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Antibiotic Susceptibilities of Two *Coxiella burnetii* Isolates Implicated in Distinct Clinical Syndromes

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Antibiotic susceptibility testing of two isolates of the Q-fever agent, *Coxiella burnetii*, was performed with recently and persistently infected L929 fibroblast cells. The two genetically distinct isolates, Nine Mile and Priscilla, are implicated in two different clinical disease syndromes, acute and chronic Q fever, respectively. We compared the efficacies of rifampin, doxycycline, and five 4-quinolone compounds (ciprofloxacin, difloxacin, ofloxacin, norfloxacin, and pefloxacin) in reducing persistent *C. burnetii* infection of L929 fibroblasts. In persistently infected cells, the Priscilla isolate was less susceptible to all antibiotics tested when compared with the Nine Mile isolate. The most effective antibiotics against the Priscilla isolate were ofloxacin, pefloxacin, and ciprofloxacin (50% inhibitory concentrations of 0.5, 2.2, and 2.5 μg/ml, respectively). In persistently infected cells, the Nine Mile isolate was highly susceptible to all antibiotics tested except doxycycline. In contrast, the Priscilla and Nine Mile isolates in recently infected cells were somewhat susceptible to doxycycline; the Priscilla isolate was significantly more susceptible to ofloxacin and rifampin in recently infected host cells than in persistently infected cells. Persistently infected L929 cells were also treated with antibiotic combinations. Although ciprofloxacin and doxycycline had no synergistic effect on the Priscilla isolate, ciprofloxacin and rifampin acted synergistically. Collectively, these in vitro results are in accord with the fact that chronic Q fever in humans is generally not successfully managed with antibiotics. They also indicate that early diagnosis may be essential and that combination antibiotic therapy that includes quinolones may be effective in treating chronic Q fever.

Coxiella burnetii is an obligate intracellular procaryotic parasite of eucaryotic cells (2). This bacterium is the etiologic agent of Q fever, a disease that typically causes an acute, febrile illness (2, 4, 5). In addition to causing acute disease, *C. burnetii* occasionally causes life-threatening chronic, relapsing endocarditic infections in humans which may involve aortic and mitral valves (16, 22, 25, 26). These two distinct disease syndromes have been attributed to the condition of the patient (22, 24–26): noncompromised or compromised patients are, in general, observed to suffer from acute or chronic Q-fever syndrome, respectively (22, 24, 26). Although chronic Q fever occurs more frequently in compromised patients, it has been thought to be the result of patient predisposition rather than a particular characteristic of the pathogen causing the infection. However, recent studies have demonstrated that the *C. burnetii* isolates associated with acute and chronic Q fever are genetically distinct (21). The Priscilla isolate of *C. burnetii* was found to contain a plasmid (designated QpRS) 2 to 3 kilobases larger than the distinct plasmid (QpH1) found in the Nine Mile isolate (21). Examination of over 20 *C. burnetii* isolates has resulted in the discovery of three general groups, one containing the QpRS-type plasmid, a second containing the QpH1-type plasmid, and a third lacking plasmids. Based on the original source, it was noted that QpRS-containing and plasmidless isolates were associated with chronically infected patients and animals, and isolates containing the QpH1-type plasmid were derived from acutely infected patients and animals. Moreover, studies comparing the virulence of the Nine Mile (acute) and Priscilla (chronic) isolates of *C. burnetii* suggest that differences in the composition and antigenicity of their surface lipopolysaccharides may account for differences in pathogenicity (14).

Antibiotic management of acute Q fever is generally successful in noncompromised patients, whereas antibiotic management of chronic Q-fever endocarditis or hepatitis is poor at best (7, 11, 18, 22, 23, 25, 27). In light of these observations, we have previously demonstrated that rifampin and several 4-quinolone antibiotics (ciprofloxacin, difloxacin, and oxolinic acid) are highly effective in reducing persistent in vitro infections caused by Nine Mile *C. burnetii* (28). In this report we show that isolates implicated in distinct clinical syndromes—acute and chronic infection—exhibit differential susceptibility to antibiotics, are more susceptible to antibiotics in early stages of persistent infection (less than 30 days), and may be more successfully managed by using antibiotics in combination.

