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## Dose Ranging and Fractionation of Intravenous Ciprofloxacin against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an In Vitro Model of Infection

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The effect of dose or dose interval on the pharmacodynamics of simulated high-dose intravenous ciprofloxacin therapy on infection due to *Pseudomonas aeruginosa* and *Staphylococcus aureus* was studied in an in vitro hollow-fiber model of infection. Simulated doses of 1,200 mg of ciprofloxacin per day as either 400 mg every 8 h or 600 mg every 12 h against *P. aeruginosa* resulted in selection of ciprofloxacin-resistant bacteria. The results with one test strain that was isolated from a patient prior to administration of intravenous ciprofloxacin demonstrated selection of a *gyrA* mutant in the model, as had occurred in vivo. A single 1,200-mg dose every 24 h did not select for bacterial resistance; however, breakthrough regrowth of ciprofloxacin-susceptible bacteria occurred. Dosages of 400 or 600 mg of ciprofloxacin every 12 h effectively reduced bacterial counts of one strain each of methicillin-susceptible or -resistant *S. aureus*, with no bacterial resistance detected at the end of experiment; in contrast, 200 mg every 12 h resulted in bacterial regrowth due to the selection of drug-resistant bacteria. These data show the need for high-dose intravenous ciprofloxacin, particularly with regimens producing high peak levels, for treatment of infections where selection for bacterial resistance is a clinical problem.

Despite significant clinical experience with ciprofloxacin and other fluoroquinolones, the optimal dose and schedule for treatment remain unknown. Elucidation of the pharmacodynamic variables most predictive of a favorable response leads to the design of optimal dosing regimens. This may be particularly important for infections due to organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, for which the MICs of most fluoroquinolones are at or slightly below the breakpoint for susceptibility and for which selection for bacterial resistance has been associated with failure to respond clinically (9, 12, 16-18, 21).

This study evaluated the effects of various doses and dosing intervals of ciprofloxacin against *P. aeruginosa* and *S. aureus* in an in vitro model of infection. Several pharmacokinetic and pharmacodynamic variables were examined to ascertain those associated with bacterial eradication and suppression of resistant bacterial subpopulations, including strains whose mechanism of resistance had been previously characterized by using specific probes.

### MATERIALS AND METHODS

**Bacteria.** All bacterial test isolates were provided by N.J.R. Two strains each of *P. aeruginosa* and *S. aureus* were studied. For all strains an inoculum of  $\sim 5 \times 10^7$  CFU/ml had mode MICs of ciprofloxacin of 1 mg/liter in cation-supplemented Mueller-Hinton broth (MHB-S), as assessed by the tube broth macrodilution method (15). *P. aeruginosa* MP001 is a pretherapy clinical isolate obtained from a patient with mediastinitis and empyema; two ciprofloxacin-resistant *gyrA* (*cfxA*) mutants (MP002 and MP003)

of this strain with ciprofloxacin MICs of 8 and 16 mg/liter, respectively, were isolated from the patient after intravenous ciprofloxacin therapy (12). *P. aeruginosa* PAO2, a well-characterized laboratory strain, was also studied in the model. Selection of ciprofloxacin-resistant mutants on agar containing 2 times the MIC of drug occurs at a frequency of  $\sim 10^{-7}$  (20). These isolates had both the *cfxA* and *cfxB* chromosomal mutations, alone or combined, as well as changes in outer membrane proteins (20).

Two strains of *S. aureus* were tested. Strain MP1200 is a methicillin-resistant strain of *S. aureus*, and MP1201 is a methicillin-susceptible strain of *S. aureus*.

**In vitro model.** Hollow-fiber bioreactor chambers (Cell-Pharm Mini-Bioreactor System; CD Medical, Inc., Miami Lakes, Fla.) were connected in series to a central compartment in an incubator as previously described (2). The volume of each peripheral chamber was 15 ml. The central compartment diluent was MHB-S; peripheral compartments contained 10% heat-inactivated human serum plus 90% MHB-S. Following a dose of ciprofloxacin, the drug was eliminated by pumping drug-free MHB-S into the central compartment at a rate adjusted to simulate the human pharmacokinetics of the drug.

The bacteria used for all experiments were prepared from inocula previously grown, aliquoted, and frozen in a mixture of brain heart infusion broth and fetal calf serum (60:40). On the day of an experiment, the frozen inoculum was thawed, diluted with fresh MHB-S, and incubated for 1 to 2 h to bring organisms into the log-linear growth phase. The bacteria were then injected into the peripheral compartments of the model and incubated for 2 h to reach an inoculum of about  $2 \times 10^6$  CFU/ml.

