In Vitro Infection Model Characterizing the Effect of Efflux Pump Inhibition on Prevention of Resistance to Levofloxacin and Ciprofloxacin in *Streptococcus pneumoniae*[▽]

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Received 22 March 2007/Returned for modification 25 April 2007/Accepted 28 August 2007

The prevalence of fluoroquinolone-resistant *Streptococcus pneumoniae* is slowly rising as a consequence of the increased use of fluoroquinolone antibiotics to treat community-acquired pneumonia. We tested the hypothesis that increased efflux pump (EP) expression by *S. pneumoniae* may facilitate the emergence of fluoroquinolone resistance. By using an in vitro pharmacodynamic infection system, a wild-type *S. pneumoniae* strain (Spn-058) and an isogenic strain with EP overexpression (Spn-RC2) were treated for 10 days with ciprofloxacin or levofloxacin in the presence or absence of the EP inhibitor reserpine to evaluate the effect of EP inhibition on the emergence of resistance. Cultures of Spn-058 and Spn-RC2 were exposed to concentration-time profiles simulating those in humans treated with a regimen of ciprofloxacin at 750 mg orally once every 12 h and with regimens of levofloxacin at 500 and 750 mg orally once daily (QD; with or without continuous infusions of 20 µg of reserpine/ml). The MICs of ciprofloxacin and levofloxacin for Spn-058 were both 1 µg/ml when susceptibility testing was conducted with each antibiotic alone and with each antibiotic in the presence of reserpine. For Spn-RC2, the MIC of levofloxacin alone and with reserpine was also 1 µg/ml; the MICs of ciprofloxacin were 2 and 1 µg/ml, respectively, when determined with ciprofloxacin alone and in combination with reserpine. Reserpine, alone, had no effect on the growth of Spn-058 and Spn-RC2. For Spn-058, simulated regimens of ciprofloxacin at 750 mg every 12 h or levofloxacin at 500 mg QD were associated with the emergence of fluoroquinolone resistance. However, the use of ciprofloxacin at 750 mg every 12 h and levofloxacin at 500 mg QD in combination with reserpine rapidly killed Spn-058 and prevented the emergence of resistance. For Spn-RC2, levofloxacin at 500 mg QD was associated with the emergence of resistance, but again, the resistance was prevented when this levofloxacin regimen was combined with reserpine. Ciprofloxacin at 750 mg every 12 h also rapidly selected for ciprofloxacin-resistant mutants of Spn-RC2. However, the addition of reserpine to ciprofloxacin therapy only delayed the emergence of resistance. Levofloxacin at 750 mg QD, with and without reserpine, effectively eradicated Spn-058 and Spn-RC2 without selecting for fluoroquinolone resistance. Ethidium bromide uptake and efflux studies demonstrated that, at the baseline, Spn-RC2 had greater EP expression than Spn-058. These studies also showed that ciprofloxacin was a better inducer of EP expression than levofloxacin in both Spn-058 and Spn-RC2. However, in these isolates, the increase in EP expression by short-term exposure to ciprofloxacin and levofloxacin was transient. Mutants of Spn-058 and Spn-RC2 that emerged under suboptimal antibiotic regimens had a stable increase in EP expression. Levofloxacin at 500 mg QD in combination with reserpine, an EP inhibitor, or at 750 mg QD alone killed wild-type *S. pneumoniae* and strains that overexpressed reserpine-inhibitable EPs and was highly effective in preventing the emergence of fluoroquinolone resistance in *S. pneumoniae* during therapy. Ciprofloxacin at 750 mg every 12 h, as monotherapy, was ineffective for the treatment of Spn-058 and Spn-RC2. Ciprofloxacin in combination with reserpine prevented the emergence of resistance in Spn-058 but not in Spn-RC2, the EP-overexpressing strain.

Streptococcus pneumoniae is the most common bacterial cause of community-acquired pneumonia. Until recently, penicillin was considered the drug of choice for the treatment of infections due to this pathogen. However, with the increasing prevalence of penicillin-resistant *S. pneumoniae*, other agents must be considered. Levofloxacin is a fluoroquinolone that is an attractive alternative because it is safe and effective for the treatment of community-acquired pneumonia due to *S. pneumoniae* and other bacterial pathogens. However, Chen et al.

(2) and Whitney et al. (17) have noted that the prevalence of quinolone-resistant *S. pneumoniae*, primarily that of ciprofloxacin-resistant strains, is rising. Recently, Davidson et al. (5) described several cases in which levofloxacin resistance in *S. pneumoniae* developed in patients who received levofloxacin as therapy for community-acquired pneumonia.

Previously, we demonstrated that the mutation frequency for resistance in a wild-type clinical strain of *S. pneumoniae* (strain Spn-058) to 3× MIC of levofloxacin was very rare, at <10⁻¹⁰. Thus, it is not surprising that we were unable to identify levofloxacin-resistant subpopulations in mice that were treated with clinically relevant levels of levofloxacin after these animals were infected with 2 × 10⁷ CFU of Spn-058. In contrast, we were able to readily generate ciprofloxacin-resistant *S. pneumoniae* mutants when infected mice were treated with a

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[▽] Published ahead of print on 10 September 2007.

dose of ciprofloxacin that simulated the area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) reported for humans treated with ciprofloxacin at 500 mg orally (p.o.) twice a day (9). One of these mutants (designated Spn-RC2) constitutively overexpressed a reserpine-inhibitable efflux pump. Sequencing of the entire open reading frames of *gyrA*, *gyrB*, *parC*, and *parE* demonstrated that there were no point mutations in these genes.

Importantly, in nonneutropenic mice infected with 2×10^7 CFU of *S. pneumoniae* strain Spn-RC2, levofloxacin therapy was associated with the expansion of *S. pneumoniae* mutants for which the MICs of levofloxacin and ciprofloxacin were increased (9). DNA sequencing demonstrated a point mutation in *parC*. MIC testing with and without reserpine showed persistent overexpression of an efflux pump. The mutation frequency of resistance to $3 \times$ MIC of levofloxacin was 2×10^{-5} for Spn-RC2, compared with $<10^{-10}$ for its wild-type progenitor. This finding suggests that efflux pump overexpression increases the likelihood of de novo generation of point mutations in the quinolone resistance-determining regions (QRDRs) of *parC* and perhaps other fluoroquinolone targets (*gyrA*, *gyrB*, and *parE*).

Brenwald et al. (1) showed that 45.4% of *S. pneumoniae* strains with decreased susceptibility to norfloxacin or ciprofloxacin overexpressed efflux pumps. These findings, in accordance with our independent findings from the mouse model, suggest that ciprofloxacin should not be used for the treatment of community-acquired upper- and lower-respiratory-tract infections, including sinusitis, community-acquired pneumonia, and acute exacerbations of chronic bronchitis. We wished to examine the role of the efflux pump in the emergence of fluoroquinolone resistance in the two strains of *S. pneumoniae* discussed above. This evaluation was conducted in vitro to remove the confounding effects of the immune system. We hypothesized that the wild-type strain Spn-058 would not become resistant to fluoroquinolones under pressure from levofloxacin because the more hydrophobic fluoroquinolones like levofloxacin are poor substrates for the pneumococcal efflux pumps, whereas a more hydrophilic quinolone, like ciprofloxacin, would allow the expansion of a resistant subpopulation (9, 14). Also, for the Spn-RC2 strain, we hypothesized that the already stable overexpression of the efflux pump would allow further emergence of resistance whether the quinolone pressure was from ciprofloxacin or levofloxacin. Finally, we hypothesized that the addition of reserpine in concentrations that would inhibit the efflux pumps would result in the suppression of resistance to both drugs.