MATERIALS AND METHODS

Source and in vitro propagation of *C. burnetii*. The phase I *C. burnetii* Nine Mile isolate was originally obtained from M. Peacock of the Rocky Mountain Laboratory, Hamilton, Mont., and the Priscilla isolate was obtained from L. Mallavia, Washington State University, Pullman. The organisms were obtained in yolk sac homogenates and were propagated in L929 cells as previously described (20). To initiate recent infection, a 50- or 200-μl sample of 20% yolk sac homogenate heavily infected with either the Nine Mile or Priscilla isolate was added to 5.0 ml of L929 cells (2.5×10^5 ml^{-1}) held in 60-mm plastic tissue culture petri dishes. The infected cells were grown in antibiotic-free Eagle minimal essential medium at 37°C in a 10% CO_2 atmosphere. After 3 days, cells infected with the Nine Mile isolate were transferred to 50-ml screw-cap Erlenmeyer flasks (10 ml of cells) and incubated at 35°C in a rotary incubator (100 rpm). The cells were pas-

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saged three times a week. Cells infected with the Priscilla isolate were maintained in petri dishes; just before antibiotic testing, infected cells (10 ml) were transferred to 50-ml Erlenmeyer flasks and passaged two to three times before testing. Host cell viability was determined by the dye-exclusion technique (15).

Determination of degree of infection. The percentage of infected cells and the degree of infection were determined as previously described (28) by direct microscopic examination of cells stained by the Gimenez technique (8). A minimum of 300 cells were examined in each prepared slide to determine the percentage of the population that was infected (1 to 50 rickettsiae per cell) and heavily infected (>50 rickettsiae per cell). Photomicrographs were made of Gimenez-stained smears. Cells were prepared for transmission electron microscopy by standard techniques and procedures described previously (1).

Antibiotic preparation and use. Ciprofloxacin (Bayer, Leverkusen, Federal Republic of Germany) and difloxacin (A-56619; Abbott Laboratories, North Chicago, Ill.) were obtained in powder form. Ofloxacin (RU-43280; Roussel UCLAF, France) and pefloxacin (Laboratoire Roger Bellon S.A., France) were kindly provided by D. Raoult, Marseille, France. Doxycycline (hydrochloride) and rifampin were from Sigma Chemical Co., St. Louis, Mo. Stock solutions of each antibiotic were freshly prepared before they were added to cell cultures. Stock concentrations varied from 5.0 to 20.0 mg/ml. Solvents used in the stock antibiotic preparations included equal volumes of 95% ethanol and dimethyl sulfoxide (rifampin), 47.5% ethanol (pH 8.0; ciprofloxacin, difloxacin, ofloxacin, and pefloxacin), and 9.5% ethanol (doxycycline). At the concentrations used, the solvents were not toxic to normal or infected L929 cells. With the exception of rifampin, all antibiotic stock solutions were sterilized via filtration (pore size, 0.22 μm). The ethanol-dimethyl sulfoxide solvent used for preparing the rifampin stock solution was also an excellent autosterilizing agent. After sterilization, antibiotic stock solutions were stored at 4°C in approximately 4-ml fractions. An appropriate volume (5 to 10 μl) of diluted antibiotic stock solution was added aseptically to 10-ml cell cultures and at subsequent cell passages to maintain the same concentration of antibiotic throughout the test period. An equal volume of solvent with no antibiotic was added to control infected cultures.

Calculation of IC. IC_{50}s and IC_{90}s (50 and 90% inhibitory concentrations) for isolates tested were determined as previously described (28) by the graphic analytical methods of Goldstein (9) and are expressed in micrograms per milliliter. The ICs were determined by measuring the decrease in percentage of cells infected after 10 days of antibiotic treatment of the persistently infected L929 cells.