Samples (1 to 1.5 ml) were collected from the central and peripheral compartments over 30 h and assayed for drug

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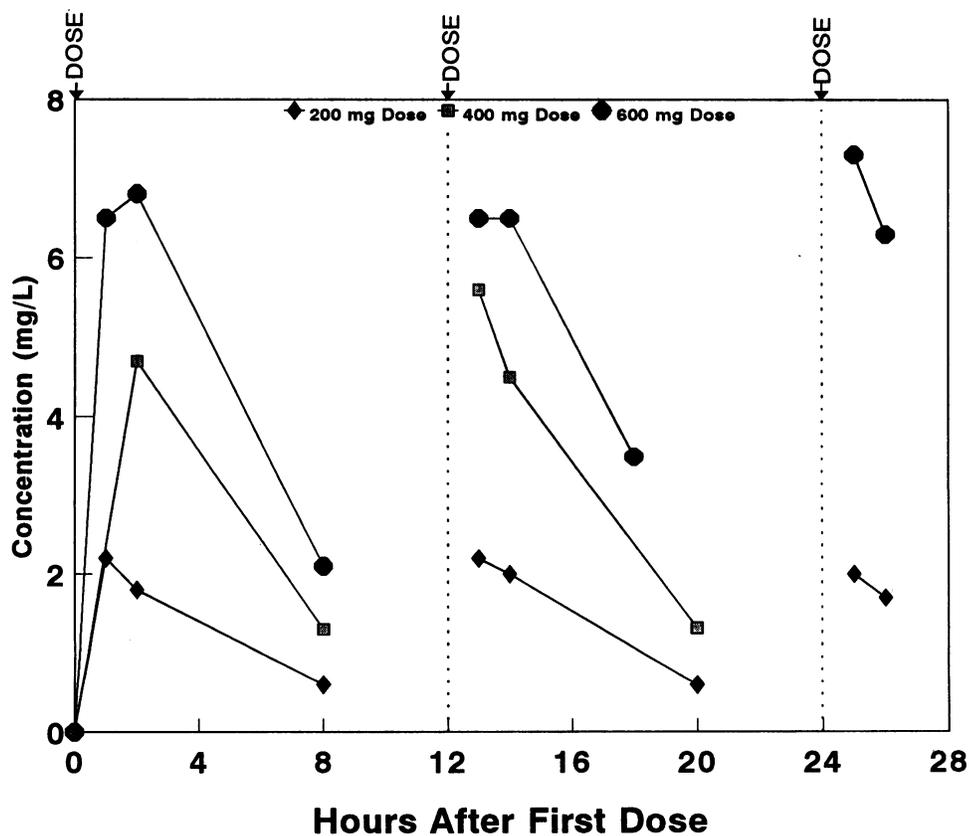
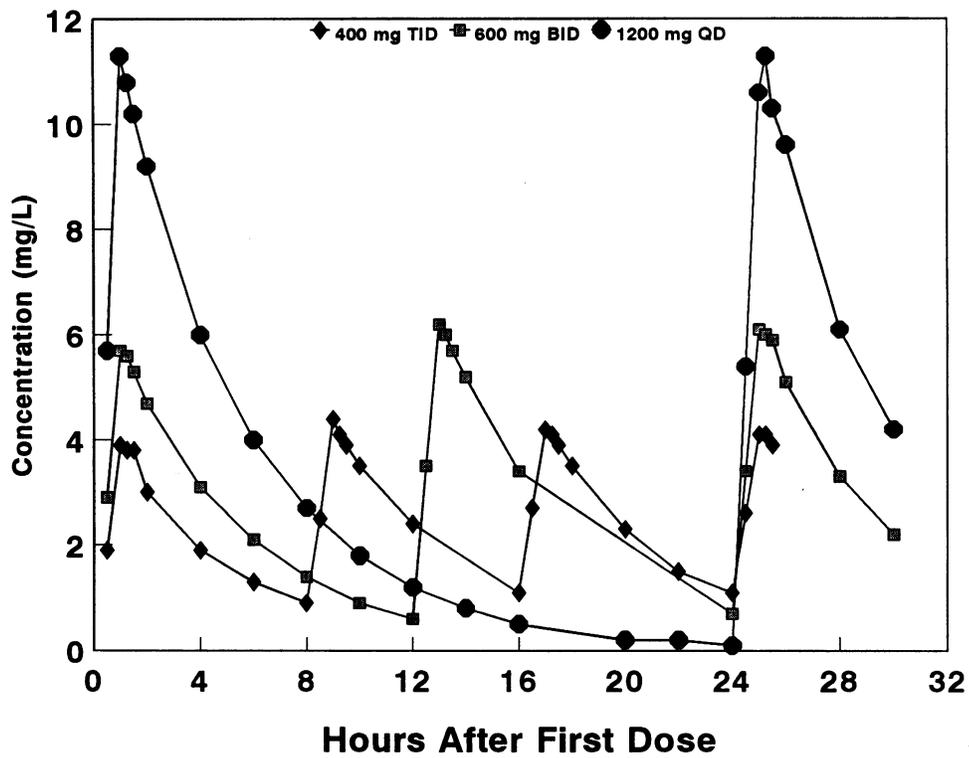


FIG. 1. Ciprofloxacin concentrations measured in the central compartment for the three dosage regimens against both strains of *P. aeruginosa* (top) and *S. aureus* (bottom). TID, every 8 h; BID, every 12 h; QD, every 24 h.

