inc., West Haven, Conn.; levofloxacin from RW Johnson Pharmaceutical Research Institute, Raritan, N.J.; sparfloxacin from Rhône-Poulenc Rorer, Collegeville, Pa.; grepafloxacin from Glaxo-Wellcome, Inc., Research Triangle Park, N.C.; and trovafloxacin from Pfizer, Inc., New York, N.Y.

**MIC determination.** MICs were determined by standardized microdilution methodology in Mueller-Hinton broth (Difco Laboratories) supplemented with 5% lysed horse blood (13). Breakpoints for all compounds except ciprofloxacin were those approved by the National Committee for Clinical Laboratory Standards (14), and susceptibility breakpoints are as follows: grepafloxacin, ≤0.5 μg/ml; sparfloxacin, ≤0.5 μg/ml; trovafloxacin, ≤1 μg/ml; and levofloxacin, ≤2 μg/ml. Strains were considered susceptible to ciprofloxacin when MICs were ≤2 μg/ml.

**Serial passages.** Glass tubes containing 1 ml of cation-adjusted Mueller-Hinton broth (Difco) supplemented with 5% lysed horse blood with doubling antibiotic dilutions were inoculated with approximately $5 \times 10^5$ CFU/ml at antibiotic concentrations from 3 doubling dilutions above to 3 doubling dilutions below the MIC of each agent for each strain. The initial inoculum was prepared by suspending growth from an overnight Trypticase soy blood agar plate (Difco) in Mueller-Hinton broth. Tubes were incubated at 35°C for 24 h. Daily passages were then performed for 50 days by taking an inoculum from the tube nearest the MIC (usually one tube below) which had the same turbidity as the antibiotic-free controls. Periodically for some of the mutants, an aliquot from a tube used as an inoculum was frozen in double-strength skim milk at −70°C for later analysis (MIC testing and sequencing of *parC* and *gyrA* genes). When the MIC for a strain increased fourfold, irrespective of the number of subcultures, passaging was stopped and strains were subcultured in antibiotic-free medium for 10 serial passages. A maximum of 50 serial passages in antibiotic were performed.

**Serotyping.** Serotyping of parent and passaged strains was performed by the standard Quellung method with sera from Statens Seruminstitut (Copenhagen, Denmark).

**PFGE.** To determine whether resistant isolates obtained at the end of serial passages were derived from those tested at the beginning of the study, the parent strains and the strains with increased MICs obtained after the last passage were tested by pulsed-field gel electrophoresis (PFGE) with a CHEF DR III apparatus (Bio-Rad, Hercules, Calif.). Bacterial cultures were grown for 6 h at 37°C (in 5% $CO_2$) in 5 ml of Todd-Hewitt broth supplemented with 5% yeast extract (BBL Microbiology Systems, Cockeysville, Md.). Cell pellets were collected by centrifugation of approximately 1.5 ml of culture for 30 s at 21,000 × *g*. Cell pellets were then resuspended in 150 μl of Pett IV buffer (10 mM Tris-HCl [pH 7.6], 1 M NaCl) and warmed to 55°C. An equal volume of 2% Incert agarose (FMC, Rockland, Maine) in distilled $H_2O$ was added to the warmed cells, and 100 μl of the cell-agarose mixture was distributed into a plug mold and allowed to solidify. Plugs were incubated for 1 h at 37°C in 1 ml of lysis buffer (6 mM Tris-HCl [pH 7.4], 1 M NaCl, 10 mM EDTA [pH 8.0], 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine) to which lysozyme (Sigma, St. Louis, Mo.) at 0.5 mg/ml and lysostaphin (Sigma) at 0.05 mg/ml were added fresh. The lysis solution was replaced with 300 μl of ESP (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% sodium dodecyl sulfate), to which proteinase K (Sigma) at a final concentration of 8 U/ml was added before use. The plugs were incubated overnight at 55°C. ESP was decanted, and the plugs were washed three times with 1 ml of TE (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA) and then stored in 1 ml of TE at 4°C. Agarose-embedded DNA was digested to completion with 20 U of *Sma*I (New England Biolabs, Beverly, Mass.) for 6 h at room temperature, and DNA fragments were separated as described by Moissenet et al. (11).

