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In Vitro Antimicrobial Susceptibility Testing of *Borrelia burgdorferi*: a Microdilution MIC Method and Time-Kill Studies

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The susceptibility of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, to various antimicrobial agents varies widely among published studies. These differences are probably due in part to variations in susceptibility testing techniques and growth endpoint determinations. We developed a microdilution method for determining the MICs of antibiotics against *B. burgdorferi*. The method incorporated BSK II medium, a final inoculum of 10^6 cells per ml, and a 72-h incubation period and was found to be simple and highly reproducible. A variety of antibiotics and strains of *B. burgdorferi* and one strain of *Borrelia hermsii* were examined by this method. MICs of penicillin, ceftriaxone, and erythromycin for the B31 strain of *B. burgdorferi* were 0.06, 0.03, and 0.03 μg/ml, respectively. We compared the MICs obtained by the microdilution method with those obtained by a macrodilution method using similar criteria for endpoint determinations and found the values obtained by both methods to be in close agreement. To further investigate the bactericidal activities of penicillin, ceftriaxone, and erythromycin against strain B31, we used subsurface plating to determine MBCs and we also performed time-kill studies. The MBCs of penicillin, ceftriaxone, and erythromycin were 0.125, 0.03, and 0.06 μg/ml, respectively. Time-kill curves demonstrated a ≥3-$\log_{10}$-unit killing after 72 h with penicillin, ceftriaxone, and erythromycin; ceftriaxone provided the greatest reduction in CFU. The described methods offer a more standardized and objective approach to susceptibility testing of *B. burgdorferi*.

The spectrum of antibiotic susceptibility of *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis, has been only partially defined. In vitro antimicrobial susceptibility studies of *B. burgdorferi* have been limited in part by the lack of standardized methods. The existing susceptibility data from different studies are difficult to compare because of differences in test conditions, inoculum concentrations, and endpoint determinations (33). *B. burgdorferi* has been reported to be most susceptible in vitro to erythromycin, azithromycin, clarithromycin, ceftriaxone, and cefotaxime (17–21, 27, 28, 35, 36). Tetracycline, doxycycline, ampicillin, and penicillin G have been reported to have less activity against the organism (18, 21, 35). The spirochetes are resistant to aminoglycosides, rifampin, trimethoprim-sulfamethoxazole, and ciprofloxacin (18, 21, 35). Wide variations in the MICs and MBCs of most antibiotics tested have been reported (26, 33). Furthermore, in vitro results have not always correlated with clinical experience (34, 41, 45). Although erythromycin has excellent in vitro activity against *B. burgdorferi*, clinically it has been less effective than tetracycline, doxycycline, or penicillin in the treatment of Lyme borreliosis (37, 41). The investigation described here was designed to develop a standardized method for in vitro susceptibility testing of borrelias. We report here the in vitro susceptibility data we obtained with a variety of antibiotics including penicillin, erythromycin, and ceftriaxone using a broth microdilution method for MICs, a subsurface plating method for MBCs, and broth macrodilution time-kill studies.

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MATERIALS AND METHODS

Spirochetes. The *B. burgdorferi* strains studied were B31 (ATCC 35210), the type strain isolated from a pool of New York *Ixodes dammini* ticks (9); Ip90, an isolate from an *Ixodes persulcatus* tick from eastern Russia (22, 23); G1, an isolate from the cerebrospinal fluid of a patient with Lyme disease in Germany (15, 38); and HB19, an isolate from the blood of a patient with Lyme disease in Connecticut (3, 40). Cultures of strains B31 and HB19 had been passed continuously from 1982 and 1983, respectively, and each had been cloned at least twice by limiting dilution (2, 8). These high-passage strains were designated B31-92 and HB19-92, respectively. The original isolates of strains B31 and HB19, which had not been passed more than three times in vitro and which were still infectious for rats (5), were designated B31-82 and HB19-83, respectively. The Ip90 and G1 strains had not been passed more than 10 times in vitro. The HS1 strain of *Borrelia hermsii* (ATCC 35209), which was isolated from a *Ornithodoros hermsi* tick in Washington State (43, 44), was also studied.

Antibiotics. The antibiotics tested were penicillin G (Eli Lilly, Indianapolis, Ind.), amoxicillin (Beecham, Bristol, Tenn.), ceftriaxone (Hoffmann-La Roche, Nutley, N.J.), cefixime (Lederle, Wayne, N.J.), cefdinir (Parke-Davis, Morris Plains, N.J.), erythromycin and clarithromycin (Abbott, North Chicago, Ill.), azithromycin (Pfizer, Groton, Conn.), chloramphenicol and doxycycline (Sigma, St. Louis, Mo.), sparfloxacin (Rhone-Poulenc-Rorer, Antony, France), and an investigational quinolone (CI 960; Parke-Davis, Morris Plains, N.J.). Antibiotics were reconstituted in the diluents recommended by the antibiotic manufacturers.

