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Spring/Summer 1997 Volume 4 • Number 1/2

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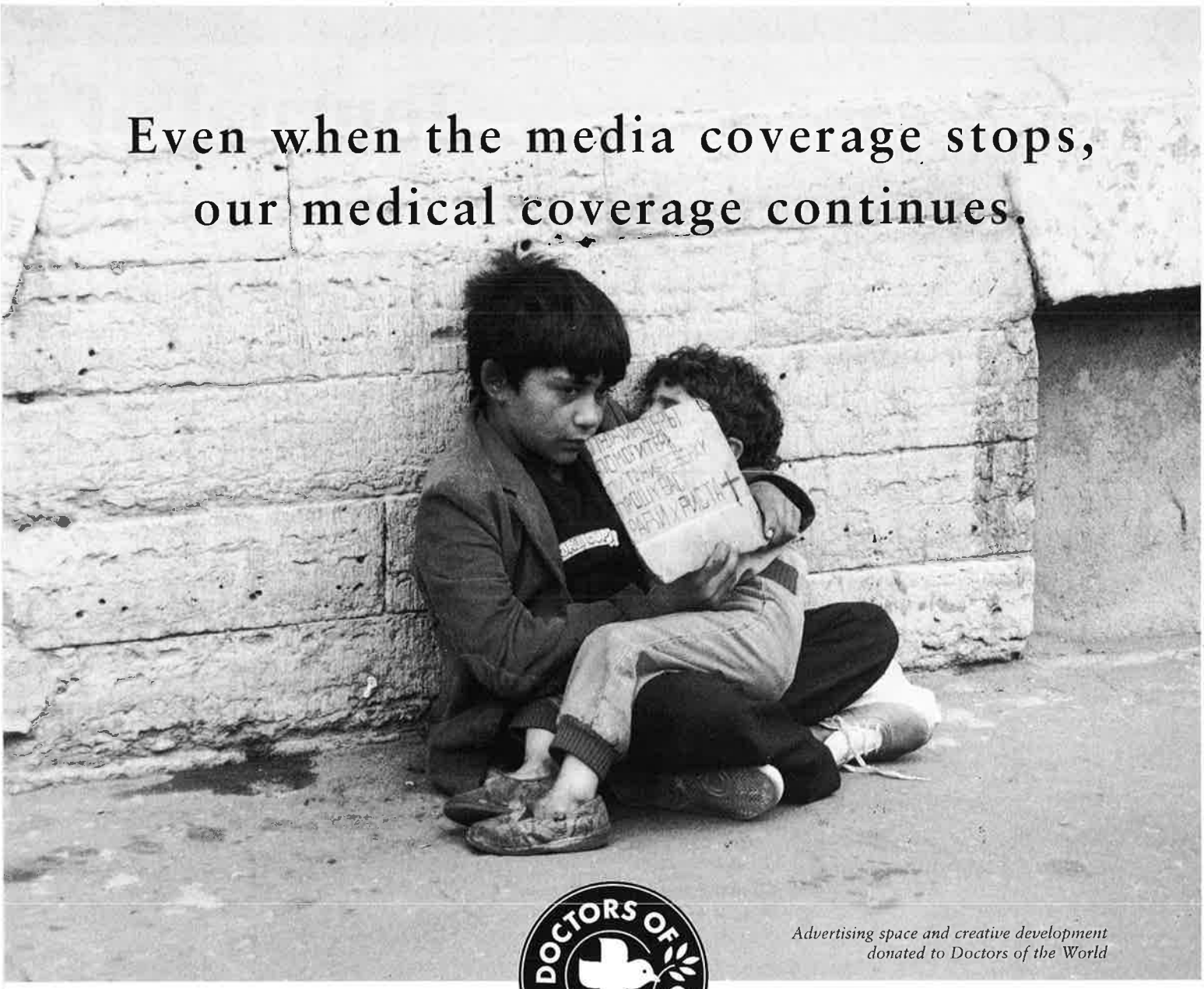

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The Borreliacidal Antibody Test: An Alternative Approach for Confirming Lyme Borreliosis

Ronald F. Schell, PhD; Steven M. Callister, PhD; Dean A. Jobe, BS, MS; and Brian K. DuChateau, PhD

ABSTRACT

False-negative and, more commonly, false-positive results continue to plague the serodiagnosis of Lyme borreliosis.