TABLE 1. Ciprofloxacin pharmacokinetic parameters in the in vitro model relative to the initial MIC, based on measured drug concentrations for different dosage regimens

Organism and regimen <sup>a</sup>	Peak/MIC <sup>b</sup>	Peak - MIC <sup>c</sup> (mg/liter)	Time > MIC <sup>d</sup> (h)		AUC > MIC <sup>e</sup> (mg · h/liter)	
			0-8 h	0-24 h	0-8 h	0-24 h
<i>P. aeruginosa</i>						
400 mg TID	4.2	3.2	7.5	23	7.9	23
600 mg BID	6	5	8	20	17	38
1,200 mg QD	11	10	8	13	40	43
<i>S. aureus</i>						
200 mg BID	2.2	1.2	5	10	2.3	4.7
400 mg BID	5.9	4.9	8	18	18.3	35.4
600 mg BID	6.8	5.8	8	24	28.6	62.8

<sup>a</sup> TID, every 8 h; BID, every 12 h; QD, every 24 h.

<sup>b</sup> Ratio of peak drug concentration to MIC.

<sup>c</sup> Difference between peak drug concentration and MIC.

<sup>d</sup> Time that ciprofloxacin concentration exceeded the MIC.

<sup>e</sup> Area under the ciprofloxacin concentration-time curve that exceeded the MIC.

(central and peripheral chambers) and bacterial (peripheral chambers) concentrations. Ciprofloxacin concentrations in samples from experiments with *P. aeruginosa* were measured by high-pressure liquid chromatography (HPLC) (10). Bioassay by a previously described method was used to measure drug concentrations in the central compartment in experiments with *S. aureus* (6). The between-day variability and accuracy of the HPLC and bioassay methods were <10%.

Bacteria were quantified by placing samples serially diluted in cold saline on drug-free Mueller-Hinton agar (MHA) plates, incubating at 37°C, and counting colonies. Samples with very low numbers of bacteria were placed in 10 ml of cold saline and then filtered with a 0.45-µm filter (Millipore Corporation, Bedford, Mass.). The filter was then placed directly on drug-free MHA and incubated. The limit of detection by this method was 10 CFU/ml.

**Dosage regimens and pharmacokinetics.** Initial experiments were conducted with a fixed daily exposure of 1,200 mg of ciprofloxacin given as 600 mg every 12 h. Subsequent reduction or fractionation of the total daily dose was based on results with this regimen; if divided doses of 600 mg every 12 h effectively reduced bacterial CFU per milliliter with no regrowth due to selection of resistant bacteria, the daily exposure would be lowered to 400 mg every 12 h and then to 200 mg every 12 h until a dose resulting in no net bacterial killing by 24 h was reached. If bacterial regrowth and/or resistance occurred following the initial regimen of 600 mg every 12 h, the total daily dose was fractionated as either 400 mg every 8 h or 1,200 mg every 24 h. Doses were given as 60-min constant-rate infusions. The elimination half-life of ciprofloxacin in the model was adjusted to 3.5 h (7).

Pharmacokinetic and pharmacodynamic parameters for each regimen were calculated by using measured ciprofloxacin concentrations in the central compartment and pretreatment MICs. Previous studies with these bioreactor units showed rapid equilibration of drug between the compartments. This allows one to focus on exposure profiles in the analogous "intravascular" compartment. The parameters calculated included the relation of the peak drug concentration to the MIC as either a ratio or a difference, the time that the ciprofloxacin concentration exceeded the MIC, and the area under the ciprofloxacin concentration-time curve that exceeded the MIC (5).

**Drug resistance.** Samples taken prior to drug exposure (time zero) and at 24 h were placed on MHA plates contain-

ing no drug or 0.5, 1, or 4 mg of ciprofloxacin per liter to quantify ciprofloxacin-resistant bacterial subpopulations. The MICs for post-treatment isolates recovered on drug-free plates also were determined by broth macrodilution.

Eight isolates of *P. aeruginosa* recovered from the model on ciprofloxacin-containing or drug-free plates prior to or 24 h after the first drug dose were probed for a *gyrA* mutation by using a broad-host-range gyrase A gene probe (19). This probe, pNJR3-2, consists of an *Escherichia coli* gyrase A gene cloned into the cosmid vector pLA2917. Previous studies of matings of NJR3-2 into resistant mutants of MP001 (selected during therapy) and PAO2 showed that it is capable of restoring pure *gyrA* (i.e., *cfxA*) mutants to wild-type susceptibility. The probe has no effect on fully susceptible or *cfxB* mutants and only partially restores susceptibility of *cfxA cfxB* double mutants of PAO2 (19).