**PCR of quinolone resistance determinants and DNA sequence analysis.** To determine whether strains that developed resistance to quinolones had alterations in topoisomerase IV or DNA gyrase compared to the parent strains, *parC*, *parE*, *gyrA*, and *gyrB* were amplified by the PCR method with the primers and cycling conditions described by Pan et al. (16). Template DNA for PCR was prepared as follows. A colony from overnight growth was lysed by incubation for 1 h at 37°C in lysis buffer (see "PFGE" above for details), and DNA was isolated by using a Prep-A-Gene kit (Bio-Rad) as recommended by the manufacturer. After amplification, PCR products were purified from excess primers and nucleotides by using a QIAquick PCR purification kit as recommended by the manufacturer (Qiagen, Valencia, Calif.) and sequenced directly by using an Applied Biosystems model 373A DNA sequencer. Mutants with mutations widely described in the literature (e.g., Ser79→Tyr or Phe in ParC and Ser83→Tyr or Phe in GyrA) were sequenced once in the forward direction. Mutants with no mutations in a particular gene or with a previously undescribed mutation were sequenced twice in the forward direction and once in the reverse direction on products of independent PCRs.

**Determination of efflux mechanism.** MICs were determined in the presence and absence of 10 μg of reserpine (Sigma) per ml as described previously (4) with 47 mutant strains for which the MIC of a particular quinolone (after 10 subcultures on drug-free medium) was at least fourfold greater than the corresponding MIC for the parent strain. An efflux mechanism was believed to be present when the MIC in the presence of reserpine was at least twofold less (1 doubling dilution) than the MIC in the absence of reserpine (tests were repeated three times).

## RESULTS

**Subculturing in subinhibitory concentrations of antibiotic.** MIC results from subculturing in subinhibitory concentrations of antibiotics are summarized in Table 1. Subculturing in amoxicillin-clavulanate led to increased MICs for only one strain, with the MIC rising from 0.015 to 0.125 μg/ml after 24 subcultures. MICs of ciprofloxacin, grepafloxacin, levofloxacin, sparfloxacin, and trovafloxacin were unaffected for this strain.

Subculturing in the presence of ciprofloxacin, levofloxacin, sparfloxacin, and grepafloxacin led to increased MICs for all 10 strains, with MICs rising from (i) 0.5 to 4.0 μg/ml to (ii) 4 to 32 μg/ml after 7 to 12 subcultures in ciprofloxacin, from (i) 0.5 to 1.0 μg/ml to (ii) 4.0 to 64 μg/ml after 14 to 49 subcultures in levofloxacin, from (i) 0.125 to 0.25 μg/ml to (ii) 1.0 to 16.0 μg/ml after 8 to 26 subcultures in sparfloxacin, and from (i) 0.06 to 0.25 μg/ml to (ii) 0.5 to 8.0 μg/ml after 5 to 23 subcultures in grepafloxacin. Subculturing in the presence of trovafloxacin led to MICs rising from (i) 0.06 to 0.125 μg/ml to (ii) 0.5 to 8.0 μg/ml for eight strains after 5 to 28 subcultures.

Resistance was stable (MICs for passaged strains remained within 1 doubling dilution of the MICs after 10 passages on antibiotic-free medium) in all cases but one. For the strain 2 mutant exposed to ciprofloxacin, the ciprofloxacin MIC reverted back to the baseline MIC of 2 μg/ml from 16 μg/ml after 10 serial subcultures in the absence of antibiotic (Table 1).

**Cross-resistance among mutants.** Of 48 mutants for which MICs of at least one of the quinolones were elevated, 33 mutants were resistant in vitro to ciprofloxacin, 31 were resistant to grepafloxacin, 34 were resistant to sparfloxacin, 28 were resistant to levofloxacin, and 9 were resistant to trovafloxacin (Table 2).

Mutant strains that were selected in ciprofloxacin were cross resistant to the other quinolones (except trovafloxacin) only when the selected strain's ciprofloxacin MIC reached 32 μg/ml (strains 1 and 8); although trovafloxacin MICs increased, strains remained susceptible. Mutant strains that were selected in grepafloxacin (MICs of ≥1 μg/ml) were also cross resistant to sparfloxacin and in most cases to ciprofloxacin, while cross-resistance to levofloxacin and trovafloxacin did not occur until the selected strain's grepafloxacin MIC reached ≥4 and ≥8 μg/ml, respectively. Mutant strains that were selected in levofloxacin (MICs of ≥4 μg/ml) were cross resistant to ciprofloxacin, grepafloxacin, and sparfloxacin in most cases (strain 5 remained susceptible to ciprofloxacin, and strain 10 remained susceptible to grepafloxacin and sparfloxacin), while cross-resistance to trovafloxacin did not occur until the selected strain's levofloxacin MIC reached ≥16 μg/ml. Mutant strains that were selected in sparfloxacin (MICs of ≥1 μg/ml) were in most cases cross resistant to grepafloxacin, while resistance to ciprofloxacin, levofloxacin, and trovafloxacin did not occur until the selected strain's sparfloxacin MIC reached ≥2, ≥2, and ≥4 μg/ml, respectively. Mutant strains that were selected in trovafloxacin (MICs of ≥2 μg/ml) were cross resistant to all the other quinolones tested. For all strains, subculturing in any

