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# Rifampin Resistance of *Legionella pneumophila* Is Not Increased during Therapy for Experimental Legionnaires Disease: Study of Rifampin Resistance Using a Guinea Pig Model of Legionnaires Disease

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**Isolates of *Legionella pneumophila* serogroup 1, obtained from guinea pigs with experimentally induced Legionnaires disease, were tested for rifampin resistance. Thirteen isolates were from animals treated with rifampin alone, four isolates were from animals treated with saline, and three isolates each were from animals treated with erythromycin or erythromycin plus rifampin; all of these isolates were derived from the same parent strain, F889. Most of the isolates were obtained from rifampin-treated animals that survived infection but had persistence of bacteria in their lungs at necropsy. No differences in rifampin agar dilution MICs were detected for the 23 isolates and parent strain that were tested. None of the 13 isolates from animals treated with rifampin alone had a high number of resistant organisms detected by using a rifampin gradient plate assay. Thirteen isolates plus the parent strain were tested by using a quantitative method of determining resistance frequency. Considerable heterogeneity among isolates was observed, but there was no evidence of increased resistance for any treatment group. The range of rifampin resistance frequencies was $10^{-7}$ to $10^{-8}$. No evidence for rifampin-induced resistance of *L. pneumophila* was found in this study.**

Rifampin is very active against *Legionella pneumophila* and other *Legionella* species in vitro and in an animal model of Legionnaires disease (7, 9–11, 17, 20, 22, 24). The use of this drug rapidly reduces the number of bacteria in the lungs of guinea pigs with experimentally induced *L. pneumophila* pneumonia and also dramatically decreases the fibrotic sequelae of pneumonia in survivors (7, 9). There is little or no evidence that the addition of other antimicrobial agents to a rifampin treatment regimen results in a better outcome in the guinea pig model (7, 10). Despite this, there has been reluctance to use rifampin alone because of concern over the rapid emergence of resistance to this drug as occurs with other organisms (1, 3, 4, 27). To determine whether rifampin resistance emerges during therapy of experimental Legionnaires disease in guinea pigs, I determined the susceptibility and frequency of resistance of *L. pneumophila* before and after treatment of infection with rifampin alone. I demonstrate that rifampin resistance does not increase during solitary rifampin therapy.

## MATERIALS AND METHODS

**Animal model.** Guinea pigs were given pneumonia with the F889 strain of *L. pneumophila* serogroup 1 as described previously (7). The pneumonia very closely resembles human disease and is fatal unless treated with appropriate antimicrobial agents. Approximately $7 \times 10^6$ CFU of bacteria was inoculated intratracheally in each animal. The *L. pneumophila* lung concentration was $\approx 10^8$ CFU/g after 24 h, and in untreated animals the bacterial count of *L. pneumophila* in the lungs rose to $\approx 10^{10}$ CFU/g at 4 days postinfection (7). Antimicrobial treatment was begun 1 day after infection and was continued for a total of 5 days; each treatment group consisted of 15 to 20 animals or about 60 animals per experiment. All drugs were administered twice daily by the intraperitoneal route. Rifampin was given in a dose of 10 mg/kg/day, and erythromycin was given in a dose of 60 mg/kg/day (7). Levels of rifampin in sera and lungs at 1 h after administration were 4 to 6 μg/ml (or μg/g) for both types of specimens; for the same time period the concentration of erythromycin in serum was 5 μg/ml and the concentration in the lung was 17 to 21 μg/g. These drug concentrations are similar to those measured in humans after the administration of the antimicrobial agent in doses suggested for the treatment of Legionnaires disease (7). About 80% of animals treated with either rifampin or erythromycin survived for 12 days after termination of antimicrobial therapy as compared with 60% treated with both drugs combined or with 5% treated with saline only. Lungs from animals dying of pneumonia or from survivors 17 days postinfection (12 days after termination of antimicrobial therapy) were cultured quantitatively for *L. pneumophila*. A sweep of confluent bacterial growth on the culture plates was processed for freezing in multiple samples in 1% skim milk. No isolate was passaged more than twice before initial freezing at −70°C.