MATERIALS AND METHODS

Microorganisms. Spn-058, a wild-type *S. pneumoniae* isolate, and Spn-RC2, an isogenic strain with stable overexpression of a reserpine-inhibitable efflux pump, were evaluated (9). Sequencing of the entire open reading frames of *gyrA*, *gyrB*, *parC*, and *parE* of both strains demonstrated no point mutations in these genes (9). The isolates were stored at -80°C in skim milk. For each experiment, a fresh sample was taken from the frozen stock and grown on blood agar plates for use in that study only.

Antimicrobial agents. Levofloxacin powder was supplied by Johnson & Johnson Pharmaceutical Research & Development (Raritan, NJ). Ciprofloxacin and reserpine were purchased from Sigma-Aldrich, Inc. (St Louis, MO). Stock solutions of levofloxacin and ciprofloxacin in sterile water were prepared and stored at -80°C . For each study, aliquots of the antibiotics were thawed and were used immediately. Reserpine solutions were prepared in dimethyl sulfoxide at 1 mg/ml

and were stored at room temperature for up to 5 days. Preliminary studies demonstrated that reserpine is stable at room temperature for at least 7 days when prepared in 100% dimethyl sulfoxide at this concentration. In Todd-Hewitt broth supplemented with 0.5% yeast extract (THB-Y), reserpine at 20 $\mu\text{g}/\text{ml}$ had a degradation half-life of 60 h at room temperature and a degradation half-life of 20 h at 35°C (data not shown).

Antibiotic susceptibility studies. Broth macrodilution MICs and minimal bactericidal concentrations (MBCs) of levofloxacin and ciprofloxacin for Spn-058 and Spn-RC2 were determined using THB-Y and Mueller-Hinton II broth supplemented with 5% lysed horse blood according to methods described by CLSI (13). Subsequently, MICs and MBCs were determined simultaneously using these media with and without the addition of 20 μg of reserpine/ml. This concentration of reserpine, alone and with subinhibitory concentrations of levofloxacin and ciprofloxacin, did not affect the growth of either *S. pneumoniae* isolate. Agar dilution susceptibility tests using Mueller-Hinton II agar supplemented with 5% lysed horse blood were conducted together with the broth susceptibility studies to correlate the MICs derived from liquid and agar media. The susceptibility studies were conducted using each of these media in duplicate on four different days.

Mutation frequency determinations. The mutation frequencies were determined by quantitatively plating suspensions of Spn-058 and Spn-RC2 onto the surface of drug-free agar and agar supplemented with $2\times$, $3\times$, and $5\times$ MICs of levofloxacin or ciprofloxacin. After 72 h of incubation at 35°C , the colonies were enumerated and the mutation frequencies were calculated by dividing the number of CFU per milliliter that grew on the surface of the drug-containing agar by the number of CFU per milliliter that grew on the surface of the drug-free agar. The mutation frequencies were determined with and without the addition of reserpine to the antibiotic-containing agar on three different days.

In vitro one-compartment pharmacodynamic system. The one-compartment pharmacodynamic system described by Ross et al. (15) was used, with modifications. For each experiment, Spn-058 or Spn-RC2 bacteria in mid-logarithmic phase were inoculated into the central compartments of several pharmacodynamic systems that were conditioned with prewarmed THB-Y for 24 h at 35°C . Antibiotic treatment was initiated 1 h later.

For studies with Spn-058, the pharmacodynamic infection systems were exposed to the following conditions for 10 days: (i) no treatment (growth control 1), (ii) reserpine with no antibiotic (growth control 2), (iii) a concentration-time profile simulating the treatment of humans with a regimen of levofloxacin at 500 mg orally (p.o.) once daily (QD), (iv) a simulated regimen of levofloxacin at 500 mg p.o. QD together with reserpine, (v) a simulated regimen of levofloxacin at 750 mg p.o. QD, (vi) a simulated regimen of levofloxacin at 750 mg p.o. QD plus reserpine, (vii) a concentration-time profile simulating the treatment of humans with a regimen of ciprofloxacin at 750 mg p.o. every 12 h, and (viii) a simulated regimen of ciprofloxacin at 750 mg every 12 h in combination with reserpine. Reserpine at 20 $\mu\text{g}/\text{ml}$ was given to the appropriate experimental systems as continuous infusions.

For pharmacodynamic studies with Spn-RC2, the effects of reserpine on the efficacy of levofloxacin and ciprofloxacin were studied separately. For 10-day studies with levofloxacin, experimental systems i through vi described above were evaluated. For 10-day studies with ciprofloxacin, experimental systems i and ii (controls without and with reserpine) and experimental systems vii and viii (ciprofloxacin without and with reserpine) were evaluated.

The concentration-time profile simulating a regimen of levofloxacin at 500 mg p.o. QD was achieved by simulating a peak non-protein-bound (free) drug concentration of 3.6 $\mu\text{g}/\text{ml}$, an AUC_{0-24} for the free drug of $36 \text{ mg} \cdot \text{h}/\text{liter}$ (based on a 30% protein binding rate), and a half-life of 7 h in serum (3). The peak free drug concentration and the AUC_{0-24} used to simulate a regimen of levofloxacin at 750 mg p.o. QD were scaled up from those used for the regimen of 500 mg QD. To achieve the targeted half-life of levofloxacin, the central compartment volume was 240 ml and drug-containing medium within this compartment was isovolumetrically replaced with drug-free medium at a rate of 0.4 ml/min. For the concentration-time profile simulating a regimen of ciprofloxacin at 750 mg p.o. every 12 h, ciprofloxacin was administered once every 12 h to achieve a steady-state AUC_{0-24} for the free drug of $25.9 \text{ mg} \cdot \text{h}/\text{liter}$, based on a 20% protein binding rate (11). The simulated peak free drug concentration and half-life for this ciprofloxacin regimen were 2.23 $\mu\text{g}/\text{ml}$ and 4 h, respectively. For the experimental systems using ciprofloxacin, the central compartment volume was 140 ml and the ciprofloxacin-containing medium was isovolumetrically replaced with drug-free medium at a rate of 0.4 ml/min. The medium exchange rate within the pharmacodynamic systems was sufficient to prevent the spontaneous lysis of the *S. pneumoniae* isolates. The actual pharmacokinetic profiles of levofloxacin and ciprofloxacin that were attained in the pharmacodynamic systems were validated by sampling the central compartment of each experimental system