TABLE 1. Resistance selection results

| Strain | Initial MIC (μg/ml)[a] | | | | | | | Selected resistance | | | Retest MIC after 10 antibiotic-free subcultures | | | | | | Mutation in QRDR[c] of: | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pen | A/C | Cipro | Grepa | Levo | Spar | Trova | Drug[a] | MIC | No. of subcultures | A/C | Cipro | Grepa | Levo | Spar | Trova | ParC | GyrA | ParE | GyrB |
| 1 | 0.01 | 0.03 | 2 | 0.25 | 1 | 0.25 | 0.125 | A/C | NR[b] | | | | | | | | ND[d] | ND | ND | ND |
| | | | | | | | | Cipro | 16 | 8 | 0.015 | 32 | 4 | 16 | 4 | 1 | D83→V | S83→F | None | None |
| | | | | | | | | Grepa | 2 | 12 | 0.015 | 8 | 2 | 2 | 2 | 0.5 | None | S83→Y | None | None |
| | | | | | | | | Levo | 8 | 21 | 0.015 | 16 | 1 | 4 | 2 | 0.25 | None | S83→F | None | None |
| | | | | | | | | Spar | 2 | 8 | 0.03 | 8 | 2 | 4 | 2 | 0.25 | None | S83→F | None | None |
| | | | | | | | | Trova | >2 | 6 | 0.015 | 64 | 8 | 32 | 16 | 8 | S79→Y | E87→K | None | None |
| 2 | 0.01 | 0.015 | 2 | 0.25 | 1 | 0.25 | 0.125 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 16 | 8 | 0.015 | 2 | 0.25 | 1 | 0.25 | 0.125 | None | None | None | None |
| | | | | | | | | Grepa | 2 | 14 | 0.015 | 2 | 2 | 2 | 2 | 0.5 | None | S83→F | None | None |
| | | | | | | | | Levo | 8 | 49 | 0.015 | 16 | 2 | 8 | 1 | 0.25 | None | None | D435→N | D435→E |
| | | | | | | | | Spar | 2 | 20 | 0.015 | 16 | 8 | 4 | 4 | 2 | S79→Y | None | None | None |
| | | | | | | | | Trova | NR | | | | | | | | ND | ND | ND | ND |
| 3 | 0.03 | 0.015 | 2 | 0.25 | 1 | 0.25 | 0.125 | A/C | 0.125 | 24 | 0.06 | 4 | 0.125 | 1 | 0.25 | 0.25 | ND | ND | ND | ND |
| | | | | | | | | Cipro | 16 | 8 | 0.015 | 16 | 0.25 | 2 | 0.25 | 0.25 | None | None | None | None |
| | | | | | | | | Grepa | >2 | 5 | 0.015 | 32 | 8 | 16 | 8 | 4 | S79→Y | E87→Q | None | None |
| | | | | | | | | Levo | >32 | 20 | 0.015 | 64 | 8 | 64 | 16 | 8 | S79→F | S83→F | None | None |
| | | | | | | | | Spar | 4 | 16 | 0.008 | 16 | 4 | 4 | 4 | 0.5 | None | S83→Y | None | None |
| | | | | | | | | Trova | 2 | 7 | 0.015 | 32 | 4 | 16 | 8 | 1 | D83→H | S83→F | None | None |
| 4 | 0.03 | 0.015 | 1 | 0.125 | 0.5 | 0.25 | 0.06 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 8 | 12 | 0.015 | 8 | 0.25 | 2 | 0.5 | 0.25 | None | None | None | None |
| | | | | | | | | Grepa | 2 | 9 | 0.015 | 8 | 4 | 4 | 4 | 1 | D83→N | S83→F | None | None |
| | | | | | | | | Levo | 8 | 19 | 0.015 | 8 | 2 | 8 | 2 | 1 | None | S83→F | D435→N | None |
| | | | | | | | | Spar | 2 | 10 | 0.015 | 2 | 2 | 1 | 1 | 0.25 | None | S83→Y | None | None |
| | | | | | | | | Trova | 1 | 15 | 0.015 | 1 | 0.25 | 2 | 1 | 1 | A115→V | S83→Y | None | None |
| 5 | 0.03 | 0.015 | 0.5 | 0.125 | 0.5 | 0.125 | 0.06 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 8 | 12 | 0.015 | 4 | 0.125 | 1 | 0.125 | 0.06 | None | None | None | None |
| | | | | | | | | Grepa | 1 | 23 | 0.015 | 1 | 0.5 | 1 | 0.5 | 0.25 | None | None | None | None |
| | | | | | | | | Levo | 4 | 24 | 0.015 | 2 | 2 | 4 | 2 | 0.5 | None | S83→F | None | None |