Broth microdilution susceptibility tests. MICs were determined by a broth microdilution method by using 96-well

round-bottom polystyrene microtiter trays (Corning Glass Works, Corning, N.Y.) Antibiotics were diluted twofold in BSK II medium (1, 2), and 100 μl of each concentration to be tested was dispensed into wells in triplicate rows. In addition, 100 μl of BSK II medium was dispensed into wells of each microtiter tray for growth controls of each strain tested and for negative controls. The ranges of antibiotic concentrations tested were as follows (in micrograms per milliliter): penicillin, 0.015 to 8; amoxicillin, 0.008 to 2.0; ceftriaxone, 0.015 to 4; cefixime, 0.015 to 4; cefdinir, 0.015 to 4; erythromycin, 0.004 to 1.0; clarithromycin, 0.0018 to 0.5; azithromycin, 0.0018 to 0.5; chloramphenicol, 0.03 to 8; doxycycline, 0.03 to 8; sparfloxacin, 0.06 to 8; and CI 960, 0.03 to 4. All wells except the negative control wells were inoculated with 10 μl of actively growing cultures of borrelias propagated in BSK II medium and were adjusted to yield a final inoculum of ca. 10^6 cells per ml, as determined by enumeration with a Petroff-Hausser chamber and phase-contrast light microscopy. Microdilution trays were sealed with sterile adhesive plastic and were incubated for 72 h at 34°C. The lowest concentration of antibiotic that showed inhibition of visual growth (a button or pellet on the bottom of the microtiter well) and lack of a color change of the BSK II medium from pink to yellow compared with the color of the growth control was interpreted as the MIC. In cases in which the BSK II medium was just beginning to change to yellow but there was visual evidence of growth, the well with the next higher concentration without evidence of growth was read as the MIC.

The MBCs of penicillin, ceftriaxone, and erythromycin were determined by a subsurface plating method performed in duplicate on 2× BSK II agar. The media for subsurface plating consisted of a 2× concentrate of BSK II medium (2× BSK II) medium without gelatin, a 3% bottom agarose, and a 2% top agarose (14, 24). Briefly, 2× BSK II medium was sterilized by passage through a nitrocellulose membrane filter (0.22-μm pore size). Rabbit serum (12% [vol/vol]; Pel Freez, Rogers, Ark.) was added, and the medium was warmed in a 37°C water bath. Under a laminar flow hood, an equal volume of autoclaved 3% agarose (Seakem LE; FMC Corp., Rockland, Maine) kept molten at 65°C was added to the 2× BSK II medium in a polystyrene tube. After the mixture was inverted several times, 8 ml was dispensed into polystyrene petri dishes (60 by 15 mm). Plates were allowed to solidify and were stored overnight at 34°C in a candle jar to prevent drying and to reduce the oxygen content of the medium before use. Molten 2% top agarose (Seaplaque; FMC Corp.) was sterilized by passage through a 0.22-μm-pore-size membrane, and a 750-μl aliquot was added to sterile 1.5-ml polystrene tubes held in a 37°C heating block. Previously prepared 2× BSK II medium with rabbit serum was warmed to 37°C, and 650 μl was added to each aliquot of molten 2% agarose. Spirochete suspensions were prepared in 2× BSK II medium, and 100 μl was added to the aliquots of molten agarose and 2× BSK II medium. The contents of the tubes were mixed well by gently vortexing the tubes, and the contents were poured immediately onto the surface of the 2× BSK II agar. Plates were rotated gently to spread the suspension evenly, the agarose was allowed to solidify, and the plates were incubated in a candle jar at 34°C. Discrete colonies were visible after 5 days and were large enough to be easily counted after 10 to 12 days (Fig. 1). The efficiency of subsurface plating was determined by successful subculturing of an inoculum of fewer than 100 cells per 100 μl, as determined by enumeration in a Petroff-Hausser chamber.

At the time that microdilution trays were inoculated, the actual inoculum density of each MIC and MBC test was determined by subsurface plating as described above. After 72 h of incubation and determination of MICs, 10 μl of each microdilution well determined to be the MIC and 10 μl of wells with concentrations greater than the MIC were mixed with 100 μl of 2× BSK II medium and was subcultured by subsurface plating. After 10 to 12 days, plates were examined visually and colonies were counted. The lowest concentration of antibiotic showing ≥99.9% killing of the final inoculum was designated the MBC (32).