Isolates recovered on drug-containing plates were passed three times on plates containing the same concentration of ciprofloxacin and once onto drug-free MHA and then transferred onto a Trypticase soy agar slant for transport and storage for probe studies. Bacterial isolates recovered on drug-free plates were passed one or two times on drug-free MHA and then transferred onto a Trypticase soy agar slant. In addition, several isolates were frozen in glycerol for longer-term storage prior to probe studies. Bacterial isolates recovered from the model were incubated with *E. coli* S17-1 containing the vector plasmid pLA2917 (negative control) or pNJR3-2 overnight in broth, and passed onto *Pseudomonas* isolation medium as previously described (19). A known *cfxA* mutant of PAO2 was used as a positive control. An isolate demonstrating an increase in ciprofloxacin susceptibility following exposure to the probe relative to that of the pretreatment isolate was judged to have a pure *gyrA* mutation.

## RESULTS

**Pharmacokinetics.** Figure 1 depicts ciprofloxacin concentrations in the central compartment of the model for the three dosage regimens tested against each strain. Drug concentrations in samples collected simultaneously from the central and all peripheral chambers of the model were with 10 to 15% of each other; thus, concentrations in the central compartment were used in pharmacokinetic-pharmacodynamic calculations. Pharmacokinetic parameters related to the MIC are shown in Table 1. As would be expected, the

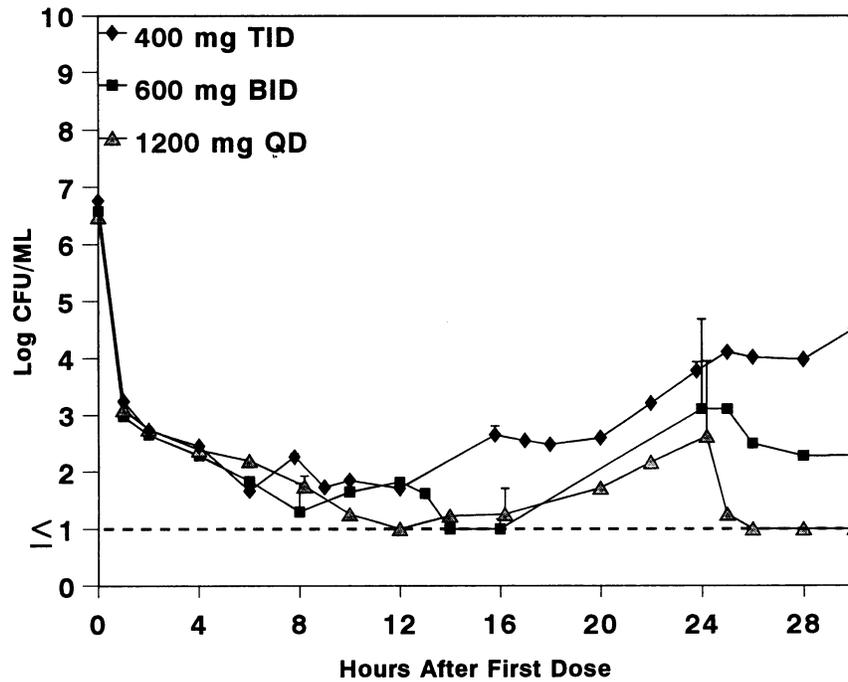
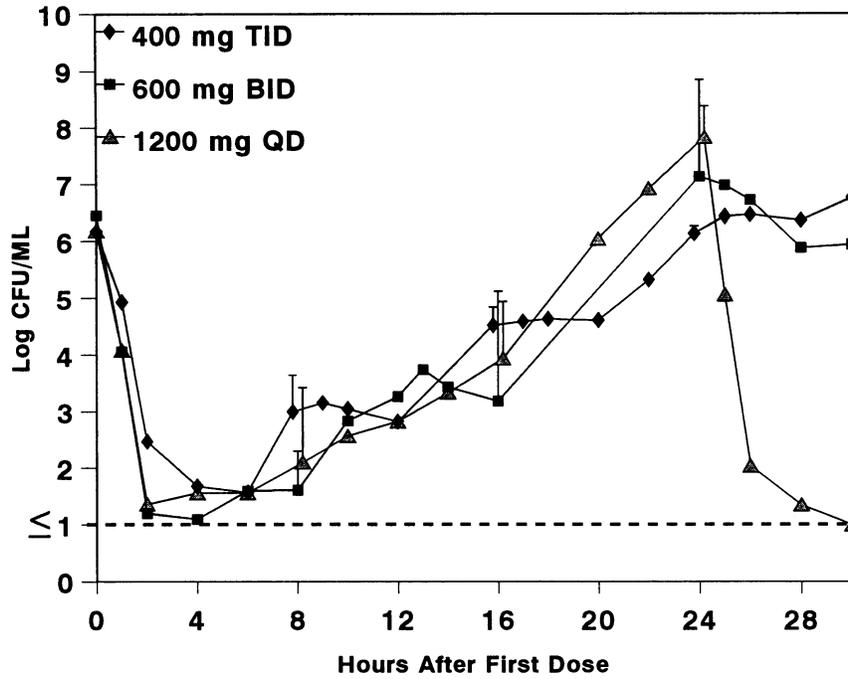