| | | | | | | | | Spar | 1 | 25 | 0.015 | 1 | 0.5 | 1 | 1 | 0.25 | None | None | None | S405→Y |
| | | | | | | | | Trova | NR | | | | | | | | ND | ND | None | ND |
| 6 | 0.03 | 0.015 | 0.5 | 0.125 | 0.5 | 0.25 | 0.125 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 4 | 7 | 0.015 | 4 | 0.25 | 2 | 0.25 | 0.25 | S79→Y | None | None | None |
| | | | | | | | | Grepa | 1 | 8 | 0.015 | 1 | 1 | 1 | 1 | 0.25 | None | None | None | G406→D |
| | | | | | | | | Levo | 4 | 14 | 0.015 | 4 | 2 | 4 | 2 | 0.25 | S79→F | None | None | D435→E |
| | | | | | | | | Spar | 4 | 9 | 0.015 | 4 | 4 | 4 | 4 | 1 | S79→Y | None | None | None |
| | | | | | | | | Trova | 1 | 28 | 0.015 | 16 | 8 | 8 | 16 | 2 | D83→G | S83→Y | R447→S | None |
| 7 | 0.25 | 0.06 | 0.5 | 0.06 | 1 | 0.125 | 0.06 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 16 | 9 | 0.06 | 8 | 0.5 | 4 | 0.25 | 0.5 | S79→F | None | None | None |
| | | | | | | | | Grepa | 0.5 | 5 | 0.06 | 2 | 0.5 | 2 | 0.5 | 0.25 | S79→F | None | None | None |
| | | | | | | | | Levo | 16 | 46 | 0.06 | 4 | 1 | 8 | 1 | 0.5 | D83→A | None | None | D435→N |
| | | | | | | | | Spar | 1 | 9 | 0.03 | 1 | 1 | 1 | 1 | 0.25 | None | None | None | None |
| | | | | | | | | Trova | 0.5 | 5 | 0.06 | 2 | 0.25 | 2 | 0.25 | 0.25 | S79→F | None | None | None |
| 8 | 0.125 | 0.06 | 4 | 0.125 | 1 | 0.25 | 0.125 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 32 | 10 | 0.03 | 32 | 4 | 4 | 2 | 1 | S79→Y | None | None | None |
| | | | | | | | | Grepa | 1 | 5 | 0.03 | 16 | 2 | 4 | 2 | 1 | S79→F | None | None | None |
| | | | | | | | | Levo | 8 | 18 | 0.06 | 32 | 4 | 8 | 2 | 0.25 | S79→Y | None | None | D435→E |
| | | | | | | | | Spar | >4 | 8 | 0.03 | 32 | 4 | 32 | 16 | 8 | S79→Y | S83→F | None | None |
| | | | | | | | | Trova | 2 | 6 | 0.03 | 32 | 4 | 16 | 8 | 4 | S79→F | S83→F | None | None |
| 9 | 0.25 | 0.25 | 1 | 0.125 | 0.5 | 0.125 | 0.125 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 8 | 10 | 0.5 | 8 | 0.25 | 2 | 0.25 | 0.125 | None | None | None | None |
| | | | | | | | | Grepa | 1 | 18 | 0.25 | 1 | 0.5 | 1 | 0.5 | 0.125 | None | None | None | None |
| | | | | | | | | Levo | 8 | 22 | 0.5 | 32 | 4 | 16 | 4 | 2 | None | S83→F | E474→K | None |
| | | | | | | | | Spar | 1 | 26 | 0.25 | 1 | 0.5 | 1 | 1 | 0.125 | None | None | None | None |
| | | | | | | | | Trova | 2 | 28 | 0.25 | 16 | 8 | 16 | 32 | 2 | D83→N | E87→K | None | None |
| 10 | 0.25 | 0.125 | 1 | 0.125 | 1 | 0.125 | 0.125 | A/C | NR | | | | | | | | ND | ND | ND | ND |
| | | | | | | | | Cipro | 8 | 7 | 0.125 | 16 | 0.25 | 4 | 0.25 | 0.25 | None | None | None | None |
| | | | | | | | | Grepa | 2 | 9 | 0.125 | 2 | 1 | 1 | 1 | 0.25 | None | None | None | None |
| | | | | | | | | Levo | 8 | 15 | 0.125 | 16 | 0.25 | 4 | 0.25 | 0.125 | None | None | None | None |
| | | | | | | | | Spar | 1 | 12 | 0.06 | 1 | 0.5 | 1 | 0.5 | 0.125 | None | None | None | None |
| | | | | | | | | Trova | 1 | 26 | 0.125 | 16 | 4 | 16 | 4 | 1 | None | S83→Y | None | None |