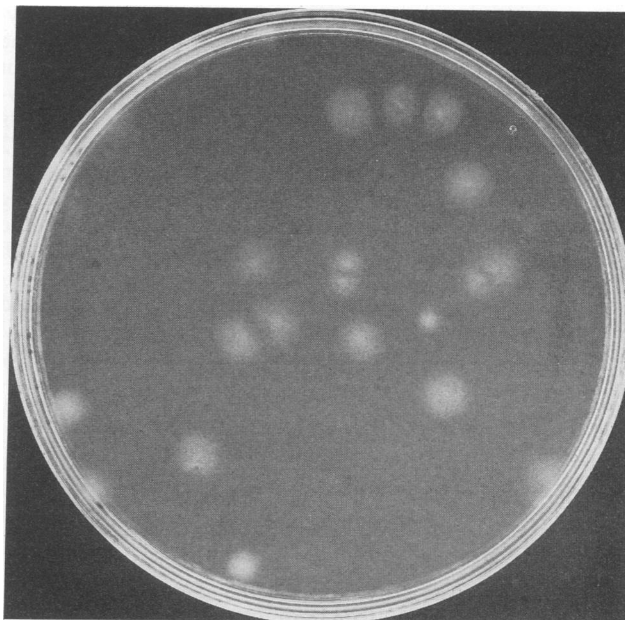

FIG. 1. Discrete *B. burgdorferi* B31 colonies on 2× BSK II agar plates (60 by 5 mm) after 10 days of incubation in a candle jar at 34°C.

Broth macrodilution methods. The MICs of penicillin, ceftriaxone, and erythromycin were also determined by a broth macrodilution method based on the method described by Barbour et al. (4). Triplicate tubes containing 5 ml of BSK II medium with the antibiotics diluted to yield the same concentrations as described above for the microdilution methods and control tubes containing no antibiotics were inoculated with B31 cells to yield a final inoculum of 10^6 cells per ml (27). Tubes were tightly capped, incubated at 34°C, and mixed by inversion at 24-h intervals. After 72 h of incubation, tubes were examined for visual inhibition of growth and a lack of color change of the BSK II medium. The MIC was defined as the lowest concentration of antibiotic showing inhibition of visible growth turbidity and a lack of color change of the BSK II medium compared with the colors of the controls. The MBCs of penicillin, ceftriaxone, and erythromycin were determined by a modification of the method previously described by Johnson and coworkers (17, 19). A total of 10% (vol/vol) of all tubes showing inhibition of growth was subcultured onto 5 ml of BSK II medium without antibiotics, and the tubes were incubated for 7 days. The MBC was defined as the lowest concentration at which no spirochetes could be detected by phase-contrast light microscopy after 7 days.

Time-kill studies. *B. burgdorferi* B31 was used to determine generation times in BSK II medium and killing rates by penicillin, erythromycin, and ceftriaxone at concentrations

representing twice the respective microdilution MICs. Polystyrene tubes containing 10 ml of BSK II medium with the antibiotic concentrations to be tested and one tube without antibiotics were inoculated with 100 μl of an actively growing culture adjusted to yield a final inoculum of ca. 10^6 cells per ml. Tubes were incubated at 34°C. At 0, 8, 24, 48, and 72 h, tubes were gently vortexed and the spirochete numbers in each tube were estimated with a Petroff-Hausser counting chamber. The estimated numbers were used to determine the appropriate dilutions needed to provide plates with countable colonies following subsurface plating. To accomplish this, 100-μl aliquots were removed from each tube and were serially diluted 10-fold in BSK II medium; 100-μl aliquots were also directly subcultured in duplicate by subsurface plating. The plates were examined and the colonies were counted after 10 to 12 days of incubation. A bactericidal effect was defined by a ≥3-$\log_{10}$-unit killing (99.9%) of the final inoculum (30). The lack of an antibiotic carryover effect was demonstrated for each antibiotic tested by subsurface plating 100 μl of the highest concentration of antibiotic tested with a known inoculum of approximately 50 to 100 B31 cells.

Antibiotic bioassay. To determine the stability of penicillin and ceftriaxone in BSK II medium after 72 h and 7 days of incubation at 34°C, agar diffusion bioassays were performed in quadruplicate with *Bacillus subtilis* ATCC 6633 as the assay microorganism (11). For analytical purposes, test concentrations were 16 μg/ml for both penicillin and ceftriaxone. Standard curves were prepared by using concentrations of 0.25, 0.5, 1, 2.5, 5, 10, and 20 μg/ml for penicillin and 2.5, 5, 10, and 20 μg/ml for ceftriaxone in BSK II medium.