Highly specific IgM or IgG borreliacidal antibodies can be detected in sera from patients with early or late Lyme borreliosis.

The use of sensitive borreliacidal antibody tests can help solve the specificity problems that continue to confound the accurate serodiagnosis of Lyme borreliosis.

Key words: Borreliacidal, *Borrelia burgdorferi*, serodiagnosis

Direct laboratory procedures for detecting *Borrelia burgdorferi* spirochetes reliably in all patients with Lyme borreliosis do not currently exist. The clinical diagnosis of Lyme borreliosis often is based primarily on exposure to *B. burgdorferi*-infected *Ixodes* spp. ticks, the presence of characteristic and protean clinical findings, and laboratory confirmation by detection of antibodies against the Lyme spirochete. Lyme borreliosis symptoms can resemble those of other clinical illnesses, including influenza, multiple sclerosis, aseptic meningitis, and rheumatoid arthritis. This ability to cause nonspecific symptoms can make an accurate clinical diagnosis of Lyme borreliosis difficult. Consequently, the physician often relies heavily on the ability of the laboratory to detect antibodies against *B. burgdorferi* accurately.

Increased reliance on serologic confirmation has focused considerable attention on the reliability of current antibody testing methods. Unfortunately, investigators repeatedly have demonstrated significant sensitivity, specificity, and reproducibility inaccuracies in current commonly used laboratory procedures. Bakken et al[1] demonstrated significant interlaboratory and intralaboratory variations as a direct result of these variables. A more recent Lyme disease testing proficiency survey with more than 500 participating laboratories confirmed the continued gross interlaboratory differences in interpretations among laboratorians.[2] These results leave little doubt that false-negative and, more commonly, false-positive results continue to plague the serodiagnosis of Lyme borreliosis.

The effects of these diagnostic shortcomings have been magnified by inappropriate testing of patients without appropriate clinical and epidemiologic indications of Lyme borreliosis.[3] These clinical and serodiagnostic inaccuracies have contributed to making the overdiagnosis of Lyme borreliosis common. Steere et al[4] illustrated this, reporting that 203 of 452 (45%) patients misdiagnosed as having Lyme disease had at least one positive diagnostic test result. Inaccurate laboratory confirmation of Lyme borreliosis has caused significant economic and health effects. Lightfoot et al[5] estimated that the incidence of false-positive serologic results in patients with nonspecific myalgia and fatigue exceeded fourfold the incidence of true-positive results in patients with nonclassical Lyme borreliosis. These researchers estimated that for each Lyme patient treated, $86 221 is spent for the treatment of patients with conditions other than Lyme borreliosis.

In an effort to improve the specificity of Lyme disease serologies, the Centers for Disease Control and Prevention (CDC) and the Association of State and Territorial and Public Health Laboratory Directors recently recommended that sera submitted for Lyme borreliosis

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serology be tested by a sensitive ELISA followed by confirmation with Western blotting of all equivocal and positive ELISA results.[6] A recent investigation by the CDC, however, demonstrated an alarming lack of specificity with this two-tiered testing approach using the currently recommended analysis and interpretive criteria.[7-9] Burkot et al[10] demonstrated false-positive results in 50% of 86 human serum samples from Papua, New Guinea, a nonendemic Lyme borreliosis area. These results accentuate the continuing need for a more specific confirmatory test for Lyme borreliosis. In addition, other approaches besides conventional serodiagnostic assays may be necessary before further significant improvements are possible.

The development and widespread availability of a specific functional assay to detect antibodies in Lyme disease sera that are lethal to *B. burgdorferi* in vitro (borreliacidal) should be helpful. The authors and others have established conclusively that sera from Lyme disease patients contains IgM or IgG borreliacidal antibodies.[11-17] Borreliacidal antibodies against *B. burgdorferi* outer surface protein (Osp)A or OspB have been demonstrated in animal sera after vaccination with these proteins,[18,19] and their existence in sera from patients with late-disseminated Lyme disease symptoms has been confirmed.[11,14-16] In addition, Ma et al[20] identified the OspA and OspB epitopes responsible for the induction of borreliacidal antibodies.

