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Impact of Resistance Selection and Mutant Growth Fitness on the Relative Efficacies of Streptomycin and Levofloxacin for Plague Therapy▽

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***Yersinia pestis*, the bacterium that causes plague, is a potential agent of biowarfare and bioterrorism. The aminoglycoside antibiotic streptomycin is the gold standard for treatment. However, this recommendation is based on scant animal and clinical data. We used an in vitro pharmacodynamic infection model to compare the efficacies of 10-day regimens of streptomycin versus the fluoroquinolone antibiotic levofloxacin for the treatment of *Y. pestis* infection and to evaluate for emergence of resistance. The human serum concentration-time profiles for standard clinical regimens of 1 g of streptomycin given every 12 h and 500 mg of levofloxacin given every 24 h were simulated. The growth fitness of drug-resistant mutants was examined in neutropenic and immunocompetent mouse thigh infection models. In the in vitro infection system, untreated bacteria grew from 10^7 to 10^{10} CFU/ml. Streptomycin therapy caused a 10^5 CFU/ml reduction in the number of bacteria over 24 h, followed by regrowth with streptomycin-resistant mutants. Levofloxacin resulted in a 10^7 CFU/ml reduction in the number of bacteria within 12 h, ultimately sterilizing the culture without resistance selection. In both the normal and neutropenic mouse infection models, streptomycin-resistant and wild-type strains were equally fit. However, 90% of levofloxacin-resistant isolates, cultured from the control in vitro infection arm, did not proliferate in the mouse models. Thus, the fluoroquinolone antibiotic levofloxacin was superior to streptomycin in our in vitro infection model. The majority of levofloxacin-resistant mutants were less fit than streptomycin-resistant and wild-type *Y. pestis*.**

As a consequence of the recurrent anthrax attacks in the United States in 2001 (22, 38) and the on-going "war on terrorism," people worldwide recognize that future attacks of bioterrorism are possible. Similar to *Bacillus anthracis* (the agent of anthrax), the bacterium *Yersinia pestis* has been weaponized and is therefore a prime candidate for future episodes of bioterror or biowarfare (1, 21). *Y. pestis* is the causative agent of plague, the illness that killed approximately 30% of all Europeans during the 14th century and more than 12 million people in India and China in the 1850s (19, 36). In nature, rodents are the natural reservoir for *Y. pestis*. The organism is perpetuated by fleas that transmit the bacterium from infected to noninfected rodents (3, 31). Humans are not part of the natural life cycle of *Y. pestis*. However, humans can be infected with *Y. pestis* through the bite of an infected flea. The bacterium is transported from the skin to local lymph nodes via the lymph system, most commonly resulting in bubonic plague. The infection can spread to the bloodstream, causing plague septicemia, and to the lungs, resulting in plague pneumonia (3, 31). Uncommonly, humans are infected by inhaling infected respiratory droplets from animals or other humans who have plague pneumonia (11, 13, 39).

If *Y. pestis* is used as a bioweapon, it is expected that victims would develop pneumonic plague with secondary septicemia as a consequence of inhaling intentionally released aerosolized bacteria (21). When therapy is delayed by as little as 18 h after the onset of symptoms, mortality from pneumonic plague approaches 100% (27, 32). Previously developed vaccines were found to be ineffective for the prevention of pneumonic plague (10, 12).

Streptomycin and the tetracyclines are the only antibiotics approved by the Food and Drug Administration (FDA) for the treatment of *Y. pestis* infections in humans. These recommendations are based on scant clinical data and several small animal studies (21). However, streptomycin is in short supply (8) and treatment outcomes for tetracyclines in animal infection models provide conflicting results (5, 34, 35). Case studies suggest that gentamicin is an acceptable substitute for streptomycin (6, 8, 21, 29), but both drugs are oto- and nephrotoxic (2, 7). Furthermore, resistance to streptomycin and the tetracyclines exists in nature or has been generated in the laboratory for strains of *Y. pestis* (18, 21, 33). Thus, there is an urgent need to identify other antimicrobial agents that are effective in the treatment of plague.