FIG. 2. Pharmacodynamics of ciprofloxacin for three different dosage regimens against *P. aeruginosa* MP001 (top) and *P. aeruginosa* PAO2 (bottom). Datum points are geometric means plus standard deviations for three replicate chambers. For abbreviations, see the legend to Fig. 1.

ratio of and difference between the peak concentration and MIC increased, the area under the curve that exceeded the MIC increased, and the time that the concentration exceeded the MIC decreased as dose size (and interval) increased. With the regimen of 400 mg every 8 h, ciprofloxacin

concentrations essentially always exceeded the initial MIC (1 mg/liter) for both strains. With the regimens of 600 mg every 12 h and 1,200 mg every 24 h, drug concentrations exceeded the initial MIC for only 80 and 54% of the dosing interval, respectively.

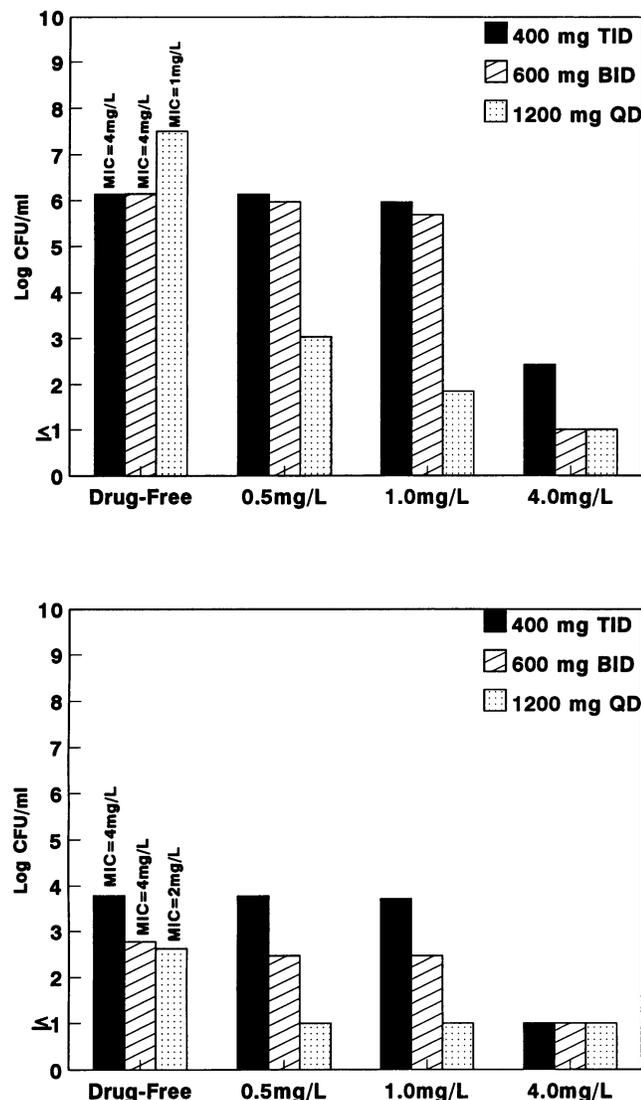


FIG. 3. Recovery of *P. aeruginosa* MP001 (top) and *P. aeruginosa* PAO2 (bottom) on MHA containing no drug or 0.5, 1.0, or 4.0 mg of ciprofloxacin per liter. Organisms were recovered at 24 h following exposure to three different ciprofloxacin dosage regimens. The broth MICs for organisms recovered on drug-free plates are also shown. For abbreviations, see the legend to Fig. 1.

Experiments with *S. aureus* utilized a fixed 12-h dosing interval; thus, changes in parameters were proportional to dose size (Table 1). With the 200-mg dose, the concentration exceeded the MIC for only 5 h of the 12-h dosing interval, whereas the 400- and 600-mg doses produced concentrations which exceeded the MIC for cumulative periods of 18 and 24 h, respectively, of a 24-h interval. The area under the curve that exceeded the MIC was severalfold higher for the 400- and 600-mg doses relative to the 200-mg dose.