RESULTS

We found that microdilution MIC determinations were simple to interpret and that the MICs were reproducible by using the criteria of lack of color change of the BSK II medium and visual inhibition of growth (a button on the bottom of the microtiter well). Our criterion for growth in the microdilution wells was based on those recommended by the National Committee for Clinical Laboratory Standards (29) for other bacteria: a definite turbidity, a single sedimented cluster (button) of organisms 2 mm in diameter or greater, or more than one cluster. Comparisons of the growth characteristics between test and growth control wells were made in determining the endpoints for each isolate tested. At concentrations of antibiotics determined to be the MIC or greater, the BSK II medium in the wells remained clear and pink. Our MICs ranged from 0.03 to 0.125 μg/ml for penicillin and 0.03 to 0.06 μg/ml for ceftriaxone for all strains of *B. burgdorferi* and one strain of *B. hermsii* tested (Table 1). Although low-passage strains of *B. burgdorferi* grew more slowly than high-passage strains, the MICs for low-passage strains were still easy to determine visually by using our criteria. We found no significant differences in the MICs of penicillin or ceftriaxone between the low- and high-passage strains of B31 and HB19 tested. The values were within 3-$\log_2$-unit dilutions, which is within the anticipated precision of a test that uses serial twofold dilutions, i.e., within plus or minus 1 dilution of a modal value. We demonstrated the reproducibility of MIC determinations with the *B. burgdorferi* B31 by testing the strain in triplicate on a minimum of five separate occasions (different days). The MICs of penicillin, ceftriaxone, and erythromycin spanned a range of only 3-$\log_2$-unit dilutions; the results were well within the anticipated precision of the test.

TABLE 1. MICs for different strains of *B. burgdorferi* and *B. hermsii*

Strain	Modal MIC (μg/ml)[a]	
	Penicillin	Ceftriaxone
B. burgdorferi		
B31-82	0.03	0.03
B31-92	0.06	0.03
Ip90	0.03	0.03
G1	0.06	0.06
HB19-83	0.06	0.06
HB19-92	0.125	0.06
B. hermsii HS1	0.03	0.03

[a] Results are based on triplicate determinations.

Penicillin, amoxicillin, ceftriaxone, erythromycin, clarithromycin, and azithromycin had the lowest MICs of the antibiotics tested against B31 (Table 2). Although *B. burgdorferi* has been reported to be resistant to ciprofloxacin (18), we tested two newer fluoroquinolones, sparfloxacin and CI 960, and found that they had some activity against B31, with MICs of 0.5 and 1.0 μg/ml, respectively. We also tested two newer oral cephalosporins, cefixime and cefdinir, and found cefixime to be more active (MIC, 0.5 μg/ml) than cefdinir (MIC, 2 μg/ml) but less active than ceftriaxone (MIC, 0.03 μg/ml).

We performed broth macrodilution susceptibility testing for two reasons: (i) to compare our results with those of other investigators who have used similar methods and (ii) to compare the results of the broth macrodilution method with those of the microdilution method we used in this study. Using the same criteria to define the MIC as described for the microdilution method (visual inhibition of growth and lack of color change of the BSK II medium), we found that the MICs of penicillin, ceftriaxone, and erythromycin were 0.03, 0.03, and 0.015 μg/ml, respectively; these values were in agreement with those obtained by the microdilution method.

The MBCs determined by the subsurface plating method described above were also simple to interpret and the results were reproducible; i.e., the ranges spanned only 2-$\log_2$-unit dilutions. The MBCs for strain B31 were 0.125 μg/ml for penicillin, 0.03 μg/ml for ceftriaxone, and 0.06 μg/ml for erythromycin. The MBCs determined by the broth macrodilution subculture method and defined by no evidence of

TABLE 2. MICs for *B. burgdorferi* B31

Antibiotic	Modal MIC (μg/ml)[a]
Penicillin G	0.06
Amoxicillin	0.06
Ceftriaxone	0.03
Cefixime	0.5
Erythromycin	0.03
Clarithomycin	0.008
Azithromycin	0.008
Cefdinir	2.0
Doxycycline	2.0
Chloramphenicol	2.0
Sparfloxacin	0.5
CI 960	1.0

[a] Results are based on triplicate determinations.

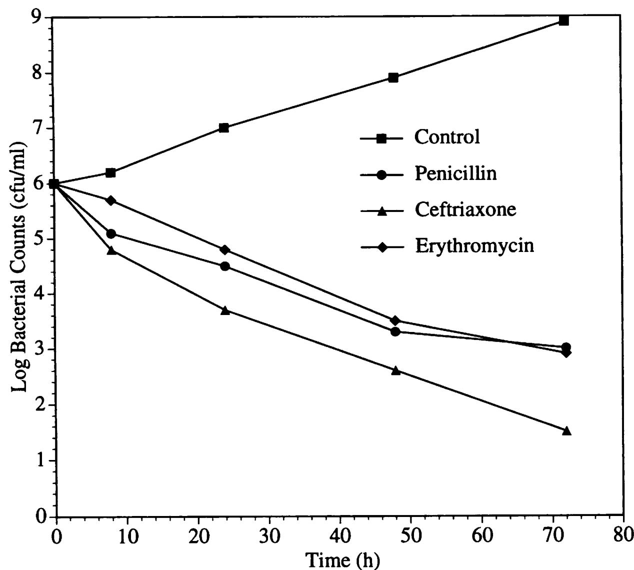

FIG. 2. Time-kill curves with *B. burgdorferi* B31. The concentrations of penicillin (0.125 μg/ml), ceftriaxone (0.06 μg/ml), and erythromycin (0.06 μg/ml) used represent twice the respective MICs.