Recently, we developed an in vitro pharmacodynamic model of *Bacillus anthracis* infection to define the efficacy of the fluoroquinolone antibiotic levofloxacin for the treatment of anthrax (14). The results of that study were central to the design of the dosing regimen that was subsequently used in a nonhuman primate model of inhalational anthrax infection that led the FDA to approve the use of levofloxacin for the treatment of people who are exposed to or have disease due to the anthrax pathogen (14, 24). In the current study, we adapted our in vitro pharmacodynamic infection model to compare the efficacies of 10-day regimens of streptomycin and levofloxacin

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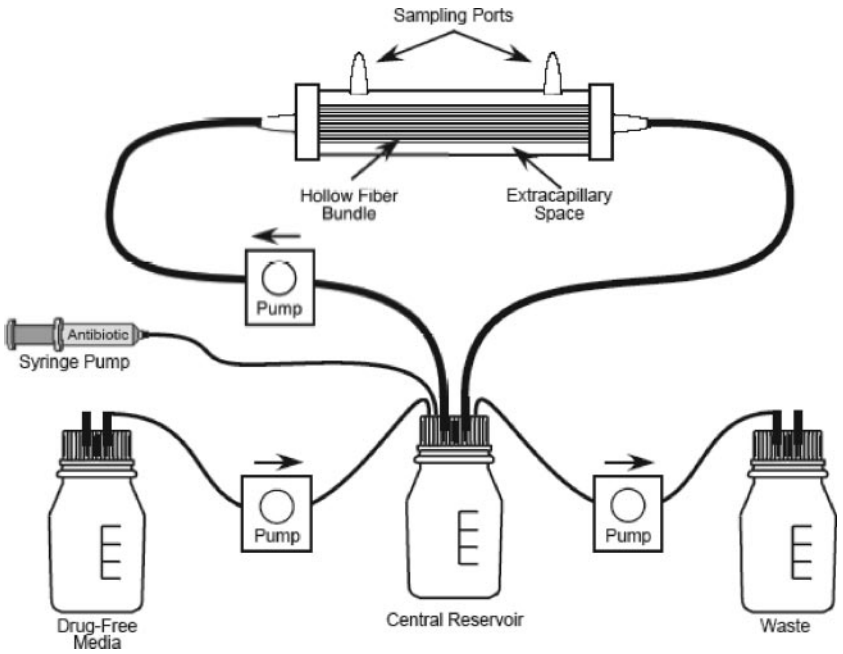

FIG. 1. Schematic of the in vitro pharmacodynamic hollow-fiber infection model.

for the treatment of *Y. pestis* infection and to examine the emergence of drug resistance during therapy. The human serum concentration-time profiles for clinical regimens of 1 g of streptomycin given intravenously (i.v.) every 12 h and 500 mg of levofloxacin given orally once every 24 h were simulated. Furthermore, we compared the growth fitness of the fully antibiotic-susceptible parent strain of *Y. pestis* with that of mutants resistant to either streptomycin or levofloxacin in neutropenic and immune normal mouse thigh muscle infection models.

(Parts of this work were presented at the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 30 October to 2 November 2004.)

MATERIALS AND METHODS

Bacterial isolate. *Yersinia pestis* ΔCO92 was kindly provided by H. Heine, United States Army Medical Research Institute of Infectious Diseases, Frederick, MD. *Y. pestis* ΔCO92 is an avirulent mutant of the fully virulent *Y. pestis* CO92 strain. It lacks the pCD1 plasmid and hence the low-calcium-response stimulon. It also lacks a capsule. *Y. pestis* ΔCO92 has in vitro growth kinetics similar to those of its wild-type progenitor (H. Heine, personal communication). Stocks of the organism were stored at −80°C in skim milk. For each experiment, a sample taken from the frozen stock was cultured on blood agar plates at 35°C for 48 h. A few colonies were then grown to the late logarithmic phase of growth (10^8 CFU/ml) in Mueller-Hinton II broth (MHB; BBL, Sparks, MD). The bacterial suspension was diluted to the desired concentration and used immediately.

Antibiotics. Stock solutions of streptomycin sulfate (Sigma-Aldrich, St. Louis, MO) and levofloxacin (a gift of Johnson & Johnson Pharmaceutical Research & Development, Raritan, NJ) were prepared in sterile water. The antibiotic solutions were filter sterilized and stored at −80°C. For each study, an aliquot of drug was thawed and diluted to the desired concentration with MHB. The drug solutions were used immediately.