**Pharmacodynamics and bacterial resistance.** (i) *P. aeruginosa*. The first dose with all regimens resulted in a marked bactericidal effect (about a 5-log reduction in CFU per milliliter) over the first 4 h in both strains (Fig. 2). With strain MP001, regrowth occurred before the second dose with all regimens; bacterial counts returned to the level of the initial inoculum by 24 h despite subsequent doses with the regi-

mens of 400 mg every 8 h and 600 mg every 12 h (Fig. 2, top). In contrast, the subsequent 1,200-mg dose given at 24 h resulted again in marked killing of this strain.

A similar but less pronounced pattern was observed with strain PAO2; initial killing with the regimens of 400 mg every 8 h and 600 mg every 12 h was followed by bacteriostasis and slight regrowth with subsequent doses (Fig. 2, bottom). However, the second 1,200-mg dose with the regimen of 1,200 mg every 24 h exerted a marked bactericidal effect against this strain; this was also observed in strain MP001.

The susceptibility of bacteria recovered at 24 h following exposure to 400 mg every 8 h or 600 mg every 12 h demonstrated that the majority of organisms were resistant to 1 mg of ciprofloxacin per liter (Fig. 3); for strain MP001, a small proportion was resistant to 4 mg/liter. The ciprofloxacin MIC (in MHB-S) for bacteria recovered on the drug-free plate was 4 mg/liter. In contrast to the every-8- or -12-h regimens, subpopulation analysis with the regimen of 1,200 mg every 24 h revealed that either only a small proportion of the inoculum was resistant to 1 mg of ciprofloxacin per liter (strain MP001; Fig. 3, top) or all organisms remained susceptible to 1 mg/liter (strain PAO2; Fig. 3, bottom). The MIC in MHB-S for bacteria recovered on drug-free medium following the single large dose did not change for strain MP001 and increased only slightly (from 1 to 2 mg/liter) for strain PAO2.

DNA gene probe analysis of isolates from both strains of *P. aeruginosa* at 24 h showed selection of pure *gyrA* mutants only in strain MP001. These mutants were recovered on the drug-free plate following the regimen of 600 mg every 12 h. In the other case they were detected in one of the three distinct colony morphology types tested from a plate containing 0.5 mg of ciprofloxacin per liter following the regimen of 1,200 mg every 24 h. No pure *gyrA* mutants were detected with the regimen of 400 mg every 8 h against this strain or with any regimen with strain PAO2, indicating that changes in susceptibility were of the *cfxB* type or perhaps were double mutations.

(ii) *S. aureus*. Dosing of 400 or 600 mg of ciprofloxacin every 12 h resulted in rapid and complete killing of both strains of *S. aureus*, with no selection for ciprofloxacin-resistant bacteria during the experiment (Fig. 4, top). However, with the regimen of 200 mg every 12 h, an initial 4- to 5-log kill was followed by bacterial regrowth within 10 h after the first dose for both strains (Fig. 4, bottom). The second 200-mg dose at 12 h produced some bacterial killing but was followed by sustained bacterial regrowth; the final dose at 24 h had no significant effect on bacterial counts.

Analysis of isolates of both strains at 24 h following the regimen of 200 mg every 12 h revealed bacterial subpopulations resistant to 1 mg of ciprofloxacin per liter (Fig. 5); a small proportion of the inoculum of the methicillin-resistant strain was also resistant to 4 mg of ciprofloxacin per liter. A fourfold rise in MIC in MHB-S (from 1 to 4 mg/liter) for bacteria recovered on drug-free plates was observed for both strains. The basis of increased resistance in these strains was not investigated.

## DISCUSSION

Although the fluoroquinolones have been established as effective agents in the treatment of a number of infections, bacterial resistance has limited their usefulness in serious infections due to certain organisms. Among gram-negative bacteria, *P. aeruginosa* has often been associated with changes in susceptibility to ciprofloxacin following therapy