The authors recently demonstrated borreliacidal antibodies against other *B. burgdorferi* proteins in patients with early localized Lyme disease.[14] In most instances, borreliacidal antibodies were detectable in serum from these patients only when *B. burgdorferi* 50772 spirochetes, which lack the genes for OspA and OspB production, were used. Using *B. burgdorferi* isolate 297, which expresses OspA and OspB, borreliacidal antibodies were detectable in only 2 of 13 (15%) sera from patients with culture-defined primary or secondary erythema migrans (EM) lesions. Agger and Case[21] also reported similar findings (11% sensitivity) using an antiquated version of the borreliacidal antibody test employing isolate 297. By contrast, borreliacidal antibodies were detectable in five of nine (56%) culture-defined patients with primary EMs and three of four (75%) patients with secondary EMs when isolate 50772 was used. Thus, the sensitivity of the borreliacidal antibody test was enhanced greatly when the spirochete was susceptible to borreliacidal activity against proteins other than OspA or OspB. When the results using each isolate were combined, borreliacidal antibodies were detectable in 77% of sera from patients with culture-defined EM lesions. The specific proteins responsible for inducing these borreliacidal antibodies shortly after infection with *B. burgdorferi* spirochetes remain unknown. Intriguing candidates include the 39 kDa protein, OspC, OspE, and OspF.[14,22,23] Regardless, these results collectively demonstrate the presence of borreliacidal antibodies in serum from patients with both early and late Lyme borreliosis sequelae.

Why use a functional assay to discern borreliacidal activity? The major benefit is the exquisite specificity of borreliacidal antibodies. False-positive results using conventional serodiagnostic tests continue to hamper efforts to improve Lyme borreliosis serology significantly. The use of viable spirochetes for the detection of antibodies that can kill *B. burgdorferi* greatly increases specificity by eliminating the detection of crossreactive or natural antibodies that bind to but are incapable of killing the Lyme disease spirochete. Other tests (Western immunoblotting, indirect fluorescent-antibody test, enzyme immunoassay) detect natural, crossreactive, or specific antibodies, and the laboratorian cannot know which are responsible for reactivity. In many instances, crossreactive antibodies (ie, anti-41 kDa) are present in the serum of patients without Lyme disease or patients with other illnesses and are detected by nonfunctional serodiagnostic assays. This is a major cause of false-positive results and subsequent overdiagnosis of Lyme disease.

The authors have demonstrated the high specificity of the borreliacidal antibodies against *B. burgdorferi* 297.[11] We determined the borreliacidal activity in 107 normal endemic and 50 nonendemic serum samples and compared the results with those obtained using a conventional indirect fluorescent-antibody test and enzyme immunoassay. The percentage of positive normal serum samples was comparable (6%) using all three assays. The indirect fluorescent-antibody assay, however, identified 41 (39%) and the enzyme immunoassay identified 47 (51%) of 104 sera from patients with antinuclear antibodies, rheumatoid factor, Epstein-Barr virus antibodies, systemic lupus erythematosus, or *Treponema pallidum* antibodies as positive. By contrast, no sera from patients with other illnesses contained borreliacidal antibodies. Sambri et al[17] confirmed these findings using *B. burgdorferi* isolate IRS. They demonstrated borreliacidal antibodies in 14 of 14 (100%) patients with varying clinical manifestations of Lyme borreliosis (six patients with EM, six with arthritis, and two with neuritis). Testing of convalescent sera was necessary before borreliacidal antibodies were detected in five patients with EM; however, this is not surprising if the test organism expressed OspA and OspB. OspA and OspB on the surface of the spirochete could hinder the interaction of borreliacidal antibodies with other *B. burgdorferi* proteins. Most importantly, these investigators confirmed the exquisite specificity of borreliacidal antibodies. Borreliacidal antibodies could not be detected in serum from 20 patients with rheumatoid arthritis and 50 with syphilis. By contrast, the indirect fluorescent-antibody test showed false-positive

reactivities in 67% and 20% of these syphilitic and rheumatoid arthritis sera, respectively.

The authors recently showed the high specificity of borreliacidal antibodies produced against other *B. burgdorferi* proteins besides OspA and OspB in serum from patients with early localized Lyme disease.[14] No borreliacidal antibodies against *B. burgdorferi* 50772 were detectable in 80 endemic normal sera. Borreliacidal activity was detectable in 1 of 17 (6%) serum samples with antinuclear antibodies. No false-positive reactivities, however, were detectable in serum from 33 patients with syphilis, mononucleosis, or rheumatoid factor.