Susceptibility studies. The susceptibility of *Y. pestis* ΔCO92 to levofloxacin and streptomycin was determined using a broth macrodilution method with MHB and an agar dilution method using Mueller-Hinton II agar (MHA; BBL, Sparks, MD) (30). Serial twofold drug dilution series were used for antibiotic concentrations of ≤1 mg/liter, and 1-mg/liter increments were used for drug concentrations of ≥1 mg/liter. The broth and agar susceptibility studies were conducted simultaneously. The final bacterial inocula were 5×10^5 CFU/ml and 10^4 CFU per spot for the broth and agar methods, respectively (30). The inocula were confirmed by quantitative cultures. The MIC was defined as the lowest concentration of antibiotic that allowed no visible growth after 48 h of incubation at 35°C. The minimal bactericidal concentrations (MBCs) were determined by inoculating the surfaces of blood agar plates with 25 μl of samples taken from the clear tubes of the macrobroth susceptibility studies. After the bacterial suspensions had fully absorbed into the agar, the surfaces were streaked. The MBC was defined as the concentration of drug that resulted in >99.9% killing of the bacterium relative to the concentration of bacterium that was present in test tubes at 0 h.

Mutation frequencies. The mutation frequencies of *Y. pestis* ΔCO92 grown with three and five times the MICs (3× and 5× MICs, respectively) of levofloxacin and streptomycin were determined by growing the organism to late-logarithmic-phase growth in MHB incubated at 35°C. Five milliliters of bacterial suspension was then cultured on the surfaces of antibiotic-free MHA plates and MHA plates that contained 3× and 5× MICs of levofloxacin or streptomycin. Duplicate cultures were incubated at 35°C for 72 h. The colonies were then enumerated, and the mutation frequencies were calculated.

Overview of the in vitro pharmacodynamic hollow-fiber infection model. The hollow-fiber bioreactor system (HFS) was originally developed for harvesting recombinant proteins. The system was later employed for cultivation of bacteria. Its use as a pharmacokinetic system that mimics human serum concentration-time profiles of drugs was first described by Blaser et al. (4) and has been modified for pharmacodynamic studies of human immunodeficiency virus (15), anthrax (14), and *Mycobacterium tuberculosis* (20) by our laboratory. The HFS allows the microorganism to grow in the peripheral compartment (extracapillary space in Fig. 1) of a hollow-fiber cartridge (Spectrum Laboratory Systems, Los Angeles, CA). The peripheral compartment is separated from the central compartment by thousands of semipermeable cellulose acetate hollow fibers (surface area, 2,100 cm^2), with pores (95% molecular size cutoff, 20 kDa) on the tubular surface that are large enough to allow nutrients, drugs, and bacterial metabolites to freely traverse into and out of the peripheral compartment but too small for bacteria to leave the peripheral compartment.

For each study, *Y. pestis* ΔCO92 was grown to mid-logarithmic phase in MHB and mutation frequencies were determined by quantitative plating of the bacterial suspension onto the surfaces of antibiotic-free MHA plates and MHA plates containing 3× and 5× MICs of levofloxacin or streptomycin. Ten milliliters of *Y. pestis* suspension, at 10^7 CFU/ml, was inoculated into the peripheral compart-

ments of three HFS cartridges that had been preconditioned with MHB for 24 h at 35°C.

Four hours after bacteria were inoculated into the HFS cartridges, treatment was initiated. In the first HFS, the free (non-protein-bound) serum concentration-time profile reported for humans given levofloxacin orally at 500 mg once every 24 h was simulated (protein binding, 30%; free peak concentration, 3.6 mg/liter; free 24-h area under the concentration-time curve, 36 mg · h/liter; and half-life [$t_{1/2}$], 7 h) (28). In the second HFS, the free serum concentration-time profile reported for humans given streptomycin i.v. at 1 g every 12 h was simulated (protein binding, 10%; free peak concentration, 38 mg/liter; free trough concentration, 2 mg/liter; and $t_{1/2}$, 2.5 h) (41). The dose of streptomycin used in this simulation is consistent with the maximum dose recommended for the treatment of human plague (i.e., 30 mg/kg of body weight per day in two divided doses). The third HFS served as a nontreatment control. The targeted pharmacokinetic profiles of drug that were attained with the HFSs were validated by sampling the central compartment of each HFS at 14 to 21 time points over the first 48 h of the study. Thereafter, trough and peak samples for streptomycin measurements were collected 30 min before and 30 min after the completion of alternating drug infusions. Preliminary studies demonstrated that the concentration-time profiles for both levofloxacin and streptomycin in the central and peripheral compartments were similar (data not shown). The drug concentrations in MHB were measured as described below.

At 0, 4, 8, 12, and 24 h and then daily for 10 days, 0.6 ml of bacterial suspension was taken from the peripheral compartment of each HFS and replaced with fresh medium. The bacteria were washed twice with normal saline to prevent drug carryover and then quantitatively cultured onto drug-free agar and agar containing 3× MIC of the treatment drug to characterize the effect of each antibiotic regimen on the drug-susceptible and -resistant bacterial populations. Organisms from the control arm were plated onto drug-free agar and both streptomycin- and levofloxacin-supplemented agars. After the plates were incubated at 35°C for 72 h, the colonies were enumerated.