TABLE 1. Mutation frequencies for Spn-058 and Spn-RC2^a

Strain	Antibiotic	Mutation frequency (mean log ₁₀ ± 1 SD), without reserpine, at:			Mutation frequency (mean log ₁₀ ± 1 SD), with reserpine, at:		
		2× MIC	3× MIC	5× MIC	2× MIC	3× MIC	5× MIC
Spn-058	Levofloxacin	-8.46 ± 0.17	-8.78 ± 0.08	<-9.60 ^b	-9.10 ± 0.25	-9.42 ± 0.22	<-9.60 ^b
	Ciprofloxacin	-5.60 ± 0.24	-5.62 ± 0.26	-8.14 ± 0.13	<-9.60 ^b	<-9.60 ^b	<-9.60 ^b
Spn-RC2	Levofloxacin	-6.41 ± 0.13	-7.85 ± 0.14	<-8.70 ^b	-8.36 ± 0.31	-8.50 ± 0.28	<-8.70 ^b
	Ciprofloxacin	-6.15 ± 0.22	-6.80 ± 0.29	<-8.70 ^b	-7.40 ± 0.22	-7.65 ± 0.25	<-8.70 ^b

^a Spn-058 and Spn-RC2 bacteria were exposed to 2×, 3×, and 5× MICs of levofloxacin and ciprofloxacin in the absence and presence of 20 µg of reserpine/ml. For Spn-058, the MIC of levofloxacin and ciprofloxacin (tested without reserpine) was 1 µg/ml. For Spn-RC2, the MICs of levofloxacin and ciprofloxacin (tested without reserpine) were 1 and 2 µg/ml, respectively. Data represent the results of three trials.

^b In three trials, no colonies were detected on agar containing this concentration of antibiotic. The mutation frequency shown is based on the highest log₁₀ number of bacterial CFU that were studied in the three trials.

at 10 to 14 time points over the first 48 h of the study by validated high-performance liquid chromatography methods. The concentrations were within 10% of the targeted values.

Reserpine was added to the appropriate pharmacodynamic systems at the beginning of each experiment to achieve a concentration of 20 µg/ml and was replenished as a continuous infusion thereafter. The concentration of reserpine in the pharmacodynamic systems was measured twice daily by a validated liquid chromatography-mass spectrometry assay.

At the beginning of each experiment and then once daily, a sample of the bacterial suspension was taken from the central compartment of each pharmacodynamic system. The bacteria were washed twice with normal saline to prevent drug carryover and were then quantitatively cultured onto drug-free agar and agar containing 2× and 3× MIC of levofloxacin or 3× and 5× MIC of ciprofloxacin to characterize the effect of each antibiotic regimen on the drug-susceptible and -resistant bacterial populations. The multiples of MICs of levofloxacin chosen were based on the results of preliminary studies which demonstrated that isolates derived from Spn-058 and Spn-RC2 that grew on agar supplemented with 2× MIC of levofloxacin for the parent strains but did not grow on agar containing 3× MIC of levofloxacin were mutants that had increased expression of efflux pumps but did not have point mutations in the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE*. However, isolates that grew on agars supplemented with 2× and 3× MIC of levofloxacin had at least one point mutation in a QRDR of these genes. For ciprofloxacin, 3× MIC was the lowest concentration of this antibiotic at which mutants with efflux pump upregulation were detected, while 5× MIC of ciprofloxacin was the lowest concentration at which we detected mutants that had at least one point mutation in the QRDR of *gyrA*, *gyrB*, *parC*, or *parE* (data not shown). Organisms from the control systems were plated onto drug-free agar and both levofloxacin- and ciprofloxacin-supplemented agars. After the plates were incubated at 35°C for 72 h, the colonies were enumerated.

Mutants cultured from drug-containing agar plates at several times during the experiments were stored at -80°C in 20% glycerol for subsequent testing of the MICs of ciprofloxacin and levofloxacin, alone and in combination with reserpine, to define the effect of each treatment regimen on the selection of *S. pneumoniae* mutants that overexpressed efflux pumps. Then a subset of these mutants was processed for PCR amplification and sequencing of the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* to characterize the effect of reserpine on the prevention of the emergence of resistance in the *S. pneumoniae* population (9).

The pharmacodynamic studies with Spn-058 and Spn-RC2 were each conducted on two separate occasions.

EtBr uptake and efflux studies. Ethidium bromide (EtBr) uptake and efflux studies as described by Gill et al. (6) were conducted, with modifications, to quantify the expression of efflux pumps by Spn-058, Spn-RC2, and several mutants of these strains that developed within the pharmacodynamic systems under suboptimal levofloxacin and ciprofloxacin regimens. The mutants evaluated had upregulated efflux pump expression, as determined by a reduction in ciprofloxacin MICs when susceptibility studies were conducted without and with reserpine. Some mutants also had a single point mutation in the QRDR of *parC* alone or together with a point mutation in *gyrA*.

To characterize the uptake of EtBr by the *S. pneumoniae* isolates, the bacterial strains were grown at 35°C to mid-logarithmic phase in THB-Y. Bacterial suspensions with a standardized initial density of 10⁷ CFU/ml (determined turbidometrically) were prepared in minimum growth uptake buffer (NaCl, 100 mM; KCl, 7 mM; NH₄Cl, 50 mM; Na₂HPO₄, 0.4 mM; Tris base, 52 mM; and glucose, 0.2%, adjusted to pH 7.5 with HCl). The bacteria were then exposed to 2 µg of

EtBr/ml, with and without the addition of 20 µg of reserpine/ml. The increase in fluorescence as EtBr entered the cells was recorded fluorometrically using a SpectraMax M5 microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 35°C. The excitation and emission wavelengths were set at 530 and 600 nm, respectively.

To quantify the efflux of EtBr from bacteria, *S. pneumoniae* isolates loaded with EtBr (in the presence of reserpine), as described above, were pelleted by centrifugation and resuspended in fresh minimal growth medium (without reserpine). The efflux of EtBr from bacterial cells was measured as a decrease in fluorescence over time.

To determine if the expression of efflux pumps was inducible, Spn-058 and Spn-RC2 bacteria from frozen stocks were grown overnight on antibiotic-free agar. A few colonies were inoculated into THB-Y containing 0.3 µg of levofloxacin/ml or 0.25 µg of ciprofloxacin/ml and grown to mid-logarithmic phase for 5 h at 35°C. This concentration of antibiotic approximated the trough concentration simulated within the pharmacodynamic infection system for the levofloxacin regimen of 500 mg QD or the ciprofloxacin regimen of 750 mg every 12 h, respectively. The bacterial suspensions were washed once to prevent drug carryover. The bacteria were resuspended in prewarmed medium to 10⁷ CFU/ml (confirmed by quantitative cultures) and were then used in the EtBr uptake and efflux studies described above to determine if antibiotic pretreatment induced efflux pump expression. Spn-058 and Spn-RC2 bacteria that had not been exposed to fluoroquinolones served as controls.

To determine if the induction of efflux pump expression was reversible, Spn-058 and Spn-RC2 bacteria from samples that were and were not pretreated with ciprofloxacin and levofloxacin for 5 h were then grown on antibiotic-free blood agar for 18 h. These strains were subsequently grown to mid-logarithmic phase in antibiotic-free THB-Y and adjusted to a concentration of 10⁷ CFU/ml (confirmed by quantitative cultures) before they were used in the EtBr uptake and efflux protocols described above.

For each bacterial isolate, these studies were conducted in triplicate on the same day and the results were averaged.

Statistical methods. The mutation frequencies and quantitative culture values are shown as means ± 1 standard deviation. The significance of differences in (i) mutation frequencies of levofloxacin and ciprofloxacin in Spn-058 and Spn-RC2, (ii) levels of EtBr uptake (at the 20-min time point) between experimental conditions, and (iii) levels of EtBr efflux (at 20 min) between experimental systems were determined by analysis of variance. If differences were identified, multiple pairwise comparisons were made using Tukey's procedure. A *P* value of <0.05 was considered significant.

