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In Vitro Susceptibilities of *Bartonella henselae*, *B. quintana*, *B. elizabethae*, *Rickettsia rickettsii*, *R. conorii*, *R. akari*, and *R. prowazekii* to Macrolide Antibiotics as Determined by Immunofluorescent-Antibody Analysis of Infected Vero Cell Monolayers

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The in vitro susceptibilities of *Bartonella* (*Rochalimaea*) *henselae*, *B. quintana*, *B. elizabethae*, *Rickettsia akari*, *R. conorii*, *R. prowazekii*, and *R. rickettsii* to different concentrations of azithromycin, clarithromycin, dirithromycin, erythromycin, and roxithromycin in Vero cell cultures were evaluated. *Bartonella* and *Rickettsia* spp. were allowed to initiate infection of the antibiotic-free Vero cell monolayers, which were maintained in 16-chamber microscope slides in the absence of antibiotics at 32°C in a CO_2-enriched atmosphere. The monolayers were then incubated for 3 h to allow for initial host cell intracellular penetration by infecting species. After inoculation, inocula were replaced and tested with media containing 12 different concentrations of each antibiotic in replicate (10 wells of each antibiotic dilution) for each species, and the monolayers were reincubated. Tetracycline served as the control. Growth status of *Bartonella* spp. and *Rickettsia* spp. was determined by evaluation of immunofluorescent staining bacilli. Five days later, when antibiotic-free, control-infected cell monolayers demonstrated significant fluorescence, media were removed for all cell monolayers, the monolayers were fixed, and all specimens were stained with standard indirect immunofluorescent antibody reagents. Fluorescent foci were enumerated by counting such foci on random fields visualized with an epifluorescence microscope. The extent of antibiotic-induced focus inhibition was recorded for each dilution of antibiotic and compared with that of an antibiotic-negative control. Effective antibiotic dilution endpoints for inhibition of *Bartonella* and *Rickettsia* proliferation, as judged by absence of increase of significant fluorescence (as compared with no-growth controls), were enumerated by determining the number of cell culture chambers at various antibiotic dilutions that were negative or positive for significant *Bartonella*- or *Rickettsia*-specific fluorescence. All of the macrolide agents tested were readily active against all three *Bartonella* organisms, and azithromycin, clarithromycin, and roxithromycin may have potential in the treatment of *Rickettsia* infections. Animal model-based clinical trials are warranted to define the specific treatment role of the newer macrolide antibiotics.

Bartonella (formerly *Rochalimaea*) *henselae* and *B. quintana*, fastidious gram-negative bacilli, are the causative agents of bacillary angiomatosis, bacillary peliosis hepatitis, and recurrent febrile disease, which occur primarily among immunocompromised patients (21, 38, 42). *B. henselae* is also the etiologic agent of cat scratch disease, another illness that occurs primarily in patients who are immunocompetent (2, 9). *B. elizabethae* has been isolated from an immunocompetent patient with infective endocarditis (8). All members of the genus *Rickettsia* are obligate intracellular bacteria. Human pathogenic rickettsial diseases have zoonotic animal reservoirs and are transmitted by arthropods, although typhus fever may be maintained in humans without an alternative vertebrate reservoir. Within the spotted fever group of rickettsia, *Rickettsia rickettsii* and *R. conorii* are the etiologic agents of Rocky Mountain spotted fever (RMSF) and Mediterranean spotted fever (MSF), respectively. *R. akari*, the agent of rickettsialpox, is also identified with the spotted fever group; however, it is taxonomically distinct from other members of this group. Of the typhus group, *R. prowazekii* causes typhus fever (also known as Brill-Zinsser disease).

Tetracycline products (e.g., tetracycline, doxycycline, and minocycline) and chloramphenicol are effective for rickettsial infections. Adverse effects from these agents may limit or contraindicate their use in certain patient populations (e.g., children under 12, pregnant women, patients with hematologic disorders). Chloramphenicol has been associated with a higher rate of mortality in patients with RMSF or MSF than has tetracycline (11, 40). While studies have assessed the activity of these antimicrobial agents against several of the rickettsial species (10, 25, 30, 34–36, 43), none have examined intracellular antibiotic action, which is thought to be more relevant to anti-rickettsial pharmacotherapy (33). Among immunocompromised patients with *B. henselae* or *B. quintana* infections, prolonged treatment with erythromycin or tetracycline appears to be effective; however, individuals must be monitored carefully for relapse at the cessation of treatment, and these antibiotics may not be universally effective against the infections (1, 22). Reports of in vitro susceptibility of these organisms to various antibiotics are limited and diverse in approach (9, 26,

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27, 31, 32). The clinical efficacy of pharmacotherapy for cat scratch disease has remained equivocal for years (23).