Agar dilution MICs were determined with mutants that were cultured from levofloxacin- and streptomycin-containing agar plates that were inoculated on days 0, 4, 6, and 10 of the in vitro study. Seven or eight isolates were studied for each time point for each experimental arm.

Antibiotic assays. (i) HPLC assay for levofloxacin. The concentration of levofloxacin in MHB was determined by high-performance liquid chromatography (HPLC), using the method of Wright et al. (40), with modifications (14). Analysis was performed using an Agilent 1100 series HPLC system equipped with a model 1046A fluorescence detector. Levofloxacin was detected fluorometrically, using excitation and emission wavelengths of 295 and 485 nm, respectively. The assay response was linear over a concentration range of 0.1 to 20 mg/liter ($r^2 > 0.99$).

(ii) Streptomycin bioassay. The concentrations of streptomycin in MHB were determined by a previously described bioassay (16), with modifications. *Bacillus subtilis* ATCC 6633 (American Type Culture Collection, Manassas, VA) spores were incorporated into Antibiotic Media 5 agar (Difco, Sparks, MD) at 10^6 CFU/ml. Forty microliters of sample or streptomycin standards (in MHB) was added to 7-mm-diameter wells that were made in the agar. The plates were incubated for 20 h at 35°C. The diameters of inhibition for samples and standards were measured. Streptomycin concentrations were calculated using the data from the curves derived from the drug standards. The zones of inhibition versus the logarithms of the drug concentrations were linear between 0.25 and 80 mg/liter ($r^2 > 0.99$).

Growth fitness of streptomycin- and levofloxacin-resistant mutants. The growth of the pan-susceptible *Y. pestis* parent strain, 10 streptomycin-resistant mutants, and 10 levofloxacin-resistant mutants was compared in both immune normal and neutropenic mouse thigh infection models as described previously (23, 25), with modifications. The streptomycin- and levofloxacin-resistant isolates were cultured from the streptomycin-treated and control experimental arms, respectively, on the 10th day of the in vitro studies. The mouse thigh infection model was used for the growth fitness studies because preliminary experiments demonstrated that the parent *Y. pestis* strain ΔCO92 did not grow in the spleens of immune normal and neutropenic mice. Briefly, a cohort of 22- to 24-g, female, outbred Swiss Webster mice (Taconic Farms, Taconic, NY) were rendered neutropenic by the administration of 150 mg/kg of cyclophosphamide, given intraperitoneally, 3 days prior to bacterial inoculation, followed by 100 mg/kg of cyclophosphamide given intraperitoneally every other day thereafter. This regimen resulted in persistent neutropenia beginning on the day the second dose of cyclophosphamide was given (data not shown). One day after the second dose of cyclophosphamide was administered, 21 groups of neutropenic mice were injected in each posterior thigh muscle with 10^6 CFU of the pan-susceptible *Y. pestis* parent strain ΔCO92, 10 *Y. pestis* strains with increased MICs to levofloxacin, and 10 *Y. pestis* mutants with increased MICs to streptomycin. In a parallel study, 21 groups of immune normal mice were injected in each posterior thigh muscle with 10^7 CFU of these 21 bacterial isolates. On days 0, 1, 2, 3, 4, 5, and 7, five mice per group were sacrificed. The infected thigh muscles were aseptically collected, homogenized, and quantitatively cultured. After the cultures had incubated at 35°C for 96 h, the colonies were counted. Agar dilution MICs were determined for 10 colonies isolated from the muscle homogenates each day to confirm that the susceptibility profiles of the organisms had not changed over the 7-day study. The animal procedures were approved by our Institutional Animal Care and Use Committee.