RESULTS

Susceptibility studies. Broth macrodilution MICs and MBCs of levofloxacin and ciprofloxacin were 1 µg/ml for Spn-058 when susceptibility studies were conducted with Mueller-Hinton II broth supplemented with 5% lysed horse blood and THB-Y. The MICs of levofloxacin and ciprofloxacin did not change when these media were supplemented with 20 µg of reserpine/ml. For Spn-RC2, the broth macrodilution MIC of levofloxacin, tested without and with 20 µg of reserpine/ml,

FIG. 1. Effect of treatment of Spn-058 with levofloxacin (levo) and ciprofloxacin (cipro), alone and in combination with the efflux pump inhibitor reserpine, on killing of the total bacterial population and on the emergence of resistance during therapy. The treatments applied to the experimental systems are shown above the panels, and the supplements added to the agar plates used to identify mutants with increased MICs to levofloxacin or ciprofloxacin are indicated next to the relevant symbols. The results (mean log₁₀ numbers of CFU per milliliter $\pm$ 1 standard deviation) from two trials are shown. Q 24 h and Q 12 h, once every 24 h and once every 12 h.

was 1 μ g/ml. For Spn-RC2, the MICs of ciprofloxacin, tested without and with reserpine, were 2 and 1 μ g/ml, respectively. The MICs of levofloxacin and ciprofloxacin for Spn-058 and Spn-RC2 that were determined by the agar dilution method were the same as those derived using the broth macrodilution

method. Reserpine did not affect the growth of the *S. pneumoniae* strains.

Mutation frequencies for Spn-058 and Spn-RC2. The frequencies of mutations in Spn-058 and Spn-RC2 conferring resistance to 2 $\times$, 3 $\times$, and 5 $\times$ MICs of levofloxacin and cipro-

TABLE 2. Levofloxacin and ciprofloxacin MICs (without and with reserpine) and QRDR mutations determined for Spn-058 mutants^a

Treatment and plate supplementation	Day 3			Day 6			Day 10		
	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation(s)
	Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin	
Control arm without reserpine									
3 $\times$ MIC of ciprofloxacin	4/1	2/1	None	4/1	2/1	None	4/1	2/1	None
5 $\times$ MIC of ciprofloxacin	8/1	2/1	None	—	—	NA	8/1	2/1	None
2 $\times$ MIC of levofloxacin	4/1	2/1	None	4/1	2/1	None	4/1	2/1	None
Control arm with reserpine									
3 $\times$ MIC of ciprofloxacin	4/1	2/1	None	4/1	2/1	None	4/1	2/1	None
5 $\times$ MIC of ciprofloxacin	8/1	2/1	None	8/1	2/1	None	—	—	NA
2 $\times$ MIC of levofloxacin	—	—	NA	—	—	NA	—	—	NA
Levofloxacin at 500 mg QD without reserpine	8/2	4/2	Ser79Tyr in ParC	8/2	4/2	Ser79Tyr in ParC	64/32	16/16	Glu85Gly in GyrA and Ser79Tyr in ParC
Ciprofloxacin at 750 mg every 12 h without reserpine	16/2	4/2	Ser79Tyr in ParC	8/2	4/2	Ser79Tyr in ParC	16/2	4/2	Ser79Tyr in ParC

^a Mutants of Spn-058 were isolated from agar plates supplemented with ciprofloxacin or levofloxacin (with or without 20 $\mu\text{g/ml}$ of reserpine) and examined for mutations in the QRDRs of GyrA, GyrB, ParC, and ParE. For Spn-058, the MIC of levofloxacin and ciprofloxacin (without and with reserpine) was 1 $\mu\text{g/ml}$. Mutants for which the MICs of levofloxacin and ciprofloxacin were increased were not detected on day 0. —, sterile agar plates; NA, not applicable.

floxacin for the parent strains are shown in Table 1. For Spn-058, for each multiple of the MIC tested, substantially more mutants were isolated from ciprofloxacin- than from levofloxacin-supplemented agar ($P < 0.01$). Reserpine reduced the frequency with which mutants were isolated from both levofloxacin- and ciprofloxacin-supplemented agar; the effect of reserpine was greater for the mutation frequencies of ciprofloxacin than for those of levofloxacin ($P < 0.02$).

In Spn-RC2, the mutation frequencies of levofloxacin were higher than those of the wild-type strain, Spn-058 (P , 0.01 at $2\times$ MIC and 0.04 at $3\times$ MIC). The mutation frequencies of ciprofloxacin for Spn-058 and Spn-RC2 were similar (P , not significant at $2\times$ and $3\times$ MIC). Reserpine decreased the mutation frequencies of ciprofloxacin and levofloxacin in Spn-RC2 ($P < 0.02$). However, the effect on Spn-RC2 was smaller than that on Spn-058.

Spn-058 and Spn-RC2 inocula used in the pharmacodynamic systems. Since the goal of this study was to determine if the inhibition of efflux pump function in *S. pneumoniae* prevents the emergence of DNA-based resistance during therapy or reestablishes antibiotic efficacy against *S. pneumoniae* strains that already overexpress efflux pumps, we wanted to use starting inocula of Spn-058 and Spn-RC2 bacteria that were unlikely to include mutants with DNA point mutations at the start of therapy. Yet we needed to start with a bacterial density that was measurable given that the limit of reproducible detection by our quantitative culture method was ≥ 50 CFU/ml of medium. Thus, for studies using our in vitro pharmacodynamic infection systems, the total load of Spn-058 and Spn-RC2 CFU inoculated into each experimental system was equivalent to 1 $\log_{10}$ CFU less than the reciprocal of the mutation frequency corresponding to $2\times$ MIC of levofloxacin or $2\times$ MIC of ciprofloxacin.

Specifically, the mutation frequency in Spn-058 conferring resistance to $2\times$ MIC of levofloxacin was $-8.46 \log_{10}$. Thus, the total bacterial load inoculated into the experimental systems for examining the efficacy of levofloxacin against Spn-058 was $7.46 \log_{10}$ CFU (or 2.88×10^7 CFU). With a central reservoir volume of 240 ml, the starting bacterial concentration in these experimental systems was approximately $5.07 \log_{10}$ CFU/ml (or 1.2×10^5 CFU/ml). A similar approach was used to determine the starting inocula for experimental systems in which levofloxacin was used to treat Spn-RC2 and in which ciprofloxacin was used to treat Spn-058 and Spn-RC2.