**Bacterial strains and growth conditions.** The identities of bacteria tested for the frequency of rifampin resistance are shown in Table 1. Ten additional isolates, each obtained from a different animal infected with the same F889 strain, were used for MIC testing and gradient plate assays but not for the quantitative determination of resistance frequency. These 10 animals were treated with rifampin alone in a dosage identical to that given to all other rifampin-treated animals. The 10 animals consisted of 8 animals that survived to day 17 postinfection and two animals that died on day 16 postinfection. Bacteria were grown on various media (detailed below) including buffered charcoal yeast extract agar supplemented with 0.1% α-ketoglutarate (BCYEα), BCYEα broth, and buffered yeast extract broth (BYEα) (8). Plates were incubated at 35°C in humidified air, while broths were

TABLE 1. Identity of *L. pneumophila* isolates

| Isolate | Code | Treatment group | Day of animal death[a] |
|---|---|---|---|
| F889 | P | None[b] | None[b] |
| F1146 | $S_1$ | Saline | 1 |
| F1147 | $S_2$ | Saline | 1 |
| F1439 | $S_3$ | Saline | 1 |
| F1440 | $S_4$ | Saline | 1 |
| F1441 | $E_1$ | Erythromycin | 8 |
| F1444 | $E_2$ | Erythromycin | 9 |
| F1447 | $E_3$ | Erythromycin | 14 |
| F1417 | $R_1$ | Rifampin | 17[c] |
| F1445 | $R_2$ | Rifampin | 6 |
| F1442 | $R_3$ | Rifampin | 6 |
| F1443 | $RE_1$ | Rifampin and erythromycin | 6 |
| F1446 | $RE_2$ | Rifampin and erythromycin | 9 |
| F1418 | $RE_3$ | Rifampin and erythromycin | 17[c] |

[a] Day postinfection.
[b] Parent strain, pretherapy isolate.
[c] Survivor to 17 days, lung culture positive for *L. pneumophila*.

incubated at the same temperature but shaken at 120 rpm in a gyratory shaking water bath.

Frozen bacteria were rapidly thawed and plated on BCYEα agar. After 2 days of incubation, multiple isolated colonies were harvested to BYEα broth, and the turbidity was adjusted to that of a 1/2 McFarland barium sulfate standard. The turbid suspension (0.1 ml) was added to BCYEα broth (10 ml) which was incubated overnight. The turbid BCYEα broth (.01 ml) was then passaged to BYEα broth (10 ml) which was incubated for about 18 h. This resulted in a suspension containing $10^8$ to $10^9$ CFU/ml. The viable bacterial counts in the BYEα broth were determined by plating 10-fold dilutions on BCYEα plates.

**Agar dilution susceptibility testing.** Agar dilution MICs of rifampin were determined as previously described by using BCYEα agar as the basal medium (8). An inoculum of $\approx 10^5$ CFU per spot was used, and plates were incubated for 2 days before the results were read. Rifampin standard powder (Merrell Dow Pharmaceuticals, Inc., Cincinnati, Ohio) was dissolved in methanol and then in 0.1 M phosphate buffer (pH 7.1). *Staphylococcus aureus* ATCC 25293 was used in each run as a control and tested on both BCYEα and Mueller-Hinton agar media containing rifampin. All testing was performed in duplicate or triplicate. Rifampin MICs were determined for the *L. pneumophila* isolates listed in Table 1 plus 10 additional isolates obtained from animals treated soley with rifampin. These tests were performed 1 to 4 months after the original isolation and freezing of the bacterial isolates. In addition, MIC testing was repeated 6 years later for the isolates listed in Table 1, which were selected for quantitative resistance frequency determinations.

**Gradient plate assays.** BCYEα plates containing a gradient of rifampin were used to estimate the frequency of rifampin resistance for 10 *L. pneumophila* isolates (not listed in Table 1) that were obtained from animals treated solely with rifampin, as well as for isolates $R_1$, $R_2$, and $R_3$ (Table 1). The gradient plates were made by pouring 10 ml of molten, cooled BCYEα agar into 100-mm-diameter petri dishes tilted at a 45° angle. The agar was then allowed to solidify, and a rifampin-containing layer of BCYEα agar was added. These gradient plates were inoculated with bacteria as soon as the top layer solidified and dried to minimize diffusion of the antimicrobial agent. The rifampin concentration in the top layer was 0.50 μg/ml.

BYEα broth containing the *L. pneumophila* isolates ($10^8$ to $10^9$ CFU/ml) was plated (0.1 ml) uniformly onto triplicate gradient plates by use of a glass rod. The number of bacterial colonies on each antibiotic-containing plate was determined at 48, 72, and 96 h. The numbers of colonies in the portion of the plates with the highest rifampin concentration were compared with the numbers of colonies in the portion of the plates with the lowest rifampin concentration. To confirm true rifampin resistance, selected colonies growing in the plate areas of high rifampin concentration were passaged twice on non-rifampin-containing BCYEα agar and then tested for rifampin MIC by using the agar dilution method.