This investigation evaluated the in vitro susceptibility of *B. henselae*, *B. quintana*, *B. elizabethae*, *R. akari*, *R. conorii*, *R. prowazekii*, and *R. rickettsii* to different concentrations of azithromycin, clarithromycin, dirithromycin, erythromycin, and roxithromycin, in Vero cell culture chamber slides, as determined by the use of an immunofluorescent antibody technique to evaluate the inhibition of *Bartonella* and *Rickettsia* proliferation.

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MATERIALS AND METHODS

Bacterial strains and cell culture preparation. The following seed stocks were obtained from the American Type Culture Collection, Rockville, Md.: *B. henselae* (Houston-1 isolate, ATCC 49882), *B. elizabethae* F9251 (ATCC 49927), *Rickettsia akari* (ATCC VR-612), *R. conorii* (ATCC VR-613), *R. prowazekii* (ATCC VR-233), and *R. rickettsii* (ATCC VR-149). *B. quintana* (OK 90-268) was an isolate from Oklahoma (42).

Bartonella spp. were plated on 5% rabbit blood heart infusion agar. After a suitable number of colonies were obtained, they were harvested with brain heart infusion (BHI) broth (BBL, Becton Dickinson and Co., Cockeysville, Md.), aliquoted in plastic cryule vials, quick frozen, and stored at −80°C. Before testing, the three *Bartonella* inocula were filtered through a 1.2-μm-pore-size filter (Sigma Chemical Co., St. Louis, Mo.) to minimize possible artifacts due to multiple organisms being associated with host cell fragments. Following filtration, the organisms were diluted in BHI broth such that 20 to 30 bacilli were observed on each microscope field of a Vero cell monolayer 2 h after inoculation.

Rickettsia spp. were grown in Vero cells and passed through a 1.2-μm-pore-size filter. On the basis of preliminary tests, dilutions of rickettsiae were cultured in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, and 2% fetal bovine serum; such a culture usually produces 5 to 10 rickettsial focus-forming units for each microscopic field examined.

Antibiotic preparation. Five different coded macrolide antibiotics (azithromycin [Pfizer Labs, Groton, Conn.], clarithromycin and erythromycin [Abbott Laboratories, North Chicago, Ill.], dirithromycin [Eli Lilly, Indianapolis, Ind.], and roxithromycin [Roussel Uclaf, Romainville, France]) and a nonantibiotic placebo (i.e., lactose) were prepared and tested with each species in a blinded fashion by study investigators. Tetracycline HCl (Abbott Laboratories, North Chicago, Ill.) served as a coded, positive antibiotic control for inhibition in each trial. After the antibiotics were dispensed in 1-ml aliquots, they were quick frozen and stored at −80°C. The stock concentration of each antibiotic was 32 μg/ml.

A coded control utilizing media but without any antibiotic (i.e., lactose) was used to provide reference numbers of foci, and a noncoded negative control (i.e., without antibiotics) was also included. After dissolution in the appropriate respective solvents, antibiotics were prepared in 1-ml aliquots at a starting concentration of 32 μg/ml. All were quick frozen and stored at −80°C. Additional noncoded positive controls for antibiotic inhibition and species growth were made with tetracycline HCl at the same concentrations as the other antibiotics, as well as media without any added drugs. Immediately before testing was initiated, the frozen drug samples were thawed and dilutions of the drugs were made with MEM–2% fetal bovine serum, as noted above.