Pharmacodynamic studies with the wild-type *S. pneumoniae* strain Spn-058. In our in vitro pharmacodynamic infection system, reserpine did not affect the growth of Spn-058, as the density of this isolate increased from $5.5 \log_{10}$ CFU/ml to approximately $8 \log_{10}$ CFU/ml within 24 h in THB-Y that was and was not supplemented with reserpine (Fig. 1B and A, respectively). The simulated regimen of levofloxacin at 500 mg QD decreased the *S. pneumoniae* population to below the sensitivity of detection of the quantitative culture assay (50 CFU/ml) by day 1, but regrowth was seen thereafter, such that the bacterial density in this experimental system matched those in the control systems on day 6 of treatment (Fig. 1C). The addition of reserpine to this levofloxacin regimen resulted in rapid killing of the total bacterial population and prevented regrowth (Fig. 1D). Levofloxacin at 750 mg QD, alone and in combination with reserpine, rapidly reduced the total Spn-058

FIG. 2. Effect of treatment of *Spn-RC2* with levofloxacin (levo), alone and in combination with reserpine, on killing of the total bacterial population and on the emergence of resistance. The treatments applied to the experimental systems are shown above the panels, and the supplements added to the agar plates for identifying mutants with increased MICs to levofloxacin are indicated next to the relevant symbols. The combined results (mean $\log_{10}$ numbers of CFU per milliliter $\pm$ 1 standard deviation) from two trials are shown. Q 24 h, once every 24 h.

population to below the sensitivity of detection of the quantitative culture assay and prevented regrowth for the duration of the 10-day studies (Fig. 1E and F).

The concentration-time profile simulating a ciprofloxacin regimen of 750 mg every 12 h was associated with an initial reduction in bacterial density by day 1, followed by rapid regrowth such that the total *S. pneumoniae* population in this experimental system was similar to those in the controls by day 2 of the study (Fig. 1G). Reserpine in combination with ciprofloxacin prevented the regrowth of *Spn-058*, resulting in the sterilization of this pharmacodynamic experimental system.

As shown in Fig. 1A and B, in the control systems, the population of mutants of *Spn-058* for which the MIC of ciprofloxacin was $3\times$ MIC for the parent strain increased from undetectable on day 0 to $\geq 3 \log_{10}$ CFU/ml by day 1. Isolates were intermittently detected on agar supplemented with $5\times$ MIC of ciprofloxacin. The density of these mutants increased in proportion to the rise in the total bacterial population. MICs

for these isolates were 4 to 8 $\mu\text{g/ml}$, decreasing to 1 $\mu\text{g/ml}$ when ciprofloxacin was evaluated in combination with the efflux pump inhibitor reserpine (Table 2). For these mutants, reserpine had less effect on the MICs of levofloxacin than it had on the MICs of ciprofloxacin. Sequencing studies showed that these strains did not have mutations in the QRDRs of *gyrA/B* and *parC/E*. Concentrations of mutants for which the MIC of levofloxacin was $2\times$ MIC for the parent strain were intermittently detected. Isolates for which MICs of levofloxacin were $\geq 3\times$ MIC for the parent strain were not detected during the 10-day study.

Figure 1G shows that the regrowth of *Spn-058* in the ciprofloxacin system was due to the rapid emergence of resistance. Mutants for which the MIC of ciprofloxacin was $\geq 3\times$ MIC for the parent strain were first detected on day 2 of treatment and completely replaced the wild-type population by day 3 of the study. Mutants for which the MICs of ciprofloxacin increased more than fivefold over the baseline also emerged during therapy,

FIG. 3. Effect of ciprofloxacin (Cipro), alone and together with reserpine, on the growth of Spn-RC2 and mutants with decreased susceptibilities to this fluoroquinolone antibiotic. The treatments applied to the experimental systems are shown above the panels, and the supplements added to the agar plates for detection of mutants with increased MICs to ciprofloxacin are indicated next to the relevant symbols. The combined results (mean $\log_{10}$ numbers of CFU per milliliter $\pm$ 1 standard deviation) from two trials are shown. Q 12 h, once every 12 h.

although they accounted for less than 1% of the total bacterial population. They were not detected until day 3 of therapy. The MICs for and the QRDR mutations identified in these mutants are shown in Table 2. Figure 1H shows that the use of reserpine in combination with ciprofloxacin at 750 mg every 12 h resulted in rapid and sustained killing of Spn-058 bacteria and prevented the emergence of resistance during therapy.

Figure 1C shows that the regrowth seen in studies with Spn-058 and the simulated levofloxacin regimen of 500 mg QD was due to the emergence of resistance during therapy. Sequencing demonstrated a Ser79Tyr mutation in ParC on day 6 and isolates with two mutations on day 10 (Table 2). For isolates with single mutations in *parC*, MICs of levofloxacin and ciprofloxacin were 4 and 8 $\mu\text{g/ml}$, respectively, decreasing to 2 $\mu\text{g/ml}$ in the presence of reserpine. For isolates with mutations in both *parC* and *gyrA*, the ciprofloxacin MIC was 64 $\mu\text{g/ml}$ and reproducibly decreased to 32 $\mu\text{g/ml}$ in the presence of reserpine in three separate trials. The addition of reserpine to levofloxacin at 500 mg QD prevented the emergence of resistance in the pharmacodynamic infection system.

Levofloxacin at 750 mg QD, with and without reserpine, rapidly killed the total Spn-058 population and did not select for resistant mutants (Fig. 1F and E, respectively).

Pharmacodynamic studies with Spn-RC2. Spn-RC2 bacteria were inoculated into the control and levofloxacin treatment systems at a concentration of $3.0 \log_{10}$ CFU/ml (the total bacterial load in 240 ml of medium was 2.5×10^5 CFU, or $5.40 \log_{10}$ CFU). This bacterial strain was also inoculated into the ciprofloxacin treatment systems at a concentration of $3.0 \log_{10}$ CFU/ml, for a total bacterial load of 1.40×10^5 CFU, or 5.15

$\log_{10}$ CFU, in 140 ml of media. The smaller medium volume was used to simulate the shorter half-life of ciprofloxacin than of levofloxacin. As the studies using Spn-058, the Spn-RC2 control systems showed an increase in mutants for which the MICs of levofloxacin and ciprofloxacin were $2\times$ and $3\times$ MICs, respectively, for the parent strain and this increase paralleled the increase in the total bacterial population (Fig. 2A and 3A, respectively). Spn-RC2 mutants for which the MICs of levofloxacin and ciprofloxacin were $3\times$ and $5\times$ MICs, respectively, for the parent strain were rarely detectable in the control systems throughout the studies. The control systems demonstrated that reserpine did not affect the growth of Spn-RC2 (Fig. 2A and B and Fig. 3A and B).

The treatment of Spn-RC2 with the simulated regimen of levofloxacin at 500 mg QD resulted in a rapid increase in bacterial density within 24 h (Fig. 2C). Regrowth was due to the emergence of levofloxacin-resistant mutants. As with Spn-058, the use of levofloxacin at 500 mg QD in combination with reserpine resulted in rapid killing of the total population of Spn-RC2 without the emergence of resistance (Fig. 2D). Levofloxacin at 750 mg QD, with and without reserpine, rapidly sterilized the pharmacodynamic system and did not select for resistance (Fig. 2F and E, respectively).

Studies that examined the effect of ciprofloxacin against Spn-RC2 were stopped after 8 days because a drug infusion syringe pump failed to infuse this antibiotic into an experimental system on day 9 of one trial and an experimental system was noted to be contaminated with a ciprofloxacin-resistant *Staphylococcus* sp. strain on day 10 of the second trial. Shortening these experiments to 8 days did not affect the study conclusions

since the ciprofloxacin regimens failed due to the emergence of drug-resistant mutants before this time point.