[a] Pen, penicillin G; A/C, amoxicillin-clavulanate; Cipro, ciprofloxacin; Grepa, grepafloxacin; Levo, levofloxacin; Spar, sparfloxacin; Trova, trovafloxacin.
[b] NR, no increase in MIC detected.
[c] Mutations refer to changes between the parent strain and the derived strain. Parent strains 4, 6, and 8 had a K137→N mutation in ParC. Parent strains 1, 3, 7, 8, and 10 had an I460→V mutation in ParE. All other parent strains had wild-type ParC, ParE, GyrA, and GyrB sequences.
[d] ND, not determined.

TABLE 2. Distribution of mutants with respect to quinolone MICs

| Quinolone | No. of mutants ($n$ = 48) for which the MIC (μg/ml) was[a]: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.06 | 0.125 | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 | 8.0 | 16.0 | 32.0 | 64.0 |
| Ciprofloxacin | —[b] | — | — | — | 8 | 7 | **5** | **7** | **11** | **8** | **2** |
| Grepafloxacin | — | 1 | 9 | 7 | **5** | **9** | **11** | **6** | — | — | — |
| Sparfloxacin | — | 1 | 8 | 5 | **9** | **10** | **7** | **3** | **4** | **1** | — |
| Levofloxacin | — | — | — | — | 11 | 9 | **13** | **5** | **7** | **2** | **1** |
| Trovafloxacin | 1 | 6 | 17 | 6 | 9 | **4** | **2** | **3** | — | — | — |

[a] Boldface represents resistance to this quinolone.
[b] —, none.

of the quinolones tested did not affect amoxicillin-clavulanate MICs.

**Serotyping and PFGE.** The 10 pneumococcal strains used comprised serotypes 1, 6A, 6B, 14, and 19F. All mutants had serotypes identical to those of the parent strains, and all but two had PFGE patterns identical to those of the parent strains. Strain 4 and strain 7, which were selected in ciprofloxacin and levofloxacin, respectively, had a one-band difference in PFGE patterns compared to the parent strains, which was most likely caused by a point mutation in one of the *Sma*I restriction sites.

**Mutations in topoisomerase IV and DNA gyrase.** Mutations in the QRDR that led to an amino acid change are listed in Table 1. Most mutations were present in ParC and/or GyrA, while few mutations were present in ParE and GyrB. Parent strains 4, 6, and 8 had a Lys137→Asn substitution in ParC compared to wild-type sequences (15, 16); however, this variation has been shown not to affect ciprofloxacin MICs (12). Additionally, parent strains 1, 3, 7, 8, and 10 had an Ile460→Val substitution in ParE. Parent strain 8 had both mutations in ParC and ParE and also had the highest ciprofloxacin MIC (4 μg/ml) of the 10 parent strains. In all other cases the amino acid sequences for ParC, ParE, GyrA, and GyrB from the parent strains were identical to the wild-type sequences (15, 16).