Immunofluorescent assay testing. For each *Bartonella* sp., 12 different concentrations (0.0156, 0.031, 0.062, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml) of each antibiotic were tested 10 times with each species. Following careful aspiration of the Vero cell supernatant medium (so as to not disrupt the confluent cell monolayers), each well of the chamber slides was inoculated with 0.25 ml of the diluted *Bartonella*-containing medium. Then, 0.25 ml of *Bartonella* inoculum was added to the wells of the chamber slides, and the slides were incubated at 35°C in a CO_2-enriched environment for 2 h, allowing time for the *Bartonella* species to establish possible attachment and entry to the host cells (3). At the end of the initial incubation period, the inocula were replaced by 0.25 ml of the antibiotic dilutions and incubated again, this time in 5% CO_2 for 5 days.

For *Rickettsia* spp., antibiotic-free Vero cells (0.25 ml of a 1.5×10^5 solution) were seeded in the wells of 16-chamber culture microscope slides (Lab-Tek; NUN Inc., Naperville, Ill.) and incubated at 37°C until confluent at three days. Slides were incubated at either 35°C for *R. prowazekii* or 32°C for the remaining species in a 5% CO_2-enriched atmosphere for 3 h, thus permitting sufficient time for rickettsiae to penetrate the cytoplasm of the host Vero cells. Inocula were carefully removed by aspiration and the monolayers were washed twice to remove rickettsiae that were partially attached or not internalized within the Vero cells. Infected monolayers were then replenished with medium containing either 8 (0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml for *R. akari*, *R. conorii*, and *R. rickettsii*) or 12 (0.015, 0.312, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml for *R. prowazekii*) different antibiotic concentrations and incubated as before at the required temperatures. Two 16-well slides were prepared for each agent, including controls, and each antibiotic dilution was tested four times.

When the noncoded, nonantibiotic control-infected cells (i.e., noncoded placebo) showed well-formed fluorescent focus formation, media were removed from all of the infected and antibiotic-treated monolayers, and the cell culture monolayers were fixed with methanol. An indirect immunofluorescence assay, using human anti-*Bartonella* sp. antibody as a primary antibody and goat anti-human immunoglobulin G (IgG) fluorescein-labeled antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) as a secondary antibody, was used to stain *Bartonella* spp. When the noncoded, nonantibiotic control-infected cells (i.e., noncoded placebo) showed well-formed *Bartonella* fluorescent focus formation, media were removed from all of the infected and antibiotic-treated monolayers, and the cell culture monolayers were fixed with methanol.

To determine the inhibition of *Bartonella* proliferation, foci from all coded specimens were stained by an indirect immunofluorescent antibody method. IgG antibodies, derived from high-titered rabbit antisera to gamma-irradiated whole *Bartonella* cells, were used as primary antibody, and fluorescein-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Inc.) was used as a secondary signal antibody. Fluorescein antibody staining produced easily visible distinct foci without background staining and with immune reagent specificity. *Bartonella* cells were harvested from the surface of commercial blood agar plates (rabbit blood heart infusion agar; BBL) and washed twice.

To determine the inhibition of *Rickettsia* proliferation, rickettsial foci from all coded specimens were stained by an indirect immunofluorescent antibody method. IgG antibodies, derived from high-titered rabbit antisera to gamma-irradiated whole *Bartonella* cells, were used as the primary antibody, and fluorescein-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Inc.) was used as a secondary signal antibody. The rabbit antibody was raised in rabbits after the rickettsiae were purified on a 66% diatrizoate meglumine and 10% diatrizoate sodium (Renografin-76; Squibb Diagnostics) isopycnic gradient to help free them from Vero host cell debris.

All of the organisms were inactivated by Co^{60} irradiation (500,000 rads) prior to immunization. *Bartonella* and *Rickettsia* antigens, sans adjuvant, were given to rabbits, and the rabbits were immunized subcutaneously on two occasions prior to serum collection, all according to an approved Centers for Disease Control and Prevention Animal Care and Use Committee protocol. The actual titers of primary antisera or fluorescein-conjugated goat anti-human IgG were determined by titration of the reagents to endpoint titers with known positive samples, then with a dilution approximately two dilutions less than the endpoint as a working dilution. Incubations of the primary and secondary antibodies were for 30 min each, with three 5-min washes with phosphate-buffered saline and a single quick dip in distilled water prior to air drying.