**Quantitative determination of resistance frequency.** Two separate experiments were performed to study resistance frequency by using a more easily and accurately quantifiable method than the gradient plate method. The first experiments were performed with isolates P, $S_3$, $S_4$, $E_1$, $R_1$, $R_2$, $R_3$, and $RE_1$; these isolates were picked for analysis to include several controls not used in the gradient plate assays and to include isolates from rifampin-treated animals that died relatively early in the course of their infection. A second experiment was performed to confirm the results of the first one and to test more isolates ($S_1$, $S_2$, $E_2$, $E_3$, $RE_2$, and $RE_3$). The frequency of rifampin resistance was determined by plating the BYEα broth-grown bacteria (0.1 ml) onto rifampin-containing (1, 2, and 4 μg/ml) BCYEα agar. Plating was done in triplicate by using an electrical turntable and a spiral inoculation method, distributing the inoculum evenly over the entire plate; $\approx 3 \times 10^7$ CFU was plated in the first study, and $\approx 2 \times 10^8$ CFU was plated in the second study. The actual viable bacterial count plated of each isolate was determined by duplicate plating (0.1 ml) of serial 10-fold dilutions of the BYEα broths onto BCYEα agar; these plates were incubated for 72 h before counting. The rifampin-containing plates were incubated for 4 days before counting colonies and were inspected by using a dissecting microscope to look for microcolonies. In one experiment, plates were incubated for an additional 2 days to detect slow-growing colonies or small-colony variants.

**Data analysis.** Resistance frequencies were determined for each isolate by dividing the mean concentration of bacteria growing on the rifampin-containing plates by the concentration of viable bacteria present in the initial inoculum. Estimates of the confidence intervals of resistance frequencies were made by assuming a Poisson distribution. Statistical comparison of normalized resistance frequencies for the different isolates was performed by using one-way analysis of variance; the Newman-Keuls test was used for post-hoc analysis (PC ANOVA; Human Systems Dynamics, Northridge, Calif.). Mean colony counts were first normalized to those of the parent strain, F889, to account for the slightly different numbers of bacteria plated for each isolate, by dividing the colony counts by the ratio of total bacteria plated for F889 to that for each isolate. The normalized colony counts were then transformed by use of a $\sqrt{x+1}$ transformation (23). The transformed data were then used in the analysis of variance testing. Regression analysis and one-way analysis of variance were used to determine the relationship between rifampin concentration and the mean frequency of resistance (PC Statistician and PC ANOVA; Human Systems Analysis).

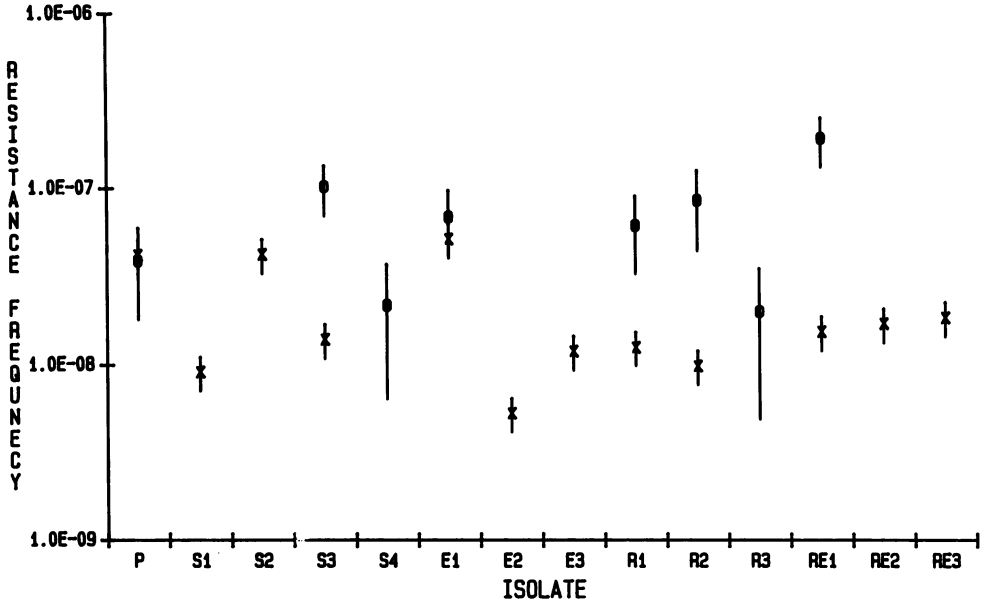


FIG. 1. Mean resistance frequencies for the *L. pneumophila* isolates listed in Table 1. Results of two separate experiments are shown, experiment 1 (●) and experiment 2 (X); note that several isolates studied in experiment 1 were restudied in experiment 2. The vertical bars represent 95% confidence intervals calculated by assuming a Poisson distribution of counts of resistant colonies. The text gives results of statistical analysis of the data points.