Mutants with double mutations of Ser79→Tyr or Phe in ParC and either Ser83→Phe or Glu87→Lys or Gln in GyrA were associated with high-level resistance to all of the fluoroquinolones tested. However, when the ParC mutation was at a position other than Ser79 (e.g., D83), trovafloxacin MICs were 1 to 2 μg/ml while the MICs of the other fluoroquinolones remained high. Strain 4 exposed to trovafloxacin had mutations of Ala115→Val in ParC and Ser83→Tyr in GyrA yet remained susceptible to all the fluoroquinolones except sparfloxacin, to which it had low-level resistance (MIC, 1 μg/ml).

Mutants with a single mutation only in ParC (Ser79→Phe or Tyr) showed various susceptibility patterns, including (i) susceptibility to all quinolones (e.g., strain 7 exposed to grepafloxacin or trovafloxacin), (ii) resistance only to ciprofloxacin (e.g., strain 6 exposed to ciprofloxacin), (iii) resistance to all quinolones except trovafloxacin (e.g., strain 8 exposed to ciprofloxacin or grepafloxacin), and (iv) resistance to all quinolones (e.g., strain 2 exposed to sparfloxacin).

Mutants with a single mutation only in GyrA (Ser83→Phe or Tyr) were associated with resistance to grepafloxacin and sparfloxacin in all cases and also with resistance to ciprofloxacin and levofloxacin in many cases. There were not any mutants with a mutation only in GyrA that were resistant to trovafloxacin.

Mutations in ParE and GyrB were observed in four and six mutants, respectively. Three of the four mutants with ParE mutations and four of the six mutants with GyrB mutations were derived from strains exposed to levofloxacin. Two mutants had a mutation only in GyrB (strain 5 exposed to sparfloxacin and strain 6 exposed to grepafloxacin). These two mutants had increases primarily in grepafloxacin and sparfloxacin MICs. Of five mutants with mutations in both (i) ParE or GyrB and (ii) ParC or GyrA (e.g., strains 4 and 6 exposed to levofloxacin), four were resistant to all of the quinolones except trovafloxacin and one was resistant to all of the quinolones. One mutant (strain 2 exposed to levofloxacin) had mutations only in ParE and GyrB (at position Asp435) and was resistant to all quinolones except trovafloxacin.

Most mutants with no mutations in ParC, ParE, GyrA, and GyrB tended to have relatively small changes in quinolone MICs (1 or 2 doubling dilutions) compared to the parent strains and remained susceptible to all or most of the quinolones tested. However, some mutants with no mutations developed resistance to some of the quinolones. For example, strains 3, 4, 5, 9, and 10 exposed to ciprofloxacin developed resistance to ciprofloxacin (MICs of 4 to 16 μg/ml), and strain 10 exposed to levofloxacin developed resistance to ciprofloxacin (MIC of 16 μg/ml) and levofloxacin (MIC of 4 μg/ml).

**Efflux mechanism.** Since several mutants were resistant to one or more quinolones and did not have any mutations in ParC, ParE, GyrA, and GyrB, we investigated the possibility that an efflux mechanism contributed to the raised MICs. This was accomplished by determining quinolone MICs in the absence and presence of reserpine (a known efflux pump inhibitor). MICs were determined for mutant strains for which the MIC of a particular quinolone (after 10 subcultures on drug-free medium) was at least fourfold greater than the corresponding MIC for the parent strain. The results are summarized in Table 3. Twenty-nine of 37 mutants had ciprofloxacin MICs that were 2 to 16 times lower in the presence of reserpine. Eight mutants had lower levofloxacin MICs, and one mutant had lower trovafloxacin MICs. In all cases the levofloxacin and trovafloxacin MICs were only 2 times lower in the presence of reserpine. No strains had lower grepafloxacin or sparfloxacin MICs in the presence of reserpine. Three different phenotypes among the mutants were observed in relation to the quinolones affected by reserpine: 20 mutants had only lower ciprofloxacin MICs, 8 mutants had lower ciprofloxacin and levofloxacin MICs, and 1 mutant had lower ciprofloxacin and trovafloxacin MICs. Reserpine lowered the ciprofloxacin MIC for all mutants that were resistant to ciprofloxacin yet had no mutations in ParC, GyrA, ParE, or GyrB (strains 3, 4, 5, 9, and 10 exposed to ciprofloxacin and strain 10 exposed to levofloxacin). Reserpine had no effect on the levofloxacin MIC for only one mutant (strain 10 exposed to levofloxacin) that had no mutations in ParC, GyrA, ParE, or GyrB.