A Zeiss Axioskope epifluorescence microscope (Carl Zeiss, Inc., Thornwood, N.Y.) equipped with a 40× objective lens was used to determine organism infection or inhibition in each well, and the findings were compared with those for controls. The number of immunofluorescent foci (i.e., clusters of multiple rickettsiae in one or more adjacent cells in the cell culture) in 25 random fields was determined for each well. Foci in each of the four wells belonging to each antibiotic dilution were counted, and the mean for fluorescent foci per 50 fields was determined. As compared to the antibiotic-negative control, the extent of antibiotic-induced focus inhibition was recorded for each dilution of antibiotic. The 10 readings of each drug dilution were averaged, and the minimal antibiotic concentration which completely inhibited growth after 5 days of incubation served as the MIC.

RESULTS

All of the agents tested in this investigation exhibited activity against all three *Bartonella* species at concentrations consistent with those achieved in human serum, with the exception of dirithromycin against *B. quintana*.

The MICs of some of the newer macrolide antimicrobial agents for the three species of *Bartonella* and representative rickettsiae are summarized in Table 1. The MICs obtained with *Rickettsia* spp. are consistent with those previously obtained via plaque and dye uptake assays (10, 25, 35, 45). Based upon this in vitro evaluation, azithromycin and clarithromycin may have potential as an alternative to tetracycline and chloramphenicol in the treatment of RMSF, and clarithromycin may be appropriate for *R. conorii* infections (i.e., MSF), especially in children and pregnant women. Azithromycin, clarithromycin, and roxithromycin appear to have potential for use in the treatment of *R. akari* and *R. prowazekii* infections.

TABLE 1. MICs

Organism	MIC (μg/ml)				
	Azithromycin	Clarithromycin	Dirithromycin	Erythromycin	Roxithromycin
B. henselae	0.0156	0.031	0.125	0.125	0.125
B. quintana	0.0312	0.0622	1.0	0.25	0.25
B. elizabethae	0.0249	0.0687	0.150	0.75	0.0625
R. akari	0.25	2	16	16	8
R. conorii	16	4	16	8	16
R. prowazekii	0.25	0.125	16	2	1
R. rickettsii	8	8	>32	16	16

As seen in the peak serum antibacterial concentration (C_{max})/MIC ratio, the pharmacokinetic parameter which serves as one positive outcome predictor of activity (see Table 2 for determinations; C_{max} was determined from human studies with multiple oral doses) (5–7, 12, 41), on this basis alone, azithromycin, clarithromycin, and roxithromycin may have the greatest potential activity against *Bartonella* spp.; however, the C_{max}/MIC ratio is based upon the use of extracellular concentrations. Based upon the use of this ratio, clarithromycin and roxithromycin have the highest potential activity against *R. rickettsii* (C_{max}/MIC = 0.35 and 0.43, respectively) and *R. conorii* (0.7 and 0.43, respectively) of all of the macrolides tested; however, these ratios may be too low to indicate clinical usefulness. Azithromycin and clarithromycin appear to have the greatest activity against *R. akari*, with azithromycin, clarithromycin, erythromycin, and roxithromycin most effective against *R. prowazekii.*

Another pharmacokinetic parameter to assess antimicrobial therapeutic outcomes is the area-under-the-plasma drug concentration versus time curve (AUC)/MIC ratio. When human AUC data are used, the results are similar to those found with C_{max} data (Table 3) (5, 6, 12, 41). The assumptions that must be acknowledged are also similar to those in the C_{max}/MIC model. When only the AUC/MIC ratio data are used, roxithromycin, clarithromycin, and azithromycin appear to be the best agents for all three *Bartonella* species. The AUC/MIC ratio data indicate that clarithromycin and roxithromycin may have the best activity against *R. conorii*, *R. prowazekii*, and *R. rickettsii*. Azithromycin, clarithromycin, and roxithromycin appear to be potentially effective against *R. akari.*