spirochetes on phase-contrast light microscopy after 7 days of incubation were significantly greater than those obtained by the subsurface plating method. The MBCs of penicillin, ceftriaxone, and erythromycin were 2, 0.125, and >1 μg/ml, respectively.

Time-kill studies with penicillin, ceftriaxone, and erythromycin at concentrations representing twice the respective MICs demonstrated a $\geq$3-$\log_{10}$-unit killing (99.9%) of the final inoculum (Fig. 2). Ceftriaxone demonstrated the greatest bactericidal effect after 72 h of incubation, with a 4.5-$\log_{10}$-unit killing, which was 1.5 $\log_{10}$ units greater than the value for penicillin or erythromycin. The generation time of strain B31 determined from the growth controls was 10.3 h.

Because we postulated that penicillin's decreased killing at 72 h relative to that of ceftriaxone might be due to penicillin's instability under test conditions, we determined the concentration of penicillin remaining after 72 h and 7 days of incubation. The bioassay demonstrated that penicillin concentrations diminished markedly during incubation at 34°C in BSK II medium. The levels of penicillin were reduced to 17% of the initial concentration after 72 h of incubation. After 7 days, the levels of penicillin were less than 2% of the initial concentration. In contrast, ceftriaxone levels were reduced to 73% of the initial concentration after 72 h and 47% of the initial concentration after 7 days.

DISCUSSION

Although penicillin, ceftriaxone, doxycycline, amoxicillin, and erythromycin have all been proven to be useful in the treatment of Lyme borreliosis (25–27, 37, 39, 41, 42), the most effective antibiotic agent and the optimal duration of therapy have not been determined conclusively (26, 33, 37). Therapy for later and more severe manifestations of Lyme borreliosis remains controversial, and management strategies are evolving (31). Antimicrobial susceptibility studies of *B. burgdorferi* can provide valuable information in the development of new therapies for Lyme borreliosis. A uniform and standardized method of antimicrobial susceptibility testing of *B. burgdorferi* has not been established. Previously described susceptibility studies of *B. burgdorferi* have predominantly used broth macrodilution methods in BSK II medium with various inoculum concentrations, incubation periods, and endpoint determinations, yielding a wide range of results for the antibiotics tested (26, 33). Preac-Mursic and colleagues (35) reported using microdilution methods in addition to macrodilution methods in susceptibility studies of *B. burgdorferi*, but they published findings obtained only by the macrodilution method.

In an effort to develop a standardized approach to antibiotic susceptibility testing of borrelias, we adapted the broth microdilution method to test the susceptibility of *B. burgdorferi* in a manner similar to that used for other bacteria (29). A variety of antibiotics from different classes, including penicillins, cephalosporins, macrolides, doxycycline, chloramphenicol, and fluoroquinolones, were examined in this manner. Among the antibiotics examined by the microdilution method in the present study, penicillin, amoxicillin, ceftriaxone, erythromycin, clarithromycin, and azithromycin had the lowest MICs for *B. burgdorferi* B31.

Berger and colleagues (7) reported that the MICs of penicillin ranged from 0.005 to 0.08 μg/ml for six different strains of *B. burgdorferi*. They defined the MIC as the lowest concentration of antibiotic in which greater than 90% of cells appeared motile, and yet the number of cells was not greater than the inoculum size after 72 h of incubation. Our MICs of penicillin ranged from 0.03 to 0.125 μg/ml for the strains of *B. burgdorferi* that we tested. Other investigators, however, have reported higher MICs, ranging from 0.5 to 8 μg/ml for penicillin and 0.25 to 1 μg/ml for amoxicillin (21, 26, 33, 35, 36), for a variety of different strains of *B. burgdorferi*. There are likely several explanations for the differences between our findings and those of other investigators. In our study, MIC determinations were made at 72 h, while the other studies determined endpoints at 4 days or longer, when concentrations of unstable antibiotics such as penicillin are likely to be significantly diminished or undetectable. Results of our bioassay demonstrated that the concentration of penicillin under test conditions markedly decreased with time. At 7 days, concentrations of penicillin were undetectable, while 47% of the initial concentration of ceftriaxone remained. When incubation periods exceed 72 h, it is likely that growth of unkilled borrelias occurs in the dilutions of antibiotics in which little or no drug remains.