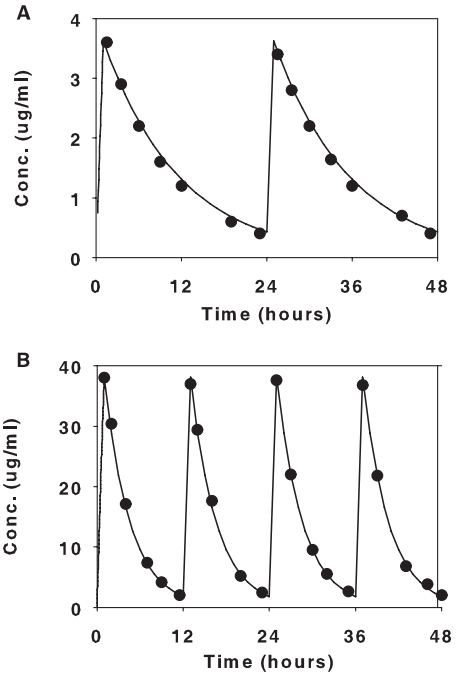

FIG. 2. Pharmacokinetic simulations for (A) levofloxacin given orally at 500 mg every 24 h and (B) streptomycin given i.v. at 1 g every 12 h. The solid lines represent the targeted antibiotic concentration-time profiles, and the circles represent the measured drug concentrations over the first 48 h of the study.

Statistical methods. Differences in bacterial densities at the end of the 10-day in vitro studies and the 7-day in vivo growth fitness studies were assessed by analysis of variance. A P value of 0.05 was considered significant. All data are presented as the mean values ± 1 standard deviation.

RESULTS

Susceptibility studies. The macrobroth MIC and MBC of levofloxacin were 0.06 and 0.125 mg/liter, respectively. For streptomycin, the MIC and MBC were both 2 mg/liter. For each antibiotic, the results for the broth macrodilution and agar-based MICs were identical.

Mutation frequencies in response to antibiotics. The mutation frequencies for levofloxacin in response to 3× and 5× MICs were −6.92 and −7.13 log CFU, respectively. For streptomycin, the mutation frequencies for these multiples of the MIC were −5.94 and −6.10 log CFU, respectively.

Effects of levofloxacin and streptomycin therapy on the total and drug-resistant bacterial populations in vitro in HFSs. The targeted human serum pharmacokinetic profiles, for levofloxacin given at 500 mg every 24 h and streptomycin given at 1 g every 12 h, were accurately simulated (Fig. 2). The density of bacteria in the control arm slowly increased from a baseline concentration of 10^7 to 10^{10} CFU/ml within 4 days and re-

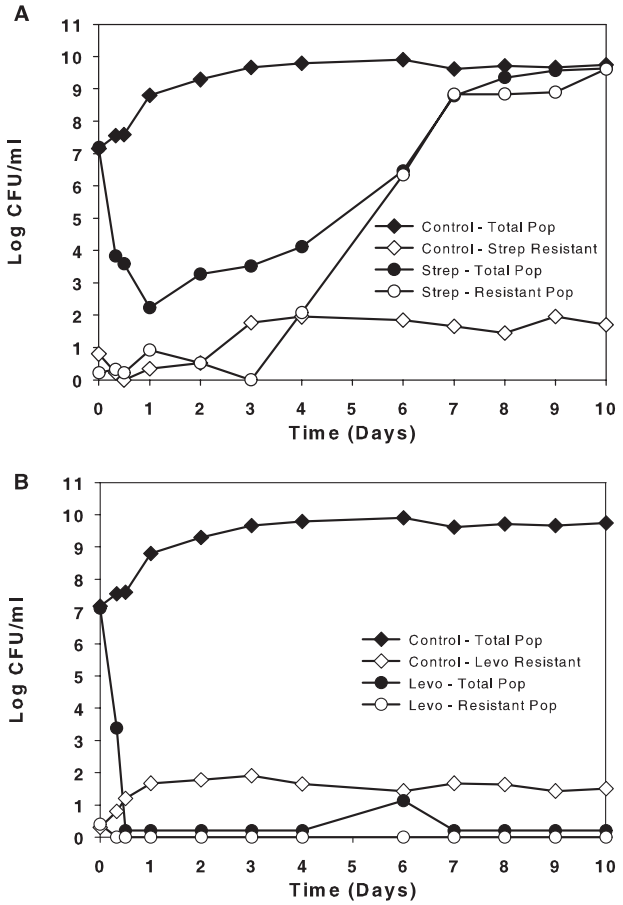

FIG. 3. Effects of streptomycin and levofloxacin therapies on the total *Y. pestis* population and the mutant populations with ≥3× MICs to (A) streptomycin and (B) levofloxacin. Streptomycin was given to simulate the human serum concentration-time profile for 1 g i.v. every 12 h, and levofloxacin dosing simulated the human serum concentration-time profile for levofloxacin given orally at 500 mg every 24 h. The studies with streptomycin and levofloxacin therapy were conducted simultaneously. The results are presented in two graphs for clarity.