The simulated regimen of ciprofloxacin at 750 mg every 12 h failed within 24 h after treatment began (Fig. 3C). The use of reserpine in combination with ciprofloxacin resulted in rapid killing of the bacterial population. Regrowth was seen beginning on day 3 of therapy, however, with the bacterial population in this treatment system approaching those in the controls by day 8 (Fig. 3D). The regrowth seen in the experimental system that was treated with ciprofloxacin alone was due to the emergence of resistance. The delayed regrowth and eventual failure seen in the ciprofloxacin-plus-reserpine system was also due to the expansion of ciprofloxacin-resistant mutants. The MICs for and the associated changes in the QRDRs of Spn-RC2 mutants that proliferated under pressure from levofloxacin and ciprofloxacin are shown in Tables 3 and 4, respectively.

EtBr uptake and efflux by *S. pneumoniae*. Relative efflux pump expression by different *S. pneumoniae* strains was determined by using EtBr uptake studies conducted with reserpine-free and reserpine-containing media and by determining the kinetics of EtBr efflux from *S. pneumoniae* in reserpine-free medium.

EtBr uptake by Spn-058, the wild-type strain, was greater than that by Spn-RC2 (Fig. 4A) ($P < 0.01$), confirming that Spn-RC2 expressed a greater number of efflux pumps at the baseline. Reserpine at 20 $\mu\text{g/ml}$ inhibited the function of efflux pumps in Spn-058 and Spn-RC2 (Fig. 4A). The results suggested that the efflux pumps in Spn-RC2 were only partially inhibited by reserpine, relative to the effect of reserpine on the efflux pumps of Spn-058 ($P = 0.04$). The efflux studies showed that EtBr was more effectively pumped out of Spn-RC2 than out of Spn-058 (Fig. 4B) ($P < 0.01$).

Induction of efflux pump expression by ciprofloxacin and levofloxacin. Pretreating Spn-058 with ciprofloxacin for 5 h was sufficient to induce increased expression of efflux pumps (Fig. 5A and B) (P of 0.02 and < 0.01 , respectively) that were inhibited by reserpine (Fig. 5A). Compared with ciprofloxacin, levofloxacin pretreatment was a relatively poor inducer of efflux pump expression by Spn-058 (Fig. 5, compare panels A and C).

For Spn-RC2, ciprofloxacin pretreatment also induced efflux pump expression to a level above the baseline (Fig. 5E and F) (P , 0.01 for both EtBr uptake and efflux). Efflux pump function in ciprofloxacin-treated Spn-RC2 bacteria was partially inhibited by 20 μg of reserpine/ml (Fig. 5E) (P , 0.02 compared with the effect of reserpine on efflux pump function in Spn-RC2 bacteria that were not pretreated with ciprofloxacin). Levofloxacin pretreatment was a poor inducer of efflux pump expression in Spn-RC2 (Fig. 5G and H) (P , not significant).

Spn-058 and Spn-RC2 isolates that were pretreated with ciprofloxacin or levofloxacin for 5 h and then grown on antibiotic-free agar for 24 h showed the same EtBr uptake and efflux kinetics as Spn-058 and Spn-RC2 isolates that had never been exposed to a fluoroquinolone antibiotic (data not shown). Thus, the induction of efflux pump expression by fluoroquinolone exposure was transient.

Efflux pump expression by mutants of Spn-058 and Spn-RC2. Mutants of Spn-058 and Spn-RC2 were collected from levofloxacin- and ciprofloxacin-supplemented agar plates on the last day of the pharmacodynamic studies (as shown in Tables 2 to 4). The isolates had been grown overnight on

TABLE 3. Levofloxacin and ciprofloxacin MICs (without and with reserpine) and QRDR mutations determined for Spn-RC2 mutants from plates supplemented with levofloxacin^a

Treatment and plate supplementation	Day 3			Day 6			Day 10		
	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation	MIC ($\mu\text{g/ml}$) without reserpine/with reserpine of:		QRDR mutation(s)
	Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin	
Control arm without reserpine 2 $\times$ and 3 $\times$ MIC of levofloxacin	—	—	NA	8/2	4/2	Ser79Tyr in ParC	8/2	4/2	Ser79Tyr in ParC
Control arm with reserpine 2 $\times$ MIC of levofloxacin 3 $\times$ MIC of levofloxacin	—	—	NA NA NA	4/1 —	2/1 —	None NA	4/1 —	2/1 —	None NA
Levofloxacin at 500 mg QD without reserpine 2 $\times$ and 3 $\times$ MIC of levofloxacin	8/2	4/2	Ser79Tyr in ParC	8/2	4/2	Ser79Tyr in ParC	64/32	16/16	Ser81Phe in GyrA and Ser79Tyr in ParC

^a Mutants of Spn-RC2 were isolated from agar plates supplemented with levofloxacin (with or without 20 $\mu\text{g/ml}$ of reserpine) and examined for mutations in the QRDRs of GyrA, GyrB, ParC, and ParE. For the parent Spn-RC2 strain, the MIC of levofloxacin (without and with reserpine) was 1 $\mu\text{g/ml}$. Mutants for which the MICs of levofloxacin were increased were not detected on day 0, —, sterile agar plates; NA, not applicable.

TABLE 4. Levofloxacin and ciprofloxacin MICs (without and with reserpine) and QRDR mutations determined for Spn-RC2 mutants from plates supplemented with ciprofloxacin^a

Treatment and plate supplementation	Day 1			Day 4			Day 8		
	MIC (μg/ml) without reserpine/with reserpine of:		QRDR mutation	MIC (μg/ml) without reserpine/with reserpine of:		QRDR mutation	MIC (μg/ml) without reserpine/with reserpine of:		QRDR mutation
	Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin		Ciprofloxacin	Levofloxacin	
Control without reserpine									
3 × MIC of ciprofloxacin	4/1	2/1	None	8/1	2/1	None	4/1	2/1	None
5 × MIC of ciprofloxacin	—	—	NA	—	—	NA	—	—	NA
Control with reserpine									
3 × MIC of ciprofloxacin	4/1	2/1	None	4/1	2/1	None	4/1	2/1	None
5 × MIC of ciprofloxacin	—	—	NA	—	—	NA	—	—	NA
Ciprofloxacin at 750 mg every 12h without reserpine									
3 × MIC of ciprofloxacin	16/2	2/2	Ser79Tyr in ParC	8/2	4/2	Ser79Tyr in ParC	64/32	16/16	Glu85Gly in GyrA and Ser79Tyr in ParC
5 × MIC of ciprofloxacin	—	—	NA	—	—	NA	16/2	4/2	Ser79Tyr in ParC

^a Mutants of Spn-RC2 were isolated from agar plates supplemented with ciprofloxacin (with or without 20 μg/ml of reserpine) and examined for mutations in the QRDRs of GyrA, GyrB, ParC, and ParE. For the parent Spn-RC2 strain, the MICs of ciprofloxacin (without and with reserpine) were 2 and 1 μg/ml, respectively. Mutants for which the MICs of ciprofloxacin were increased were not detected on day 0. Note that the time points are different from those reported in Tables 2 and 3. —, sterile agar plates; NA, not applicable.