In addition, our MIC determinations were made by visual examination for inhibition of growth turbidity and the lack of color change of the BSK II medium in microtiter wells compared with the colors of the controls. Other studies have relied on microscopic examination for the loss of motility of borrelias in the presence of different concentrations of antibiotics or failure to grow borrelias on broth subculture for MIC determinations (7, 21, 27, 34–36). When we examined, by phase-contrast light microscopy, borrelias from microdilution wells containing concentrations of penicillin determined to be the MIC or greater, we were still able to detect occasional motile spirochetes at 72 h (data not shown).

It is also possible that the differences in our MICs of penicillin compared with those obtained by other investigators could be accounted for by differences in the broth microdilution and macrodilution methods. However, a high degree of correlation between MICs determined by broth macrodilution and those determined by broth microdilution

has been demonstrated in previous studies, with MICs varying by no more than 1 dilution for most antibiotics and most organisms tested (13). When we used the same inoculum density, the same incubation temperatures and times, and the same criteria to define MICs by a broth macrodilution method as we used for the microdilution method, we found that the MIC results between the methods were in close agreement. The microdilution method had the advantages of requiring less preparation time and less medium, and it lent itself well to the testing of multiple isolates simultaneously. We also discovered that microdilution trays could be prepared, frozen at −70°C, and thawed when needed without significant variations in MICs from those in trays that had not been frozen (data not shown).

Previously reported MBC determinations for *B. burgdorferi* have also used broth macrodilution methods. MBCs have been determined by the absence of spirochetes on microscopic examination after incubation periods of up to 3 weeks and/or the absence of spirochetes in BSK II medium subcultures after incubation periods of up to 3 weeks. The MBC determinations made by these methods require virtually 100% killing of the final inoculum, which is a rigorous requirement of any antibiotic. We used a subsurface plating technique similar in principle to the guidelines for determining the bactericidal activities of antimicrobial agents against other bacteria, as recommended by the National Committee for Clinical Laboratory Standards (30). The MBC was defined as a $\geq$3-$\log_{10}$-unit (99.9%) killing of the final inoculum (30, 32). We found the MBCs of penicillin (e.g., 0.125 μg/ml) and erythromycin (0.06 μg/ml) to be 1 dilution greater than the respective MICs. The MIC and the MBC of ceftriaxone were identical, i.e., 0.03 μg/ml.

Time-kill curve techniques have been shown with other microorganisms to have the best correlation with cure, as demonstrated in animal models, and with clinical outcomes (6, 10, 12, 16). Following the general guidelines recommended by the National Committee for Clinical Laboratory Standards (30), we were able to demonstrate a $\geq$3-$\log_{10}$-unit (99.9%) killing of the final inoculum with penicillin, erythromycin, and ceftriaxone at concentrations representing twice the MIC. The time-kill curve data obtained for penicillin, erythromycin, and ceftriaxone would also support our MBC data for these antibiotics. Ceftriaxone demonstrated the greatest bactericidal effect after 72 h, with a 1.5-$\log_{10}$-unit greater killing of the final inoculum than that of penicillin or erythromycin. A likely explanation for the decreased killing of penicillin relative to that of ceftriaxone was the significantly diminished levels of penicillin with time.

Our methodology for time-kill studies of borrelias is unique in that we used subsurface plating techniques. Subculturing by subsurface plating allowed us to demonstrate viable organisms after 72 h of incubation with concentrations of antibiotics greater than the MICs, even when no spirochetes or only nonmotile spirochetes were seen on examination of treated cultures by phase-contrast light microscopy (data not shown).

In summary, our data indicate that a microdilution method with BSK II medium, a final inoculum of 10^6 cells per ml, and incubation for 72 h holds promise for standardization of antibiotic susceptibility testing of borrelias. Time-kill studies and MBCs determined by subsurface plating can also provide valuable information regarding the activities of antibiotics against *B. burgdorferi*. In addition, we demonstrated that penicillin does, indeed, have good in vitro bactericidal activity against *B. burgdorferi*, but its instability under previously published test conditions must be taken into consideration. We believe that the susceptibility methods described here provide a more standardized and more objective means of examining borrelias. These methods should be used to explore the activities of additional antimicrobial agents and a larger number of *B. burgdorferi* strains.