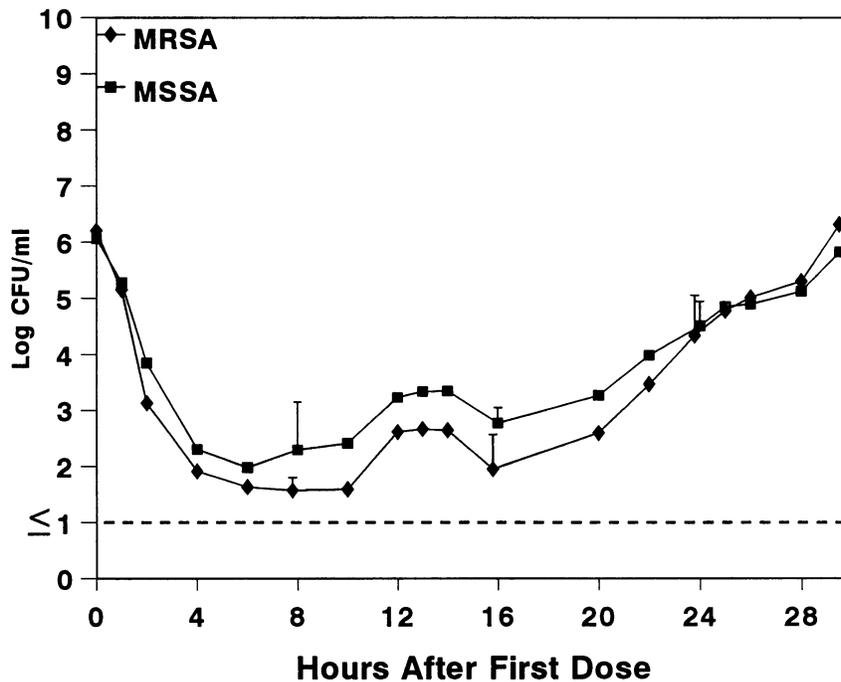
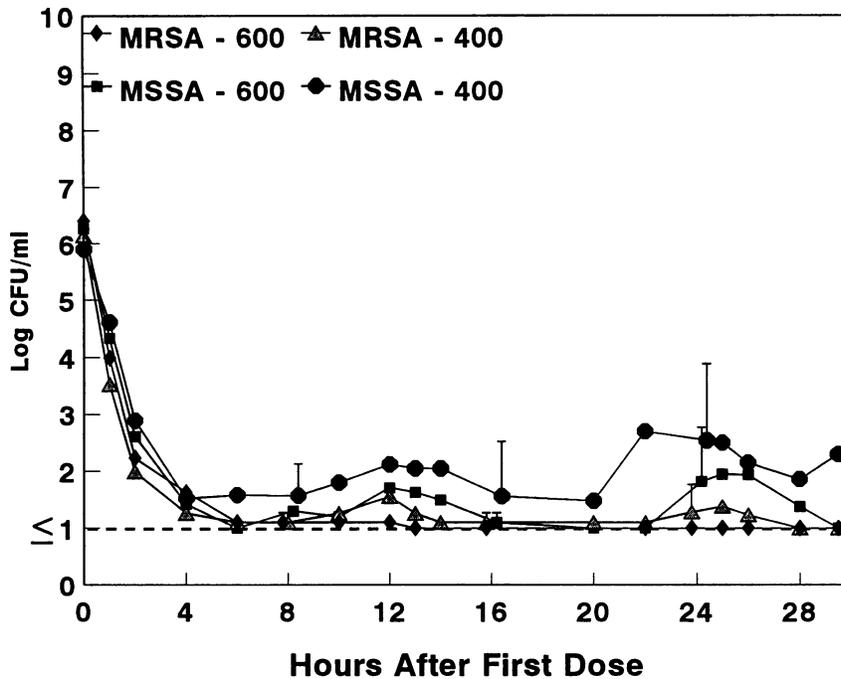


FIG. 4. Pharmacodynamics of ciprofloxacin following 400 or 600 mg every 12 h (top) and 200 mg every 12 h (bottom) against a methicillin-susceptible (MSSA) and a methicillin-resistant (MRSA) strain of *S. aureus*. Datum points are geometric means plus standard deviations for three replicate chambers.

(12, 14, 16, 21, 22); this has been a particular problem with intravenous dosage regimens of 200 to 300 mg twice daily (6, 17). The mechanism of resistance in gram-negative bacilli seems to be of two types. Alterations in DNA gyrase (*gyrA* mutation) occur at a frequency of  $10^{-7}$  and are associated

with a 4- to 16-fold increase in the MIC compared with that for wild-type strains (22). Bacteria may also develop changes in outer membrane permeability to ciprofloxacin, thus limiting access of the drug to the cytoplasmic target site (3). Resistance of methicillin-susceptible and -resistant strains of

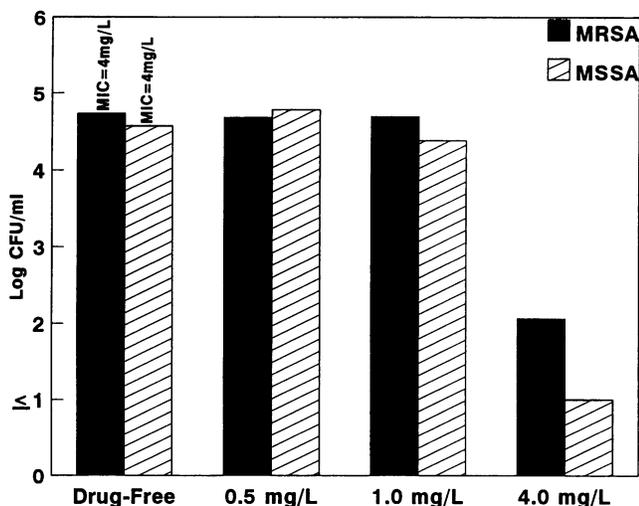


FIG. 5. Recovery of *S. aureus* on agar plates containing no drug or 0.5, 1.0, or 4.0 mg of ciprofloxacin per liter from two replicate chambers. Organisms tested were those recovered at 24 h following exposure to 200 mg of ciprofloxacin every 12 h. The broth MICs for organisms recovered on drug-free plates are also shown. For abbreviations, see the legend to Fig. 4.