RESULTS

Antibiotics previously shown to be effective in controlling the Nine Mile isolate of *C. burnetii* within persistently infected L929 fibroblast cells were tested for their efficacies in the control of the Priscilla isolate of *C. burnetii*. For comparison, concurrent, duplicate cultures of both the Priscilla and Nine Mile isolates were maintained and tested. The L929 cells were persistently infected with either Nine Mile or Priscilla *C. burnetii* for a minimum of 400 days before testing. Antibiotic efficacies were determined by direct microscopic examination of Gimenez-stained cells and subsequently by calculating the percent reduction (or nonreduction) of infection. For each experimental and control infected flask, a total of 300 cells were examined each time the flasks were sampled. All experiments were performed with concurrent, duplicate cultures so that the comparisons made would be those observed under identical culture conditions. In all experiments performed, the viabilities of treated and control infected L929 cell populations were greater than 95%. The maximum antibiotic concentrations used were 5 μg/ml (the 4-quinolones), 1.0 μg/ml (rifampin), and 20 μg/ml (doxycycline). These experimental antibiotic concentrations were used because (with the exception of doxycycline) they had previously been shown to be effective in controlling the Nine Mile isolate (28). Previously we reported that doxycycline at a concentration of 10 μg/ml was only somewhat effective in reducing Nine Mile *C. burnetii* infection in L929 cells (28); therefore, a maximum concentration of 20 μg/ml was used in these studies. In addition, the concentrations of antibiotics used approach the upper range of physiological levels observed in vivo (10).

TABLE 1. Efficacies of antibiotics in the reduction of infection of L929 cells by Nine Mile and Priscilla isolates of *C. burnetii*[a]

Antibiotic	% of cells infected in experimental/control cultures			
	Day 0		Day 10	
	Nine Mile	Priscilla	Nine Mile	Priscilla
Doxycycline[b]	92/92	89/88	34/88	72/84
Rifampin[c]	90/92	93/92	4/88	54/88
Difloxacin	92/95	92/91	2/91	61/90
Ciprofloxacin	92/93	88/88	1/93	23/84
Pefloxacin	92/92	90/93	1/95	21/86
Norfloxacin	92/95	92/90	6/92	20/90
Ofloxacin	92/93	89/89	2/93	14/85

[a] Values represent average percentages of infected cells obtained from concurrent, duplicate experimental and control cultures, each having three independent sets of 100 cells counted at each sample time. Results with the quinolones are from experiments with the maximum concentration of 5 μg/ml. At the onset of these studies, L929 cells used were persistently infected for >1,000 days with the Nine Mile isolate or for >400 days with the Priscilla isolate.

[b] Results are from the maximum concentration tested (20 μg/ml).

[c] Results are from the maximum concentration tested (1 μg/ml).

Comparison of the effect of antibiotics on the Priscilla and Nine Mile isolates within persistently infected cells. The efficacies of all antibiotics tested in the control of both *C. burnetii* isolates are summarized in Table 1. As in our previous report (28), the 4-quinolone antibiotics ciprofloxacin and difloxacin were highly efficacious against the Nine Mile isolate. In addition, the 4-quinolone compounds ofloxacin, pefloxacin, and norfloxacin were highly effective against the Nine Mile isolate (IC_{90}s of 0.7, 1.4, and 4.6 μg/ml, respectively). Rifampin was the most efficacious drug against Nine Mile *C. burnetii*, having an IC_{90} lower than 0.1 μg/ml. Doxycycline produced only moderate reduction of infection even at 20 μg/ml. In contrast, the Priscilla isolate exhibited a significantly lower susceptibility to all antibiotics tested. The most effective drug in reducing Priscilla persistent infection was ofloxacin, followed distantly by pefloxacin, norfloxacin, and ciprofloxacin (IC_{50}s of 0.5, 2.2, 2.4, and 2.5 μg/ml, respectively). Difloxacin was the least effective quinolone antibiotic against the Priscilla isolate, achieving neither 50 nor 90% inhibition at the maximum concentration tested. Neither rifampin nor doxycycline treatment of Priscilla isolate-infected cells resulted in 50% reduction of infection at the maximum concentrations tested (reductions of 40 and 19%, respectively). No antibiotic tested caused a 90% reduction of the Priscilla isolate; IC_{90}s

TABLE 2. IC_{50}s and IC_{90}s obtained for antibiotics in the reduction of infection of L929 cells by the Nine Mile and Priscilla isolates of *C. burnetii*[a]