mained at this concentration for the remainder of the 10-day study (Fig. 3). Without antibiotic pressure, streptomycin- and levofloxacin-resistant mutants grew in proportion to the total bacterial population. Streptomycin therapy resulted in a 10^5 CFU/ml decrease in the total bacterial population by 24 h. However, this was followed by regrowth, with the total bacterial density matching those of the control arm by day 7. Regrowth was due to organisms that were resistant to streptomycin (Fig. 3A). In contrast, levofloxacin therapy was associated with a $>10^6$ CFU/ml decrease in bacterial density, to below the lower limit of detection of the assay (40 CFU/ml of medium). This occurred within 12 h of initiation of therapy (Fig. 3B). Levofloxacin therapy did not select for resistance over the 10-day study. There was a single blip in bacterial counts in the levofloxacin treatment arm on day 6 of the 10-day treatment course. These organisms were found to be susceptible to levofloxacin. At the end of the 10-day study, the entire volume of bacterial suspension in the levofloxacin-treated arm was cultured. These cultures were sterile.

MICs were determined for isolates cultured on agar supplemented with 3× MIC of levofloxacin or 3× MIC of streptomycin during the 10-day study (Table 1). In the streptomycin treatment arm, a mixture of small-colony variants (with MICs equivalent to that of the pan-susceptible parent strain upon susceptibility testing) and larger colonies with increased MICs to this aminoglycoside were cultured from streptomycin-supplemented agar during the first 3 days of therapy. By day 4, only rapidly growing colonies with normal morphologies were isolated from streptomycin-containing agar plates. The MICs to streptomycin increased with the duration of therapy (Table 1). In the levofloxacin treatment arm, mutants with increased MICs to this drug were isolated on antibiotic-supplemented agar only prior to the initiation of therapy on day 0 and grew in numbers consistent with the mutational frequency to resistance. Mutants with increased MICs to levofloxacin and streptomycin were cultured from the placebo arm throughout the study.

Growth fitness of drug-resistant mutants in mice. Growth of the pan-antibiotic-susceptible *Y. pestis* parent isolate was compared with that of 10 levofloxacin-resistant mutants (MIC of 1 mg/liter) and 10 streptomycin-resistant isolates (MIC of 8 mg/liter) in neutropenic and immune normal mouse thigh infection models. The mouse thigh infection model was used for the growth fitness studies because preliminary experiments demonstrated that the *Y. pestis* parent strain ΔCO92 does not grow in the spleens of immune normal and neutropenic mice. The growth of the 10 streptomycin-resistant mutants was similar to that of the pan-drug-susceptible parent strain in both mouse thigh infection models. In contrast, 9 of the 10 levofloxacin-resistant isolates were less fit than the parent strain. The growth curves for the parent strain, 2 of the 10 streptomycin-resistant mutants, and 3 of the 10 levofloxacin-resistant mutants in neutropenic and immune normal mice are shown in Fig. 4A and B. The growth curves for the eight streptomycin-resistant isolates not shown were similar to that of the parent

TABLE 1. Streptomycin and levofloxacin MICs of *Y. pestis* mutants selected on agar plates supplemented with 3× MIC of streptomycin or levofloxacin during the 10-day in vitro study

Treatment arm	Antibiotic	MIC (mg/liter) for parent strain	MIC (mg/liter) for mutants[a]			
			Day 0	Day 4	Day 6	Day 10
No treatment	Levofloxacin	0.06	0.25–0.5	0.25–1	0.5–1	0.5–1
	Streptomycin	2.0	6–8	6–8	6–8	6–8
Streptomycin treatment	Streptomycin		2#–8	2#–8	8–16	8–32
Levofloxacin treatment	Levofloxacin		0.25–1	—	—	—

[a] For each time point and treatment arm, the MIC range is reported for seven randomly tested isolates. —, antibiotic-supplemented plates were sterile; #, small-colony variants.