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REFERENCES

1. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:71–75.
2. **Barbour, A. G., W. Burgdorfer, S. F. Hayes, O. Perer, and A. Aeschlimann.** 1983. Isolation of a cultivable spirochete from *Ixodes ricinus* ticks of Switzerland. Curr. Microbiol. **8**:123–126.
3. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface of Lyme disease spirochetes. Infect. Immun. **45**:94–100.
4. **Barbour, A. G., W. J. Todd, and H. G. Stoenner.** 1982. Action of penicillin on *Borrelia hermsii*. Antimicrob. Agents Chemother. **21**:823–829.
5. **Barthold, S. W., K. D. Moody, and D. S. Beck.** 1990. Susceptibility of laboratory rats to isolates of *Borrelia burgdorferi* from different geographic areas. Am. J. Trop. Med. Hyg. **42**:596–600.
6. **Bayer, A. S., and K. Lam.** 1985. Efficacy of vancomycin plus rifampin in experimental aortic-valve endocarditis due to methicillin-resistant *Staphylococcus aureus*: in vitro-in vivo correlations. J. Infect. Dis. **151**:157–165.
7. **Berger, B. W., M. H. Kaplan, I. R. Rothenberg, and A. G. Barbour.** 1985. Isolation and characterization of the Lyme disease spirochete from the skin of patients with erythema chronicum migrans. J. Am. Acad. Dermatol. **13**:444–449.
8. **Bundoc, V. G., and A. G. Barbour.** 1989. Clonal polymorphisms of outer membrane protein OspB of *Borrelia burgdorferi*. Infect. Immun. **57**:2733–2741.
9. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216**:1317–1319.
10. **Chandrasekar, P. H., L. R. Crane, and E. J. Bailey.** 1987. Comparison of the activity of antibiotic combinations in vitro with clinical outcomes and resistance in serious infections caused by *Pseudomonas aeruginosa* in nonneutropenic patients. J. Antimicrob. Chemother. **19**:231–329.
11. **Chapin-Robertson, K., and S. C. Edberg.** 1991. Measurement of antibiotics in human body fluids: techniques and significance, p. 295–366. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 3rd ed. The Williams & Wilkins Co., Baltimore.
12. **Drake, T. A., and M. A. Sande.** 1983. Studies of the chemotherapy of endocarditis: correlation of in vitro, animal model, and clinical studies. Rev. Infect. Dis. **5**(Suppl. 2):S345–S351.
13. **Gerlach, E. H.** 1972. Microdilution. I. A comparative study, p. 63–76. *In* A. Balows (ed.), Current techniques for antibiotic susceptibility testing. Charles C Thomas, Springfield, Ill.
14. **Hinnebusch, J., and A. G. Barbour.** Linear and circular plasmid copy number in *Borrelia burgdorferi*. J. Bacteriol., in press.
15. **Huppertz, H. I., V. Sticht-Groh, and T. Schwan.** 1986. Borderline antibody response in initial stages of lymphocytic meningitis does not rule out borreliosis. Lancet **ii**:1468–1469. (Letter.)
16. **Jackson, G. G., and L. J. Riff.** 1971. Pseudomonas bacteremia: pharmacologic and other bases for failure of treatment with gentamicin. J. Infect. Dis. **124**:S185–S191.
17. **Johnson, R., C. Kodner, and M. Russell.** 1987. In vitro and in vivo susceptibility of the Lyme disease spirochete, *Borrelia burgdorferi*, to four antimicrobial agents. Antimicrob. Agents Chemother. **31**:164–167.
18. **Johnson, R. C.** 1989. Isolation techniques for spirochetes and their sensitivity to antibiotics in vitro and in vivo. Rev. Infect. Dis. **11**(Suppl. 6):S1505–S1510.
19. **Johnson, R. C., C. B. Kodner, P. J. Jurkovich, and J. J. Collins.**

1990. Comparative in vitro and in vivo susceptibilities of the Lyme disease spirochete *Borrelia burgdorferi* to cefuroxime and other antimicrobial agents. Antimicrob. Agents Chemother. **34:**2133–2136.