Antibiotic	IC (μg/ml) for *C. burnetii* isolate			
	Nine Mile		Priscilla	
	50%	90%	50%	90%
Doxycycline	9.5	NA[b]	NA	NA
Rifampin	0.08	0.3	NA	NA
Difloxacin	0.5	1.6	NA	NA
Ciprofloxacin	0.5	1.7	2.5	NA
Pefloxacin	0.6	1.4	2.2	NA
Ofloxacin	0.3	0.7	0.5	NA
Norfloxacin	1.1	4.6	2.4	NA

[a] At the onset of these studies, L929 cells used were persistently infected for >1,000 days with the Nine Mile or for >400 days with the Priscilla isolate.
[b] NA, Inhibition not achieved.

of antibiotics against the Priscilla agent are therefore stated as being not achievable with the maximum antibiotic concentration tested. A summary of the IC_{50}s and IC_{90}s of all antibiotics tested against the two persistently infecting *C. burnetii* isolates is shown in Table 2. Clearly, the Priscilla isolate is much less susceptible to antibiotics that are highly effective in reducing the level of persistent infection caused by the Nine Mile isolate. Results from this study lend support to our previous report (28) that, at the concentrations tested, the quinolone antibiotics are apparently rickettsicidal. This can be concluded from the fact that the reductions in percentage of infection observed within quinolone-treated persistently infected cells occurred at rates that exceed the rates of reduction that would have been observed in the case of mere dilution of statically inhibited rickettsiae through the process of host cell division.

Comparison of the effect of antibiotics on the Priscilla and Nine Mile isolates within recently infected cells. L929 cells recently infected (22 days) with either the Priscilla or Nine Mile isolate of *C. burnetii* were exposed to antibiotics to determine whether they exhibited antibiotic susceptibilities that were different from those of their counterparts in persistently infected cells. Our results (Table 3) show that both *C. burnetii* isolates in recently infected cells (<30 days) were significantly more susceptible to ofloxacin, rifampin,

TABLE 3. Comparison of drug efficacies in the elimination of the Priscilla or Nine Mile isolate of *C. burnetii* from recently and persistently infected L929 cells

C. burnetii isolate and antibiotic	% of cells infected in experimental/control cultures[a]	
	Recently infected (22 days)	Persistently infected (≥400 days)
Priscilla		
Ofloxacin (5 μg/ml)	15/88	35/90
Rifampin (1 μg/ml)	14/90	53/88
Doxycycline (10 μg/ml)	40/88	86/89
Nine Mile		
Ofloxacin (5 μg/ml)	2/92	2/91
Rifampin (1 μg/ml)	1/90	2/90
Doxycycline (10 μg/ml)	20/90	56/91

[a] Values are mean results compiled from duplicate experiments, each of which was sampled independently in triplicate. Experimental cultures were exposed to antibiotics for 10 days; control cultures were exposed to corresponding antibiotic solvents only for 10 days.

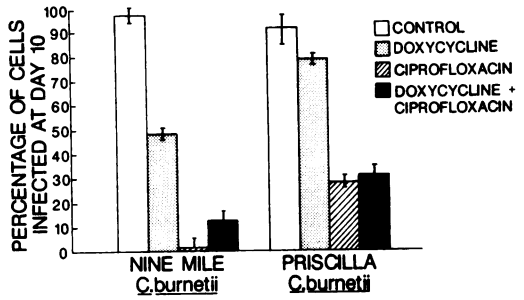

FIG. 1. Effect of doxycycline (10 μg/ml) and ciprofloxacin (5 μg/ml), alone and in combination, on L929 cells persistently infected with either Nine Mile or Priscilla *C. burnetii*. All cultures were greater than 90% infected on day 0. At day 0 cultures had been persistently infected with the Priscilla or Nine Mile isolate for 618 or 1,448 days, respectively.

and even doxycycline than were their persistently infecting counterparts (>400 days); this was especially true of the Priscilla isolate.