*S. aureus* to ciprofloxacin and other fluoroquinolones is an increasing problem which has limited the use of these agents in the treatment of serious infections due to these organisms (13, 20).

In view of the clinical problem of bacterial resistance with fluoroquinolone therapy of infection due to these species, we studied the role of pharmacokinetics in the selection of resistance. The dosage regimens tested higher simulated levels of exposure of ciprofloxacin than have been studied systematically to date in humans. Our studies included experiments with isolates of *P. aeruginosa* whose genotypic and phenotypic bases for resistance following exposure to ciprofloxacin were previously well characterized. One of the strains tested in our model was a pretherapy isolate recovered from a patient whose failure to respond to intravenous ciprofloxacin therapy was proven to be due to selection of a drug resistant *gyrA* mutant of the same strain (12, 16). Studies using the model with the pretherapy isolate of this strain (M0001) showed that a high-dose regimen of 1,200 mg of ciprofloxacin per day given as either 400 mg every 8 h or 600 mg every 12 h resulted in the selection of ciprofloxacin-resistant bacteria with a *gyrA* mutation, as had been described previously for this organism (12). While the scope of comparisons is indeed limited, these data suggest that the endpoints observed in the in vitro model for a dosage regimen may forecast the response to human infection with a similarly large bacterial inoculum (5).

The killing of *P. aeruginosa* by ciprofloxacin was initially rapid and then slow prior to regrowth due to bacterial resistance (2). This biphasic killing pattern has been noted previously in experiments using static concentrations of drug (4, 23). Although adaptive resistance has not been described for fluoroquinolones, Wolfson et al. have described selection of partially tolerant mutants of *E. coli* in vitro following exposure to norfloxacin (23).

Although bacterial regrowth was observed within 6 to 8 h with all of the dosage regimens for *P. aeruginosa*, the regrowth that occurred following exposure to a single

1,200-mg dose was due not to ciprofloxacin-resistant bacteria but to breakthrough growth of susceptible bacteria. This was evident by the marked bactericidal effect of a subsequent dose given at 24 h. Therefore, for these strains of *P. aeruginosa*, exposure to 1,200 mg of ciprofloxacin per day given as any regimen in the model was incapable of preventing the selection of drug-resistant bacteria or causing a net reduction of bacterial counts over a 24-h period. Bacterial regrowth occurred because of selection of ciprofloxacin-resistant bacteria with regimens using more frequent dosing (400 mg every 8 h or 600 mg every 12 h) or survival of small numbers of ciprofloxacin-susceptible bacteria which persisted and regrew with the regimen of 1,200 mg every 24 h during prolonged periods of low concentrations of drug.

Studies with a methicillin-susceptible and a methicillin-resistant strain of *S. aureus* demonstrated the rapid selection of resistance to ciprofloxacin with the low dosage (200 mg every 12 h) but not with the regimens of 400 or 600 mg every 12 h. Whether these higher-dosage regimens will result in a reduced frequency of resistance when continued over a longer period of exposure (as is the case with treatment of human infection) clearly requires further study.

Although previous studies of fluoroquinolones against gram-negative bacteria in vitro (1, 6) and animal (8, 11) models of infection and in humans (15) have examined the relation between certain pharmacokinetic parameters and outcomes, the results from these studies have at times appeared to conflict with each other. However, the apparent discrepancies between studies are often resolved when one considers differences in study design, selection of improper pharmacokinetic models, and use of a large bacterial inoculum to measure the selection of bacterial resistance as an endpoint (5). However, the weight of evidence indicates that high peak concentrations relative to the MIC is the parameter associated with improved outcome, particularly when selection for bacterial resistance leads to failure in clinical or experimental infection (1, 4, 8). As with previous studies with fluoroquinolones in this model, peak concentration/MIC ratios of less than 10 were associated with selection of drug-resistant populations of *P. aeruginosa* (1, 6). The finding that shorter dosage intervals with a fixed dosage level result in lower, sustained drug levels closer to the MIC is not surprising, since these regimens most closely resemble laboratory procedures to select for drug-resistant mutants in vitro.

The data from the current study show the importance of high-dose therapy resulting in an increased exposure of bacteria to ciprofloxacin during the early stages of therapy (e.g., the first 8 h) for the suppression of ciprofloxacin-resistant bacteria. Our system is set up to test the worst-case example, in which no host defenses are present, and results when intact host defenses are present may be different. Further studies evaluating the safety and efficacy of higher doses in humans are required.

#### ACKNOWLEDGMENTS

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