## RESULTS

**Agar dilution MICs.** Agar dilution MICs of rifampin for all the isolates ranged from 0.03 to 0.06 μg/ml. Repeat testing in 1990 of isolates $P_1$, $S_2$, $S_4$, $R_1$, $R_2$, $R_3$, $E_1$, and $RE_1$ gave results identical to those obtained 6 years previously. Rifampin was antagonized by BCYEα medium as judged by the MICs for the control *S. aureus* strain when tested on BCYEα (0.25 μg/ml) and Mueller-Hinton (0.03 μg/ml) media.

**Gradient plate assays.** The number of colonies growing in the portion of the gradient plates with the highest rifampin concentration was relatively low and did not apparently vary among the 13 different isolates tested. From 2 to 10 rifampin-resistant colonies were found for each strain. The approximate frequency of resistant colonies growing on these plates ranged from $10^{-7}$ to $10^{-8}$. The bacterial colonies growing in the areas of high rifampin concentration were highly and stably resistant to the drug. Rifampin MICs for these resistant colonies, after two passages on non-antimicrobial-agent-containing BCYEα medium, ranged from 1 to ≥128 μg/ml, with a mean and median of 20 and 64 μg/ml, respectively.

**Quantitative resistance frequency determinations.** The first experiment showed that all isolates contained rifampin-resistant colonies in low numbers (Fig. 1, ●). No increase in colony numbers was observed after prolonged incubation nor were microcolonies observed by use of a dissecting microscope. There was no correlation between the rifampin concentration of the plates (1, 2, or 4 μg/ml) and the numbers of resistant colonies ($r^2 = 0.02$, $P = 0.56$, by regression analysis; $F_{(2,21)} = 0.196$, $P = 0.18$, by analysis of variance). The data were therefore pooled with respect to antibiotic concentration to determine the mean frequency of resistance for each isolate. The mean frequencies of resistance ranged from $1.0 \times 10^{-7}$ to $8.4 \times 10^{-8}$ for the various isolates and differed more than expected by chance alone ($F_{(7,16)} = 7.1$, $P < 0.001$, by analysis of variance). Isolate $RE_1$ contained significantly more resistant colonies than did all other isolates tested ($P = 0.01$ to 0.05, by Newman-Keuls test, depending on comparison). Isolate $S_3$ contained significantly more resistant colonies than did isolates $S_4$ and $R_3$ ($P = 0.05$). The other isolates were not significantly different from each other ($P > 0.05$). None of the three isolates from animals treated with rifampin alone contained more resistant colonies than did the other isolates.

The resistance frequency experiment was repeated with isolates P, $S_3$, $E_1$, $R_1$, $R_2$, and $RE_1$; six previously untested isolates were included as additional controls ($S_1$, $S_2$, $E_2$, $E_3$, $RE_2$, and $RE_3$) (Fig. 1, X). As in the first experiment, there was no correlation of frequency of resistance with concentration of antimicrobial agent ($r^2 < 0.001$, $P = 0.56$, by regression analysis; $F_{(2,33)} = 0.21$, $P = 0.20$, by analysis of variance), so the resistance frequency data were pooled, with respect to antibiotic concentration, for each isolate. The mean frequencies of resistance differed more than expected by chance alone ($F_{(11,24)} = 16.2$, $P < 0.001$, by analysis of variance). In this experiment, isolates determined in the first experiment to contain significantly more resistant colonies actually contained fewer such colonies than did the other isolates tested. In fact, isolates $RE_1$ and $S_3$ expressed resistance significantly less frequently than did isolates $P_1$, $S_2$, and $E_1$ ($P \leq 0.01$, by Newman-Keuls test). These latter three isolates expressed resistance significantly more often than did all other isolates ($P \leq 0.01$); they were not significantly different from each other. Isolate $E_2$ expressed resistance less frequently than did isolates $RE_1$, $RE_2$, and $RE_3$ ($P \leq 0.05$). There were no other significant differences among the isolates.

If the data from both experiments are grouped by the four types of animal treatment, there is no apparent difference between treatment types and frequency of resistance of the bacterial isolates obtained from the animals ($F_{(3,50)} = 1.82$, $P$

= 0.15, by analysis of variance). This is true whether or not the parent strain is included in the saline treatment group.