Combination antibiotic treatment. Antibiotics having different targets were paired for experimentation aimed at revealing possible synergy in the control of persistent infection of L929 cells by both isolates of *C. burnetii*. The antibiotics used had been previously demonstrated to be at least somewhat effective in controlling the Nine Mile *C. burnetii* isolate in vitro (28). Ciprofloxacin-rifampin (10:1) and ciprofloxacin-doxycycline (1:2) were used in combination at concentrations that were previously shown to be effective (28). The combination of doxycycline and ciprofloxacin did not result in synergy when tested against the Priscilla isolate (Fig. 1). However, when ciprofloxacin and rifampin were combined (10:1), synergistic effects were seen in the reduction of infection caused by the Priscilla isolate (Fig. 2 and 3). Independently, ciprofloxacin and rifampin resulted in the reduction of Priscilla-infected cells by 36 and 10%, respectively; in combination, however, they caused a 91% reduction. None of the antibiotic combinations produced synergistic effects on the Nine Mile isolate. In fact, doxycycline and rifampin apparently delayed the effect of ciprofloxacin during the first 5 days of treatment (data not shown).

DISCUSSION

C. burnetii, the etiologic agent of Q fever, was until recently thought to be monogenotypic: acute and chronic

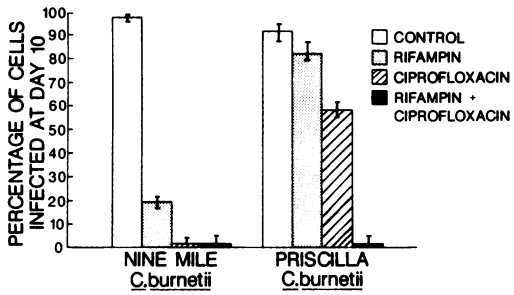

FIG. 2. Effect of rifampin (0.5 μg/ml) and ciprofloxacin (5 μg/ml), alone and in combination, on L929 cells persistently infected with either Nine Mile or Priscilla *C. burnetii*. All cultures were greater than 90% infected on day 0. At day 0 cultures had been persistently infected with the Priscilla or Nine Mile isolate for 534 or 1,376 days, respectively.