20. **Johnson, R. C., C. Kodner, M. Russell, and D. Girard.** 1990. In-vitro and in-vivo susceptibility of Borrelia burgdorferi to azithromycin. J. Antimicrob. Chemother. **25**(Suppl. A):33–38.
21. **Johnson, S. E., G. C. Klein, G. P. Schmid, and J. C. Feeley.** 1984. Susceptibility of the Lyme disease spirochete to seven antimicrobial agents. Yale J. Biol. Med. **57:**549–553.
22. **Jonsson, M., L. Noppa, A. G. Barbour, and S. Bergstrom.** Heterogeneity of outer membrane proteins in *Borrelia burgdorferi*: comparison of *osp* operons of three isolates of different geographic origins. Infect. Immun., in press.
23. **Kryuchechnikov, V. N., E. I. Korenberg, S. V. Scherbnkov, Y. V. Yovalevsky, and M. L. Levin.** 1988. Identification of *Borrelia* isolated in the USSR from *Ixodes persulcatus* schulze ticks. J. Microbiol. Epidemiol. Immunobiol. **12:**41–44.
24. **Kurtii, T. J., U. G. Munderloh, R. C. Johnson, and G. G. Ahlstrand.** 1987. Colony formation and morphology in *Borrelia burgdorferi*. J. Clin. Microbiol. **25:**2054–2058.
25. **Liu, N. Y. H. Dinerman, R. E. Levin, et al.** 1989. Randomized trial of doxycycline versus amoxicillin/probenecid for the treatment of Lyme arthritis: treatment of non-responders with iv penicillin or ceftriaxone. Arthritis Rheum. **32**(suppl.):S32. (Abstract.)
26. **Luft, B. J., P. D. Gorevic, J. J. Halperin, D. J. Volkman, and R. J. Dattwyler.** 1989. A perspective on the treatment of Lyme borreliosis. Rev. Infect. Dis. **11**(Suppl.6):S1518–S1525.
27. **Luft, B. J., D. J. Volkman, J. J. Halperin, and R. J. Dattwyler.** 1988. New chemotherapeutic approaches in the treatment of Lyme borreliosis. Ann. N.Y. Acad. Sci. **359:**352–361.
28. **Mursic, V. P. B. Wilske, G. Shierz, M. Holmburger, and E. Suss.** 1987. In vitro and in vivo susceptibility of *Borrelia burgdorferi*. Eur. J. Clin. Microbiol. **6:**424–426.
29. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. Approved standard. NCCLS document M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
30. **National Committee for Clinical Laboratory Standards.** 1991. Methods for determining bactericidal activity of antimicrobial agents. NCCLS document M26-T. National Committee for Clinical Laboratory Standards, Villanova, Pa.
31. **National Institutes of Health.** 1991. State-of-the-Art Conference. Diagnosis and treatment of Lyme disease. Clin. Courier **9:**1–8.
32. **Pearson, R. D., R. T. Steigbigel, H. T. Davis, and S. W. Chapman.** 1980. Method for reliable determination of minimal lethal antibiotic concentrations. Antimicrob. Agents Chemother. **18:**699–708.
33. **Philipson, A.** 1991. Antibiotic treatment in Lyme borreliosis. Scand. J. Infect. Dis. Suppl. **77:**145–150.
34. **Preac-Mursic, V., K. Weber, H. W. Pfister, B. Wilske, B. Gross, A. Baumann, and J. Prokop.** 1989. Survival of *Borrelia burgdorferi* in antibiotically treated patients with Lyme borreliosis. Infection **17:**355–359.
35. **Preac-Mursic, V., B. Wilske, and G. Shierz.** 1986. European *Borrelia burgdorferi* isolated from humans and ticks: culture conditions and antibiotic susceptibility. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **263:**112–118.
36. **Preac-Mursic, V., B. Wilske, G. Schierz, E. Suss, and B. Gross.** 1989. Comparative antimicrobial activity of the new macrolides against *Borrelia burgdorferi*. Eur. J. Clin. Microbiol. Infect. Dis. **8:**651–653.
37. **Rahn, D. W., and S. E. Malawista.** 1991. Lyme disease: recommendations for diagnosis and treatment. Ann. Intern. Med. **114:**472–481.
38. **Rosa, P. A., and T. G. Schwan.** 1989. A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction. J. Infect. Dis. **160:**1018–1028.
39. **Steere, A. C., J. Green, R. T. Schoen, E. Talyor, G. J. Hutchinson, D. W. Rahn, and S. E. Malawista.** 1985. Successful parenteral penicillin therapy of established Lyme arthritis. N. Engl. J. Med. **312:**869–874.
40. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308:**733–740.
41. **Steere, A. C., S. E. Malawista, J. H. Newman, P. N. Spieler, and N. H. Bartenhagen.** 1980. Antibiotic therapy in Lyme disease. Ann. Intern. Med. **93**(Part 1):1–8.
42. **Steere, A. C., A. R. Pachner, and S. E. Malawista.** 1983. Neurologic abnormalities of Lyme disease: successful treatment with high-dose intravenous penicillin. Ann. Intern. Med. **99:**767–772.
43. **Stoenner, H. G.** 1974. Biology of *Borrelia hermsii* in Kelly medium. Appl. Microbiol. **28:**540–543.
44. **Thompson, R. S., W. Burgdorfer, R. Russell, and B. J. Francis.** 1969. Outbreak of tick-borne relapsing fever in Spokane County, Washington. JAMA **210:**1045–1050.
45. **Wretlind, B., R. C. Johnson, K. Hansen, and V. Preac-Mursic.** 1991. Antibiotic susceptibility of *Borrelia burgdorferi* in vitro and in animal models. Scand. J. Infect. Dis. Suppl. **77:**143–144.