DISCUSSION

Traditional in vitro studies to determine antibiotic activity are performed by assessing the extracellular medium supernatant concentration to determine a MIC. The C_{max}/MIC or AUC/MIC ratio may then be used as an indicator of in vivo activity. It must be assumed the antibiotic can achieve required inhibitory concentrations at the site of infection. When treating intraphagocytic bacterial infections such as those caused by *Bartonella* spp. and *Rickettsia* spp., an additional barrier in the form of the phagocytic cell membrane must be breached in order to eradicate the infection. Common antibiotics such as penicillins, cephalosporins, and aminoglycosides do not effectively penetrate this phagocytic membrane, while fluoroquinolones, tetracyclines, and macrolides do penetrate. The same antibiotic may achieve different concentrations within different phagocytic cell types (16, 17, 39), but this intracellular antibiotic concentration is only one of the many important variables contributing to the efficiency of bacterial killing. Indirect effects that may increase the efficiency of bacterial killing can occur when the antibiotic interacts with the phagocyte through mechanisms that increase the efficiency of phagocytic killing (14, 18). Other variables affecting membrane penetration are pH (20), temperature (10, 19, 25), bacteria (13, 17, 28, 37, 39), and extracellular calcium concentrations (29).

Both erythromycin and doxycycline, the traditional pharmacotherapies used to treat *Bartonella* infections in immunocompetent patients, achieve good membrane penetration and concentrate within the cytoplasm of phagocytic cells. These agents readily enter macrophages and leukocytes and achieve high intracellular concentrations; however, different species of *Bartonella* may behave differently within the endothelial cell or other cell types. Traditionally, tetracycline or chloramphenicol has been used to treat rickettsial infections. Both antibiotics achieve good membrane penetration and concentrate within the cytoplasm of phagocytic cells. Macrolides, especially the newer agents, also penetrate eukaryotic membranes and achieve high intracellular concentrations (4, 15, 20). However, different species of rickettsiae behave differently within the endothelial cell. Within the first 10 h of infection, *R. rickettsii* organisms multiply and spread with apparent ease from host cell to host cell without immediate host cell lysis (19, 44). Because a delay in initiation of antirickettsial pharmacotherapy is associated with an increase in mortality (11, 34), antimicrobial agents with a more rapid penetration rate and a higher intracellular concentration may provide an added therapeutic advantage. Other species of rickettsiae grow and accumulate within the original infected cell, do not cross host cell

TABLE 2. C_{max}/MIC ratios

Organism	Ratio (C_{max} [μg/ml]/MIC [μg/ml])[a]				
	Azithromycin (12)[b]	Clarithromycin (6)	Dirithromycin (41)	Erythromycin (7)	Roxithromycin (5)
B. henselae	26.3 (0.41/0.0156)	91.0 (2.82/0.031)	3.84 (0.48/0.125)	24.0 (3.0/0.125)	55.2 (6.9/0.125)
B. quintana	13.14 (0.41/0.0312)	45.34 (2.82/0.0622)	0.48 (0.48/1.0)	12.0 (3.0/0.25)	27.6 (6.9/0.25)
B. elizabethae	16.47 (0.41/0.0249)	41.04 (2.82/0.0687)	3.21 (0.48/0.15)	4.0 (3.0/0.75)	110.4 (6.9/0.0625)
R. akari	1.64 (0.41/0.25)	1.41 (2.82/2)	0.03 (0.48/16)	0.19 (3.0/16)	0.86 (6.9/8)
R. conorii	0.03 (0.41/16)	0.7 (2.82/4)	0.03 (0.48/16)	0.19 (3.0/16)	0.43 (6.9/16)
R. prowazekii	1.64 (0.41/0.25)	22.56 (2.82/0.125)	0.03 (0.48/16)	1.5 (3.0/2)	6.9 (6.9/1)
R. rickettsii	0.05 (0.41/8)	0.35 (2.82/8)	0.01 (0.48/32)	0.19 (3.0/16)	0.43 (6.9/16)

[a] All drugs were used at a dose of 500 mg except roxithromycin (150 mg).
[b] Reference from which C_{max} data were derived.