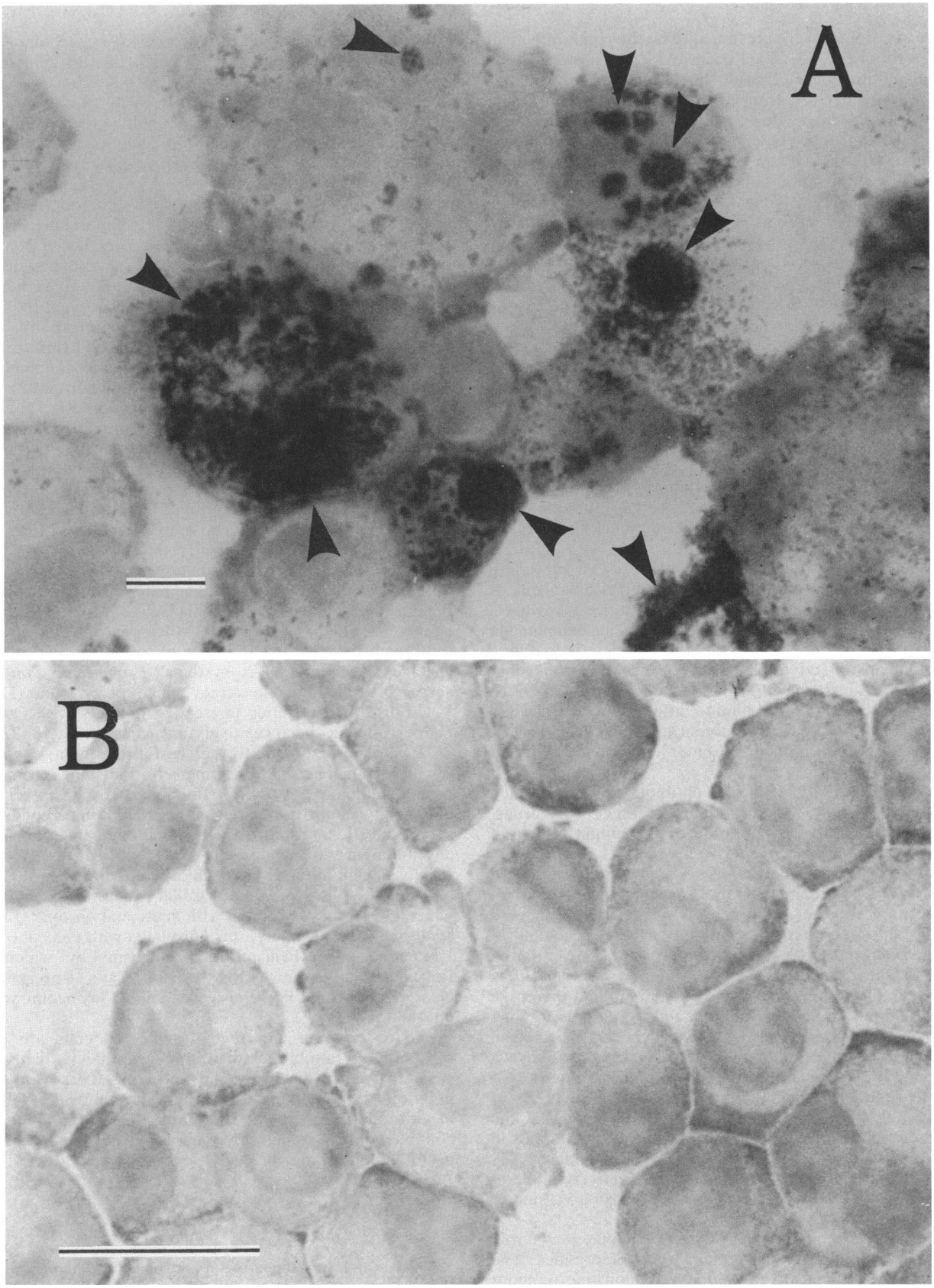

FIG. 3. Photomicrographs illustrating the effect of combination antibiotic treatment on cells persistently infected with Priscilla *C. burnetii*. Heavily infected L929 cells (A) showed little or no sign of infection after 10 days of treatment (B) with rifampin (0.5 μg/ml) and ciprofloxacin (5 μg/ml). On the first day of treatment (day 0), the cell populations had been persistently infected for 534 days. Arrowheads point to concentrations of *C. burnetii* (hundreds of organisms). Bars, 5 μm.

disease syndromes were presumed to be the result of patient predisposition (22, 25, 27). Recent evidence shows distinct plasmid and lipopolysaccharide differences between isolates of *C. burnetii* derived from animals and humans (14, 21). Other workers have demonstrated the Nine Mile and Priscilla isolates to be equally infectious but different in their abilities to induce fever in laboratory animals (14). Collectively, these data support the hypothesis (21) that different isolates may be implicated in different disease manifestations such as acute or chronic Q fever. Our current studies indicate that, in addition to exhibiting genotypic and surface antigenic differences, the two *C. burnetii* isolates under investigation, Nine Mile and Priscilla, also exhibit differences in antibiotic susceptibility. The observation that the Nine Mile isolate, implicated as a cause of acute Q fever, was highly susceptible to a broad range of antibiotics correlates with the apparent clinical success observed in the treatment of primary acute Q fever (28). In contrast, the Priscilla isolate, implicated as a cause of chronic Q fever in goats (21)—and possibly humans—exhibited significant resistance to all antibiotics that were effective against the Nine Mile isolate. This observation is in agreement with the fact that chronic Q fever in humans, including Q-fever endocarditis, is not successfully managed with either antibiotic treatment or a combination of antibiotic treatment and surgical procedures (7, 18, 22, 24, 26, 27).

The underlying causes of the antibiotic susceptibility differences between the Nine Mile and Priscilla isolates observed in these studies remain to be determined; however, there are several possibilities that are now being examined in our laboratory. There is no common characteristic of the drugs that are effective against the Nine Mile isolate but ineffective against the Priscilla isolate; doxycycline, rifampin, and the quinolones bind to different targets, directly affect different processes, are not strikingly similar in structure or charge, and appear to enter bacteria by different means.