## DISCUSSION

Rifampin therapy alone did not result in an increase of bacterial resistance. In fact, no change in resistance was noted for the 23 *L. pneumophila* isolates obtained from animals treated with or without rifampin. No change in rifampin MICs from that for the parent strain was observed for any of the derivative isolates. Several reports of rifampin resistance of *Legionella* species and other bacteria document that the rifampin MICs for the resistant strains are much higher than those for the parent strains as was the case for the *L. pneumophila* isolates that grew on the gradient and resistance frequency assay plates (1, 3, 6, 14, 17, 18). It is therefore unlikely that significant rifampin resistance was undetected by the MIC determinations performed in this study.

To detect more subtle changes in rifampin resistance, larger numbers of bacteria were plated on antibiotic-containing plates. By using two different methods, one semiquantitative and the other quantitative, no significant increase in rifampin resistance was observed for *L. pneumophila* isolates from animals treated with rifampin alone. The gradient plate assays showed that the 13 bacterial isolates obtained from different animals treated with rifampin alone did not differ significantly in the number of rifampin-resistant colonies present. These studies were expanded by use of the quantitative determination of rifampin resistance frequency.

The first resistance frequency experiment showed that some isolates apparently contained significantly more rifampin-resistant mutants than did others. These differences were unlikely to have been detected by the gradient plate method, although the estimated range of resistance frequencies from the gradient plate experiments is identical to that found for the more accurate method. Since the isolates with the apparently greater resistance frequencies were obtained from animals treated with regimens other than rifampin alone, a second study was done with more isolates from these other groups. The second experiment showed similar heterogeneity but did not demonstrate any increase in resistance frequency for any antibiotic-treated group.

The apparently discrepant results between the first and second resistance frequency experiments and the significant differences among resistance frequencies for isolates within these experiments are to be expected (5, 12). This is because mutation frequency is not predictable by use of a Poisson distribution model and may occur at considerably higher or lower rates in repeated experiments. This nonadherence to a Poisson distribution was described in a classical paper by Luria and Delbrück, demonstrating that mutational events in bacteria are not adaptive ones but rather occur spontaneously and at random (12). Thus, the fluctuations in resistance frequency seen for the various isolates are simply a reflection of the randomness of rifampin resistance mutations. If the range of the resistance frequencies of bacterial isolates obtained from the saline-treated animals is taken as representative of these random events, a more accurate estimate of the fluctuations of natural resistance frequency is obtained. Ths range overlaps those observed for the resistance frequencies of *L. pneumophila* isolates obtained from animals treated with any of the antibiotic regimens studied (Fig. 1); this overlap is confirmed by the results of analysis of variance when the data are grouped by treatment type. Fluctuations in resistance frequency of non-antibiotic-exposed bacteria have been noted for other organisms such as *Mycobacterium tuberculosis* (5). The range of frequencies of rifampin resistance for most non-antimicrobial-agent-exposed populations of bacteria is of the same order of magnitude observed in this study, $10^{-7}$ to $10^{-8}$ (5, 14, 18, 25, 27). This range of resistance frequencies is also similar to that found by Moffie and Mouton for *L. pneumophila* (17).

There are several possible explanations for why rifampin resistance did not develop in this animal model. It may be because of the very low MICs of rifampin for *L. pneumophila*, because of the high intracellular concentration of the drug, wherein the bacteria reside, because of the short duration of therapy, because of very rapid decline in bacterial numbers in the lungs of rifampin-treated animals, or because of a combination of all of these factors. Some of these factors have probably been of importance for the development of rifampin resistance in other animal models of infection and for clinical trials with rifampin as prophylaxis or therapy (1, 3, 13, 19, 27). Regardless, it appears as if the resistance to rifampin by some bacteria has been uncommon in situations where the drug has been used extensively (2, 26).

The clinical implications of this study are uncertain because it is difficult to know how much can be extrapolated from this animal model to the treatment of human Legionnaires disease. Rifampin is currently used by some in combination with another antimicrobial agent for therapy of human Legionnaires disease (15). At least two failures of combined rifampin and erythromycin therapy in humans have been reported; posttherapy isolates from these patients did not become resistant to rifampin (16, 21). Levels of rifampin in sera and lungs in the guinea pigs used in this study were similar to those found in humans, but whether lung clearance of *L. pneumophila* is similar in rifampin-treated humans is unknown (7). Regardless, these results cast doubt on the general theory that solitary rifampin therapy promotes resistance to the antibiotic and demonstrates that such phenomena may be pathogen and therapy specific.

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