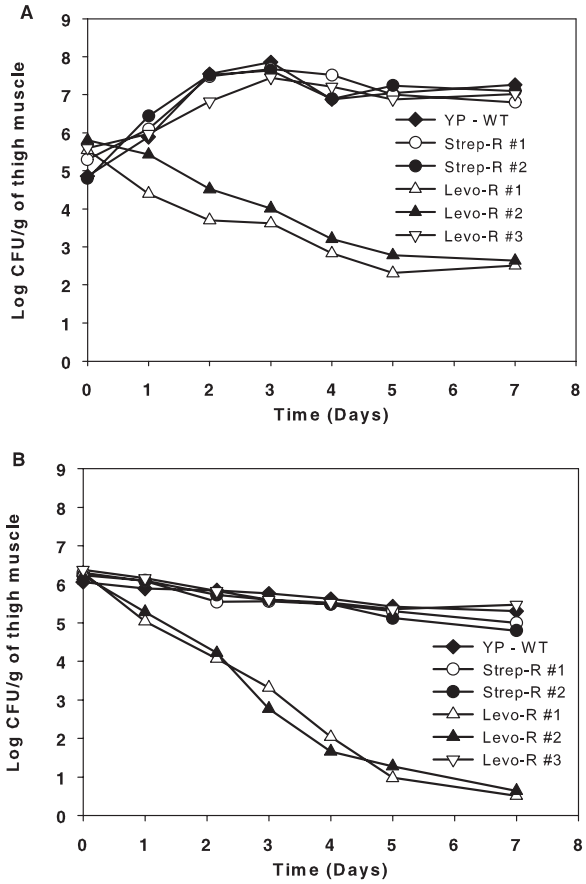

FIG. 4. Growth fitness of the parent *Y. pestis* strain ΔCO92 (streptomycin MIC, 2 mg/liter; levofloxacin MIC, 0.06 mg/liter), 2 of 10 streptomycin-resistant mutants (streptomycin MIC, 8 mg/liter), and 3 of 10 levofloxacin-resistant (MIC, 1 mg/liter) mutants in (A) neutropenic and (B) immune normal mouse thigh infection models. The error (standard deviation) bars are not shown for clarity. At the 7-day time point, the difference in bacterial densities in tissues of mice infected with the parent strain and each of the less fit levofloxacin-resistant mutants was significant ($P < 0.01$).

isolate. The growth curves for the seven other levofloxacin-resistant strains were similar to those of the less fit mutants shown in Fig. 4A and B. The differences between experimental groups on day 7 for both infection models were significant ($P < 0.01$ by analysis of variance) for these less fit mutants compared with the parent strain. Susceptibility studies conducted on isolates cultured from homogenates of thigh muscles over the 7-day studies demonstrated that the MICs had not changed over time (data not shown).

DISCUSSION

In this study, we found that streptomycin, the "gold standard" for the treatment of plague, was inferior to a clinical dose of levofloxacin. Streptomycin rapidly selected for resistant mutants during therapy, while levofloxacin rapidly sterilized the infection model. Importantly, streptomycin-resistant mutants were as fit as the parent strain in neutropenic and immune normal mice. In contrast, 90% of the levofloxacin-resistant mutants were less fit than the parent isolate. The mechanism by which levofloxacin decreased the growth fitness of these mutants requires further investigation.

Streptomycin is currently considered the drug of choice for the treatment of infections due to *Y. pestis*, based on scant clinical data and several small animal studies (21). Without antibiotic therapy, the mortality rate associated with bubonic plague is about 40%, whereas the mortality rates of untreated septicemic and pneumonic plague approach 100% (12). Case studies in the United States report that the fatality rates for antibiotic-treated bubonic, septicemic, and pneumonic plague are 14%, 22%, and 57%, respectively (12). Mortality rates approaching 100% are seen for people with plague pneumonia in whom therapy is delayed by as little as 18 to 24 h after the onset of disease symptoms (27). Mortality from plague is due to overwhelming infection, leading to septic shock and organ failure. However, the extent to which selection of resistance during therapy contributes to treatment failure is unknown, since susceptibility studies have been reported only for *Y. pestis* isolates that were cultured from patients either prior to or shortly after therapy was initiated (27). We are unaware of studies in which susceptibility studies were conducted on *Y. pestis* isolates that were collected from people after they had failed antibiotic therapy.