## DISCUSSION

The increased incidence of drug-resistant pneumococci observed in recent years may be due to selective pressure caused by drug abuse. In this study we obtained only one mutant whose amoxicillin-clavulanate MIC increased fourfold (still susceptible; MIC, 0.125 μg/ml) after serial passages in subinhibitory concentrations of amoxicillin-clavulanate. A study by Pankuch et al. (18) had similar findings. However, azithromycin readily selected for resistant mutants (18). In the present study we were readily able to select for quinolone-resistant *S. pneumoniae* mutants after serial passages in subinhibitory concentrations of quinolones. Among the quinolones, exposure to trovafloxacin selected for resistance in the least number of strains (8 versus 10). Additionally, trovafloxacin retained the greatest in vitro potency against mutants resistant to the other quinolones. A previous study by Gootz et al. found that trovafloxacin selected for first-step mutants less frequently than

TABLE 3. Effect of reserpine on quinolone MICs[a]

| Strain no. | Selecting quinolone | MIC (μg/ml)[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Cipro | Cipro + reserp | Levo | Levo + reserp | Trova | Trova + reserp |
| 1 | Cipro | 32 | 8 | 16 | 8 | —[c] | — |
| | Grepa | 8 | 1 | — | — | — | — |
| | Levo | 16 | 4 | — | — | — | — |
| | Spar | 8 | 2 | — | — | — | — |
| | Trova | 64 | 16 | — | — | — | — |
| 2 | Grepa | 2 | 1 | — | — | — | — |
| | Levo | 16 | 2 | — | — | — | — |
| | Spar | 16 | 4 | — | — | — | — |
| 3 | Cipro | 16 | 1 | — | — | — | — |
| | Grepa | 32 | 16 | — | — | — | — |
| | Levo | 64 | 32 | — | — | — | — |
| | Spar | 16 | 2 | — | — | 0.5 | 0.25 |
| | Trova | 32 | 16 | — | — | — | — |
| 4 | Cipro | 8 | 2 | 2 | 1 | — | — |
| | Grepa | 8 | 4 | — | — | — | — |
| 5 | Cipro | 4 | 0.5 | — | — | — | — |
| | Levo | 2 | 1 | — | — | — | — |
| 6 | Cipro | 4 | 1 | — | — | — | — |
| 8 | Cipro | 32 | 4 | 4 | 2 | — | — |
| | Grepa | 16 | 2 | — | — | — | — |
| | Levo | 32 | 4 | — | — | — | — |
| | Spar | 32 | 8 | 32 | 16 | — | — |
| | Trova | 32 | 8 | — | — | — | — |
| 9 | Cipro | 8 | 1 | 2 | 1 | — | — |
| | Levo | 32 | 4 | 16 | 8 | — | — |
| | Trova | 16 | 4 | 16 | 8 | — | — |
| 10 | Cipro | 16 | 1 | 4 | 2 | — | — |
| | Levo | 16 | 2 | — | — | — | — |
| | Trova | 16 | 8 | — | — | — | — |

[a] Cipro, ciprofloxacin; Grepa, grepafloxacin; Levo, levofloxacin; Spar, sparfloxacin; Trova, trovafloxacin; reserp, reserpine. Strain numbers refer to the parent strain of the derived mutant, and the selecting quinolone was the quinolone in which the strain was subcultured at subinhibitory concentrations.

[b] MICs of the mutants after 10 subcultures on drug-free medium in the presence and absence of 10 μg of reserpine per ml.

[c] —, MIC not determined or there was no difference between the MICs in the presence and absence of reserpine.

ciprofloxacin (8). Levofloxacin selected for a fourfold or greater increase in MICs for all 10 pneumococcal strains, but in contrast to the case for the other quinolones, the minimum number of subcultures required for this to occur was 14 (i.e., longer than for other quinolones). This may have therapeutic significance.