Decreased susceptibility to the antibiotics by the Priscilla isolate may be due to an alteration in the target of the drugs. For example, a mutation within the alpha subunit protein of DNA gyrase (topoisomerase II) could result in decreased binding of the quinolone antibiotics and thus a decreased inhibition of DNA-related metabolism. A similar explanation may account for the resistance of the Priscilla isolate to rifampin, due to a possible alteration of the beta subunit of the core bacterial RNA polymerase enzyme (13). Mutation within the gene encoding the beta subunit may lead to a decreased affinity for the binding of rifampin and thereby a lessened inhibitory effect. Rifampin and the quinolones may be especially susceptible to resistance via this mechanism due to their very specific binding affinities. In our studies we found that the Priscilla isolate exhibited an increased resistance to both the 4-quinolones and rifampin. In terms of statistical probability, it would be very unlikely that the Priscilla isolate concurrently possesses distinct mutations in the genes encoding DNA gyrase and DNA-dependent RNA polymerase (alpha and beta subunits, respectively). Therefore, it is likely that these antibiotics are resisted via one or more other mechanisms.

The unique plasmid in the Priscilla isolate may play a role in resistance; however, that remains to be determined. The plasmid may encode enzymes that act upon one or more antibiotics, resulting in their inactivation. However, the broad-scale differences in antibiotic susceptibilities between the Nine Mile and Priscilla isolates would suggest that this possible enzyme-mediated antibiotic inactivation is not the sole mechanism by which the Priscilla isolate may resist antibiotics. Another means by which the Priscilla isolate may resist antibiotics is through an alteration of its cell cycle, resulting in a prolonged growth rate and a less pronounced effect than that on rapidly growing cells. In addition, because of rapid half-life decay of many antibiotics, the chance of inhibiting sensitive processes in slowly growing organisms is diminished.

Because of the broad-scale nature of antibiotic resistance in the Priscilla isolate and the fact that the unique Priscilla plasmid possesses only 2 to 3 kilobase pairs more DNA than does the Nine Mile plasmid, it is unlikely that several distinct resistance mechanisms are encoded within its plasmid. Another explanation for the general resistance to antibiotics in the Priscilla isolate may be decreased permeability. Nalidixic acid, tetracyclines, aminoglycosides, and various other antibiotics are often resisted by bacteria whose membrane structure has a lessened permeability to one or more antibiotics (3, 6, 17). It is reasonable to hypothesize that the Priscilla isolate may possess such a modification of its membrane structure (outer membrane, inner membrane, or both). Furthermore, being an obligate intracellular parasite, *C. burnetii* may present an additional problem to successful antibiotic treatment by exploiting the cell membrane of its host as an additional barrier to antibiotic entry. It has been demonstrated that cells infected with *C. burnetii* and other rickettsiae have modified cell membranes (12, 19). A related possibility for antibiotic resistance may result from a change in permeability within the Priscilla isolate, which may lead to an increased efflux of antibiotics when concentrations within the organism reach levels sufficient to induce one or more specific antibiotic export systems. *Escherichia coli* possesses such an inducible tetracycline export system (3).

The use of antibiotics in treating patients with Q-fever endocarditis has been relatively unsuccessful (7, 18, 22, 24, 26, 27). Even in apparently successful treatment, cessation of conventional antibiotic regimens (tetracyclines, trimethoprim-sulfamethoxazole, etc.) frequently results in relapse, eventually leading to a variety of life-threatening conditions such as aortic and mitral valvular incompetence, hepatitis, and pulmonary incapacitation. It is clear from our in vitro results that differences in antibiotic susceptibility do exist between the Nine Mile and Priscilla isolates of *C. burnetii*, which have been associated with acute and chronic Q-fever syndromes, respectively; this correlates with clinical observations. The mechanism or mechanisms by which the Priscilla isolate achieves this resistance are at present unclear; further investigation is essential and is ongoing within our laboratory.

The observation that recently infected cells are more susceptible to antibiotics than long-term-infected cells suggests that early diagnosis and antibiotic therapy may be important in preventing subsequent chronic disease. These in vitro data also indicate that quinolones or quinolones in combination with other antibiotics such as rifampin may be useful in controlling chronic Q fever.

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