In a mouse model of plague septicemia, Bonacorsi et al. (5) administered a single dose of streptomycin to mice 24 h after they were infected i.v. with 400 CFU of a fully virulent strain of *Y. pestis*, an inoculum that was >20 times the 50% lethal dose. Before antibiotic therapy was initiated, the concentration of bacteria in mice increased from approximately 10^1 to 10^4 CFU/spleen. Streptomycin therapy decreased the concentration of bacteria to approximately 10^1 CFU/spleen 8 h after drug administration, but regrowth (to 10^4 CFU/spleen) was seen 24 h after the drug was administered. Susceptibility testing was conducted on "a sample" (5) of the latter bacteria isolated from antibiotic-free agar plates. These organisms remained susceptible to streptomycin. It is likely that the starting inoculum and, consequently, the concentration of bacteria in spleens at the initiation of therapy were below the mutation frequency for resistance of the *Y. pestis* isolate. Therefore, it is unlikely that the mice were inoculated with streptomycin-resistant mutants prior to the start of therapy. Also, the short, 1-day treatment course made it unlikely that resistant mutants would be identified. In our study, streptomycin-resistant mutants were not readily detected until the fourth day of streptomycin therapy. Furthermore, since streptomycin has a 25-min serum $t_{1/2}$ in mice, we estimate that the concentration of streptomycin fell below the MIC for the *Y. pestis* strain within 2 h after drug administration. This duration of antibiotic exposure would provide little antibiotic pressure for selection of resistance. Therefore, it would be expected that the regrowth that was seen after the aminoglycoside had cleared from the animals would be due to streptomycin-susceptible bacteria. A higher starting inoculum could not be examined by Bonacorsi et al. because the wild-type *Y. pestis* strain used was highly virulent in their mouse infection model. In contrast, in our in vitro infection model, the mutation frequency for 3× MIC of streptomycin of 8.7×10^{-5} CFU suggests that approximately 115 CFU of streptomycin-resistant mutants were instilled in the infection system with the starting inoculum of 1×10^7 CFU/ml (1×10^8 CFU/system). These mutants were amplified under streptomy-

cin pressure and replaced the streptomycin-susceptible population as the drug-susceptible microorganisms were killed by antibiotic therapy. Notably, in monkey models of plague septicemia and pneumonia in which subjects were challenged with large inocula of a fully virulent strain of *Y. pestis*, two of seven monkeys failed streptomycin therapy due to the emergence of streptomycin-resistant mutants (26), showing that our in vitro model can be predictive of in vivo outcomes. The monkey study (26) and our study show that prevention of emergence of resistance during therapy is important because streptomycin-resistant mutants can lead to treatment failure. Furthermore, people with plague pneumonia may transmit streptomycin-resistant organisms to other individuals via infected respiratory droplets. The person-to-person dissemination of drug-resistant mutants may have a profound impact on the efficacies of mass treatment efforts that would be implemented in response to an act of bioterrorism or biowarfare.

There are no clinical studies evaluating the role of fluoroquinolone antibiotics for the treatment of infections due to *Y. pestis*. However, in vitro studies demonstrate that fluoroquinolones have potent activity against *Y. pestis* (17, 37). Animal studies provide similar results, showing that the fluoroquinolones ciprofloxacin and ofloxacin are as effective as or more effective than streptomycin in the treatment of pneumonic and septicemic plague (9). Importantly, these studies used antibiotic doses that achieved serum drug exposures in mice that were higher than those reported for humans who were given clinically prescribed dosages of these medications (28, 41). The activities of newer fluoroquinolones, including levofloxacin and moxifloxacin, have not been reported. Simulating the human concentration-time profile for the clinically prescribed regimen of levofloxacin given orally at 500 mg every 24 h in our pharmacodynamic infection model, we demonstrated that levofloxacin was superior to i.v. streptomycin in the treatment of infection due to *Y. pestis*. While streptomycin therapy selected for mutants that were resistant to streptomycin, levofloxacin was able to rapidly kill both the wild-type isolate and mutants with levofloxacin MICs as high as 1 mg/liter. Moreover, many of the levofloxacin-resistant mutants were less fit than the streptomycin-resistant mutants and the parent *Y. pestis* strain ΔCO92. Taken together, these findings suggest that levofloxacin-resistant mutants should have less impact than streptomycin-resistant mutants on treatment outcomes. Further studies are needed to evaluate this hypothesis using fully virulent wild-type strains of *Y. pestis*.

Based on our data, levofloxacin should be evaluated further for the treatment of plague. It offered effective therapy for treatment of infection due to the parent *Y. pestis* strain and, in contrast to streptomycin, did not select for resistance during therapy. Furthermore, it has other advantages over streptomycin. First, the aminoglycoside streptomycin is both ototoxic and nephrotoxic (2, 7). In contrast, extensive clinical use of levofloxacin has proven that this drug has an excellent safety profile and is well tolerated by humans. Second, the supply of streptomycin is limited (8), while levofloxacin is readily available. This factor makes it impractical to rely on streptomycin for large-scale treatment and prophylaxis of plague in a bioterror or biowarfare scenario. Third, streptomycin must be administered i.v. or intramuscularly and is injected twice daily. Levofloxacin is available as both oral and i.v. formulations and is administered once daily. These properties of levofloxacin should increase patient acceptance and compliance with antibiotic therapy if future studies prove that levofloxacin is useful for the prevention and treatment of plague, especially if the drug were to be given to a large population of people in response to an act of bioterror or biowarfare.

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