TABLE 3. AUC/MIC ratios

Organism	Ratio (AUC [μg · h/ml]/MIC [μg/ml])[a]				
	Azithromycin (12)[b]	Clarithromycin (6)	Dirithromycin (41)	Erythromycin (7)	Roxithromycin (5)
B. henselae	205.13 (3.2/0.0156)	219.35 (6.8/0.031)	26.96 (3.37/0.125)	86.4 (10.8/0.125)	453.6 (56.7/0.125)
B. quintana	102.56 (3.2/0.0312)	109.32 (6.8/0.0622)	3.37 (3.37/1.0)	43.2 (10.8/0.25)	226.8 (56.7/0.25)
B. elizabethae	128.51 (3.2/0.0249)	98.98 (6.8/0.0687)	22.47 (3.37/0.15)	14.4 (10.8/0.75)	907.2 (56.7/0.0625)
R. akari	12.8 (3.2/0.25)	3.4 (6.8/2)	0.21 (3.37/16)	0.67 (10.8/16)	7.09 (56.7/8)
R. conorii	0.2 (3.2/16)	1.7 (6.8/4)	0.21 (3.37/16)	0.67 (10.8/16)	3.54 (56.7/16)
R. prowazekii	12.8 (3.2/0.25)	54.4 (6.8/0.125)	0.21 (3.37/16)	5.4 (10.8/2)	56.7 (56.7/1)
R. rickettsii	0.4 (3.2/8)	0.85 (6.8/8)	0.1 (3.37/32)	0.67 (10.8/16)	3.54 (56.7/16)

[a] See Table 2, footnote *a*.
[b] Reference from which AUC data were derived.

membranes readily, and spread to other cells, presumably as the architecture of the host cell disintegrates; therefore, the antibiotic penetration rate into the infected cell may be important for rapid containment of certain rickettsial infections. In this investigation, the rickettsiae were able to internalize within the host cells prior to antibiotic administration, thus providing a discrimination between extracellular and intracellular antimicrobial activity. Further, studies that document the achieved peak antibiotic intracellular concentration and the rate of that achievement (i.e., intracellular AUC/MIC ratio) may help to determine the optimum dosing schedule necessary to maintain intracellular MIC required for clinical efficacy.

This in vitro model for antimicrobial activity was based upon the ability of the antibiotic to inhibit *Bartonella* growth in a cell culture environment. Therefore, this model is significantly different from another in vitro model, which investigated the ability of single antibiotic dilutions to render *Bartonella* noninfectious (31). These results were consistent with those previously obtained via plaque and dye uptake assays.

On the basis of this in vitro evaluation, clarithromycin, roxithromycin, and azithromycin may have potential as an alternative to tetracycline and chloramphenicol in the treatment of infections caused by *R. akari* or *R. prowazekii*, cat scratch disease or bacillary angiomatosis, especially in children or during pregnancy. Additionally, azithromycin and clarithromycin may have potential use in the treatment of RMSF. As only one isolate of each species was tested, subsequent studies that employ more isolates may be needed to clarify further the overall efficacy of these macrolide agents. However, several of the antibiotics tested have similar, rickettsial genus-wide activity, suggesting that differences between isolates of a given species would be expected to be minimal with *Rickettsia* spp. Animal models to evaluate antimicrobial pharmacotherapy have proven unreliable (24). The definitive duration of antimicrobial pharmacotherapy for specific patient subpopulations (e.g., immunocompetent, immunosuppressed, human immunodeficiency virus positive) remains to be determined, but suggested regimens have been proposed (1, 22).

At present, data regarding the intracellular antimicrobial concentrations in *Bartonella*- and *Rickettsia*-infected endothelial cells are nonexistent. In traditional studies where extracellular pathogens have been examined, antibacterial activity has been shown to correlate with serum drug concentrations. This extracellular concentration, however, may not correlate as well with intracellular pathogens such as *Bartonella* or *Rickettsia* spp. or with some of the new macrolides that exhibit a large volume of distribution into cells and tissue. Studies to assess the drug activity of macrolide agents with *Bartonella* spp. or *Rickettsia* spp. in both environments are indicated. Until future studies determine the true intracellular (i.e., endothelial) MIC and C_{max} for *Bartonella* spp. and *Rickettsia* spp., we must continue to rely on plasma concentrations and assume that adequate phagocytic membrane penetration occurs. Because in vitro antimicrobial data cannot demonstrate a direct clinical correlation to the selection and use of antimicrobial pharmacotherapy, animal and human clinical trials are needed to determine the specific role that these agents may play in the treatment of these infections.

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