Gene sequencing of the QRDRs of *parC* and *gyrA* in this study has shown, as in previous studies (8, 10, 12, 15, 16, 21), that the mutations associated with quinolone resistance were Ser79→Phe or Tyr and Asp83→Gly or His in ParC and Ser83→Phe or Tyr and Glu87→Lys in GyrA. Mutations not previously described for *S. pneumoniae* that were also associated with quinolone resistance were Asp83→Ala, Asn, or Val in ParC and Glu87→Gln in GyrA. For the majority of the quinolone-resistant mutants, resistance occurred by stepwise selection in which low-level resistance was associated with a mutation in either ParC or GyrA (depending on the primary target of the quinolone used as the selecting agent) and high-level resistance was associated with mutations in both ParC and GyrA. Gootz et al. previously reported that mutants with double mutations in ParC and GyrA were resistant to trovafloxacin (MIC of 4 to 16 μg/ml) (8). In this study we observed the same trovafloxacin MICs for mutants with double mutations when the ParC mutation was at Ser79; however, when the ParC mutation was at another position (e.g., D83) the trovafloxacin MICs were 1 to 2 μg/ml. Another ParC mutation not previously described but which was not associated with resistance was Ala115→Val. The fact that Val is very similar to Ala probably accounts for the lack of association with resistance to quinolones; however, the possibility that Ala115 is not a critical residue for quinolone resistance also should be considered.

Gene sequencing of the QRDRs of *parE* and *gyrB* in this study has shown, as in previous studies by Pan et al. (16) and Perichon et al. (19), that mutations at Asp435 are associated with quinolone resistance. In some cases mutations in ParE and GyrB may play a major role in quinolone resistance, as evidenced by one mutant in this study (strain 2 subcultured in levofloxacin) that had mutations at Asp435 in both ParE and GyrB and was resistant to all of the quinolones except trovafloxacin. In contrast, mutations in the QRDR of GyrB at Ser405 or Gly406 did not appear to be important in quinolone resistance, since they were not associated with any resistance.

It has been previously reported by Pan and Fisher that the primary target of ciprofloxacin is ParC (17). In this study all mutants with a mutation(s) in topoisomerase IV and/or DNA gyrase derived from subculturing in ciprofloxacin had mutations first appear in ParC. In contrast, mutants derived from subculturing in any of the other quinolones (including sparfloxacin, which Pan and Fisher [17] reported targeted GyrA) had mutations first appear in some cases in ParC and in other cases in GyrA. Interestingly, all mutants with a mutation at Asp435 in ParE and/or GyrB were selected by subculturing in levofloxacin, which suggests that ParE and GyrB may be important targets of levofloxacin. The broad-spectrum activity against pneumococci (compared to ciprofloxacin) of grepafloxacin, levofloxacin, sparfloxacin, and trovafloxacin may be due in part to their ability to act on more than one target.

As in other studies, mutations in ParC, ParE, GyrA, and GyrB were not associated with all increases in MICs, as some mutants for which MICs of quinolones were increased did not have any mutations in ParC, ParE, GyrA, and GyrB. Clearly, other mechanisms of fluoroquinolone resistance exist in *S. pneumoniae*. Baranova and Neyfakh (3) and Brenwald et al. (4) have recently provided evidence for a multidrug transporter that may be responsible for ciprofloxacin and norfloxacin resistance. Also, there may exist an efflux mechanism similar to NorA in *Staphylococcus aureus* (23). In this study we observed 29 mutants for which MICs of at least one of the quinolones were lower in the presence of reserpine, which suggests the involvement of an efflux mechanism. Additionally there may be more than one type of efflux mechanism involved, since there were three different phenotypes observed among the mutants in relation to the quinolones affected by reserpine.

The clinical significance of the findings of this study is uncertain. While we were readily able to select for quinolone-resistant *S. pneumoniae* mutants after serial passages in subinhibitory concentrations of quinolones, the incidence of quinolone resistance in naturally occurring strains is extremely low despite the fact that quinolones such as ciprofloxacin and ofloxacin have been used clinically for over 10 years. However, many of the same mutations in ParC and GyrA observed in the in vitro-selected quinolone-resistant mutants have been observed by us in ciprofloxacin-resistant *S. pneumoniae* clinical isolates (data not shown). A better understanding of the mechanisms

of resistance should help to keep the levels of quinolone resistance in clinical isolates low.

This study indicates that in contrast to the case for amoxicillin-clavulanate, sequential subculture in subinhibitory concentrations of all quinolones tested led to substantially increased MICs. Oral drugs such as cephalosporins and macrolides have been shown to select for resistance in the pneumococcus (18), and the potential for this to occur with quinolones has been demonstrated in this paper. In order to help minimize the emergence of quinolone-resistant pneumococci, we feel that these findings emphasize the need for cautious and judicious use of broad-spectrum quinolones in the treatment of community-acquired respiratory tract infections.

## ACKNOWLEDGMENT

This study was supported by a grant from SmithKline Beecham Laboratories, Collegeville, Pa.

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