antibiotic-free agar plates before they were stored at −80°C. To evaluate efflux pump function, these isolates were taken from frozen stocks and grown at 35°C for 24 h on antibiotic-free agar before they were examined in the EtBr uptake and efflux assays. Four of six isolates of Spn-058 that grew on ciprofloxacin-supplemented agar (and had no mutations in *gyrA/B* and *parC/E*) expressed slightly higher levels of efflux pumps than the parent, wild-type Spn-058 strain. The EtBr assay results for one mutant and the wild-type strain are shown in Fig. 6A. The MICs for two of six of the isolates were equivalent to those for the control, wild-type strain, and the efflux pump activities of these isolates were also equivalent to those of the control (data not shown). An isolate derived from Spn-058 that had a single mutation in *parC* and a separate strain with single mutations in both *parC* and *gyrA* demonstrated increased efflux pump expression compared with the wild-type Spn-058 isolate (Fig. 6B). The first mutant had a stable level of expression of efflux pumps that was slightly higher than that of the parent strain (*P*, not significant), while the second mutant of Spn-058 had a stable level of expression of efflux pumps that was similar to that of Spn-RC2 (*P*, 0.02 compared with the parent Spn-058 isolate). A mutant of Spn-RC2 that had single mutations in both *parC* and *gyrA* demonstrated greater efflux pump function than the parent Spn-RC2 strain (*P* = 0.03). Reserpine increased the uptake of EtBr by all of the *S. pneumoniae* strains evaluated (*P*, <0.01 compared to the same strains that were not exposed to reserpine).

DISCUSSION

Previously, in a nonneutropenic mouse thigh infection model, we demonstrated that the first step in quinolone resistance for Spn-058 was the overexpression of an efflux pump that was reserpine inhibitable (9). In several studies with this wild-type strain, the emergence of resistance under pressure from levofloxacin was not seen. However, the use of ciprofloxacin as the therapeutic agent was immediately followed by resistance emergence. In fewer than 10% of the instances, the recovered strains were shown to have a target site mutation in *parC*. In all other instances, no target site mutations were identified in *parC/E* and *gyrA/B* but the change in the MIC was ameliorated by the addition of the efflux pump inhibitor reserpine. The use of the pump-overexpressing strain Spn-RC2 in the animal model allowed immediate further emergence of resistance to both levofloxacin and ciprofloxacin. The resistant mutants had single point mutations in *parC* and, based on a reduction of ciprofloxacin MICs in the presence of reserpine, continued to express a reserpine-inhibitable efflux pump.

Given that the animal model was not neutropenic, the present project used an in vitro pharmacodynamic infection system to remove the issue of the host immune system and clearly demonstrate the central role of the efflux pump in the process of the emergence of resistance to fluoroquinolones in *S. pneumoniae*. The addition of reserpine to demonstrate the effect of pump blockade is something that could also be effectively accomplished with this in vitro pharmacodynamic system.

For the wild-type strain, Spn-058, studies with a one-compartment pharmacodynamic system showed that levofloxacin at 500 mg QD and the highest Food and Drug Administration-

FIG. 4. Results from EtBr uptake (A) and efflux (B) studies of Spn-058 and Spn-RC2. The combined results (means $\pm$ 1 standard deviation) of three trials are shown. Fluorescence intensity is expressed in relative units.

approved dose of ciprofloxacin (750 mg every 12 h) were both associated with the emergence of resistance. However, the emergence of resistance occurred 3 days later with levofloxacin therapy than with ciprofloxacin treatment, despite the higher starting inoculum used in the levofloxacin-treated system. The addition of reserpine to each of these fluoroquinolone regimens or the use of levofloxacin at 750 mg QD, as monotherapy, prevented resistance. For Spn-RC2, an isogenic strain with constitutive overexpression of efflux pumps, studies using the pharmacodynamic system showed that levofloxacin at 500 mg QD was again associated with the emergence of resistance and that resistance was prevented when this regimen was used in combination with reserpine and when 750 mg of levofloxacin/day was used as monotherapy. Ciprofloxacin at 750 mg every 12 h also resulted in resistance in Spn-RC2. However, the addition of 20 μ g of reserpine/ml to this ciprofloxacin regimen only delayed the onset of resistance. Mutants that arose under suboptimal ciprofloxacin and levofloxacin regimens had both a stable increase in efflux pump expression and a mutation in *parC* alone or together with a mutation in *gyrA*. These findings show that with these fluoroquinolone antibiotics, efflux pump expression in *S. pneumoniae* was requisite for the subsequent acquisition of point mutations in *parC* and *gyrA* and that the inhibition of efflux pump function reduced the emergence of resistance. These studies also show that the efflux pumps of *S. pneumoniae* were saturable since levofloxacin at 750 mg QD, as monotherapy, prevented the emergence of resistance in both strains.

Our results were concordant with those of Lacy and colleagues (10), who treated four *S. pneumoniae* strains for which the MICs of levofloxacin and ciprofloxacin were 1 to 4 μ g/ml for up to 48 h in an in vitro infection model. In their study, the simulated exposure to ciprofloxacin at 400 mg administered intravenously every 12 h resulted in the emergence of resistance in all of the evaluated *S. pneumoniae* isolates within 24 h of treatment initiation. The simulated regimen of levofloxacin at 500 mg QD did not amplify resistant populations in three of the strains investigated. In the case of their most resistant isolate (for which the MIC of levofloxacin was 2.0 mg/liter), the bacterium persisted despite 2 days of treatment, suggesting the early amplification of resistant populations, as was clearly seen in our experiment (Fig. 1C).

The antibiotic susceptibilities, mutation frequencies, and

EtBr study results provide insights into the central role of efflux pump function in the emergence of fluoroquinolone resistance in *S. pneumoniae* and the differential effect of levofloxacin and ciprofloxacin on efflux pump expression. In the present study, in which serial twofold dilutions were employed in antibiotic susceptibility testing, the finding that the MICs of ciprofloxacin but not the MICs of levofloxacin for Spn-RC2 decreased with reserpine suggests that ciprofloxacin is a better substrate than levofloxacin for these efflux pumps. In previous studies, in which 0.2- μ g/ml increments of levofloxacin were used, the MICs of levofloxacin for Spn-RC2 decreased from 0.8 to 0.6 μ g/ml in each of four trials when susceptibility testing was conducted without and with reserpine (9). These findings suggest that the efflux pumps are more effective at removing ciprofloxacin than levofloxacin from the cytosol of *S. pneumoniae*.

This conclusion is supported by the fact that ciprofloxacin is more hydrophilic than levofloxacin (9, 14). A hydrophilic compound would be expected to traverse the water-filled transmembrane channels of efflux pumps more readily than more hydrophobic molecules.

The EtBr assays show that ciprofloxacin is also a more potent inducer of efflux pump expression than levofloxacin. These observations have important implications for the mutation frequencies of resistance to these antibiotics. Since ciprofloxacin is a better inducer of efflux pump expression than levofloxacin, the mutation frequencies of resistance in Spn-058 were higher for ciprofloxacin than for levofloxacin and were similar to those seen in the stably efflux pump-overexpressing strain Spn-RC2. Markham (12) demonstrated that the addition of reserpine to static cultures markedly diminished the selection of *S. pneumoniae* mutants resistant to ciprofloxacin at 3 $\times$ baseline MIC (45-fold reduction) and 4 $\times$ baseline MIC (complete suppression). In the present study, the inhibition of efflux pump function in Spn-058 with reserpine decreased the mutation frequencies of ciprofloxacin and levofloxacin to similar, lower values, underscoring the impact of efflux pump expression on the potential for the emergence of resistance during therapy. Since levofloxacin was a relatively poor inducer of efflux pump expression, the mutation frequencies of levofloxacin were higher for Spn-RC2 than for Spn-058, suggesting that levofloxacin would more readily select for resistance in Spn-RC2—as was seen previously in our mouse infection model (9).

FIG. 5. EtBr uptake and efflux in Spn-058 and Spn-RC2 bacteria that were (+) and were not (–) pretreated with ciprofloxacin (cipro) and levofloxacin (levo). The combined results (means $\pm$ 1 standard deviation) of three trials are shown. Fluorescence intensity is expressed in relative units. w/o; without.

The mechanism by which efflux pump expression leads to the acquisition of DNA point mutations in *parC* and *gyrA* of *S. pneumoniae* has not been defined. Cirz et al. (4) showed that ciprofloxacin-mediated DNA damage in *Escherichia coli* caused the derepression of the SOS-regulated polymerases Pol II, Pol IV, and Pol V, which, in turn, induced DNA mutations that resulted in fluoroquinolone resistance. We speculate that the upregulation of efflux pumps reduces the concentration of

various fluoroquinolones to different extents within the cytosol of *S. pneumoniae* to permit the bacterium to survive within the antibiotic milieu. However, the residual concentration of the fluoroquinolone within the microorganism may be sufficient to cause DNA damage that leads to the production of Pol polymerases. These proteins cause error-prone replication which, in turn, allows rapid resistance to emerge in *S. pneumoniae*, even when the starting bacterial population burden is substan-

FIG. 6. EtBr uptake studies to assess efflux pump function in mutants of Spn-058 that did not have point mutations in the QRDRs of *gyrA/B* and *parC/E* (A) and in mutants of Spn-058 and Spn-RC2 with *parC* and *gyrA* mutations (B) that were isolated from the pharmacodynamic systems after exposure to suboptimal ciprofloxacin and levofloxacin therapies. These strains were grown on drug-free agar before they were evaluated in the EtBr uptake studies. The combined results (means $\pm$ 1 standard deviation) of three trials are shown. Fluorescence intensity is expressed in relative units. w/o, without. -, without.

tially lower than the reciprocal of the mutation frequency for resistance. Since ciprofloxacin is more effectively pumped out of *S. pneumoniae* and is a better inducer of efflux pump expression than levofloxacin, one would expect a higher mutation frequency rate to be associated with ciprofloxacin than with levofloxacin.

The EtBr studies suggest that the success or failure of reserpine in preventing resistance in *S. pneumoniae* isolates treated with ciprofloxacin and levofloxacin is determined by the residual efflux pump function associated with its use. For example, ciprofloxacin and, to a lesser extent, levofloxacin were able to induce increased efflux pump expression in Spn-058. However, 20 μ g of reserpine/ml was able to reduce the efflux pump function associated with these antibiotics to levels that were similar to those in reserpine-treated Spn-058 bacteria that had never been exposed to these fluoroquinolones (Fig. 5A and C). The EtBr uptake and efflux studies showed that Spn-RC2 had a higher baseline level of efflux pump expression than its wild-type progenitor, Spn-058, but 20 μ g of reserpine/ml did reduce efflux pump function in Spn-RC2 isolates to levels similar to those in Spn-058 (Fig. 4A). Also, 20 μ g of reserpine/ml was able to reduce the level of efflux pump function in Spn-RC2 bacteria that were treated with levofloxacin down to the levels seen in reserpine-treated Spn-RC2 bacteria that had never been exposed to levofloxacin (Fig. 5G). However, ciprofloxacin upregulated efflux pump expression in Spn-RC2 to levels that were only partially inhibited by reserpine (Fig. 5E). The different degrees of efflux pump inhibition by reserpine offer a likely explanation for why reserpine was

successful in preventing resistance in Spn-058 and Spn-RC2 isolates that were treated with a simulated regimen of levofloxacin at 500 mg QD and in Spn-058 isolates that were treated with a simulated regimen of ciprofloxacin at 750 mg every 12 h in the pharmacodynamic systems and why reserpine only delayed the onset of resistance in Spn-RC2 isolates that were treated with the same ciprofloxacin regimen.

Higher concentrations of reserpine could not be examined in the pharmacodynamic systems, mutation frequency studies, and EtBr studies because the 20- μ g/ml concentration of reserpine was at the limit of solubility of this drug in THB-Y. Also, we did not examine the effect of higher concentrations of levofloxacin on the induction of efflux pump expression in the EtBr uptake studies. However, the pharmacodynamic studies demonstrated that simulated exposures to levofloxacin at 750 mg QD reproducibly prevented resistance in both Spn-058 and Spn-RC2. This result suggests that higher concentrations of levofloxacin than those evaluated in the EtBr experiments continued to be poor inducers of increased efflux pump expression and that these pumps are saturable under this levofloxacin regimen. Since treatment failure was seen with the highest Food and Drug Administration-approved dose of ciprofloxacin but treatment success was seen with the highest approved dose of levofloxacin, our study results suggest that levofloxacin at 750 mg/day, as monotherapy, should be preferred over ciprofloxacin for the treatment of *S. pneumoniae* respiratory tract infections.

It is clear that efflux pumps play a critical role in the pathway to fluoroquinolone resistance in pneumococci. In the present

study, all of the isolates that had point mutations in *parC* and *gyrA* also had stable overexpression of efflux pumps. However, this study also showed that ciprofloxacin and, to a lesser extent, levofloxacin were able to transiently upregulate efflux pump function in Spn-058 and Spn-RC2. It is likely that most efflux pump overexpression in other *S. pneumoniae* strains is inducible and unstable, in contrast to the stable overexpression seen in Spn-RC2. This scenario would account for the large fraction of isolates that do not demonstrate stable efflux pump overexpression when exposed to fluoroquinolones, even ciprofloxacin (1). However, the induction of these pumps would serve the same purpose, allowing time for the production of a target site mutation, even when the bacterial population may be relatively small and below the reciprocal of the mutation frequency. This induction would allow the ultimate emergence of resistance with a *parC* or *gyrA* target site mutation. The withdrawal of drug pressure would then allow the downregulation of the pump, resulting in an isolate with a target site mutation but without stable overexpression of efflux pumps. Indeed, we have seen a transient increase in efflux pump expression in response to fluoroquinolone exposure in other bacterial species. In our mouse thigh infection model, for example, *Pseudomonas aeruginosa* treated with levofloxacin demonstrated efflux pump overexpression under quinolone pressure, but we did not identify target site mutations (8). A similar finding was observed with another quinolone tested against the same *Pseudomonas* isolate in our hollow fiber infection model (16), as well as in an evaluation of moxifloxacin in an in vitro model of tuberculosis in our laboratory (reference 7 and unpublished data from our laboratory). Efflux pump expression, either through induction or stable overexpression, may be the ultimate pathway to target site mutation and resistance, especially when bacterial burdens do not substantially exceed the reciprocal of the mutation frequency of resistance. The subject of efflux pumps is an important area that requires further exploration.

ACKNOWLEDGMENT

This work was supported by a grant from Ortho McNeil Janssen Scientific Affairs, LLC.

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