These results leave little doubt that a borreliacidal antibody test will be valuable for confirming Lyme borreliosis. The authors have developed a flow cytometric procedure to maximize the sensitivity of borreliacidal antibody detection.[12] *B. burgdorferi* organisms are incubated with Lyme disease serum and complement for 16 to 24 hours, stained for 1 minute with acridine orange, and analyzed by flow cytometry. Killing by borreliacidal antibodies causes a significant increase in acridine orange uptake by dead, blebbed organisms. Borreliacidal activity then can be detected objectively and accurately by monitoring fluorescence intensity with the flow cytometer. A flow cytometer measures the fluorescence intensity of individual spirochetes as they pass through a laser light. Thus, small numbers of spirochetes can be evaluated to enhance the detection sensitivity greatly, compared with borreliacidal assays that require visual interpretations.

Using flow cytometric determination of borreliacidal activity against as few as 10^4 spirochetes, the authors demonstrated the sensitivity of this procedure using sera from 49 patients in Westchester County, NY, with culture- or case-defined EM lesions.[14] Flow cytometric borreliacidal antibody detection was almost threefold more sensitive than results using an enzyme immunoassay (72% versus 28%) when *B. burgdorferi* isolates 297 and 50772 were used. These results demonstrated the enhanced sensitivity of the flow cytometric borreliacidal antibody test for detecting a highly specific functional antibody response compared with detection of many types of antibodies using a conventional enzyme immunoassay, which used much higher amounts of antigen. These data also demonstrate the likelihood that increased use of sensitive borreliacidal antibody tests could help solve the specificity problems that continue to confound the accurate serodiagnosis of Lyme borreliosis.

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A Macrodilution Well Method for Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Determination of Antimicrobials Against *Borrelia burgdorferi* in vitro

Ilse Wendelin, PhD; Robert Gasser, MD, PhD; and Emil C. Reisinger, MD

ABSTRACT

The conventional broth macrodilution tube method to test minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antimicrobials against *Borrelia burgdorferi* in vitro is cumbersome and requires large amounts of material. An economical microdilution method has been described only for MIC determination. This microdilution MIC method was combined with a subsurface plating method for MBC determination.

We have developed an economical and simple macrodilution well method for both MIC and MBC determination using 24-well tissue culture plates with volumes of 2 mL per well. MIC was determined after 4 days of incubation; MBC determination subcultures were incubated for 3 weeks. The 24-well method was highly reproducible; MIC and MBC results were well within the anticipated precision of a twofold dilution test. MIC and MBC values obtained by the 24-well method were identical with those obtained by the tube method.

Our results demonstrate that the 24-well method is appropriate for MIC and MBC determination and for testing antibiotic synergism by the checkerboard method. The macrodilution 24-well method combines the advantages of the macrodilution tube method (MIC and MBC) and the microdilution well method (economical and easy handling).

Key words: *Borrelia burgdorferi,* MIC, MBC, 24-well method, checkerboard

INTRODUCTION

Borrelia burgdorferi, a fastidious spirochete, requires a complex broth and microaerophilic conditions for growth in vitro.[1-3] Antimicrobial susceptibility testing of *B. burgdorferi* usually has been performed by broth macrodilution using tightly capped tubes filled with medium to about 90% of their capacity.[4-11] This method can yield minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values; however, the use of screw-capped tubes is cumbersome and requires large amounts of media. Several reports describe the use of 2-mL vials[12,13] or the use of plates with wells appropriate for 2 mL[14] or 200 μL medium, respectively.[5] An economical and simple microdilution method has been developed for MIC determination, but not for MBC determination.[15] This MIC microdilution method was combined with a subsurface plating method for MBC determination.[15-17] When MICs are determined by a broth dilution method, and, subsequently, MBCs are determined by a subsurface plating method, however, the growth conditions for the fastidious borreliae are changing within the test. We developed an easy and economical broth macrodilution method for MIC, MBC, and checkerboard testing using 24-well tissue culture plates.

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MATERIALS AND METHODS

B. burgdorferi strains

B. burgdorferi-type strains ATCC 35210 (B31, isolate from *Ixodes scapularis* ticks), ATCC 35211 (isolate from *Ixodes ricinus* ticks), and ATCC 53899 (isolate from cerebrospinal fluid) were purchased from the German Collection of Microorganisms, Braunschweig. The strains TX 532 passage 4 (isolate from cat fleas) and TX 1574 passage 6 (isolate from *Amblyomma americanum* ticks) were provided by J. Rawlings, Austin, Tex. The strain PKo (skin isolate) was provided by V. Preac-Mursic, Munich, Germany.

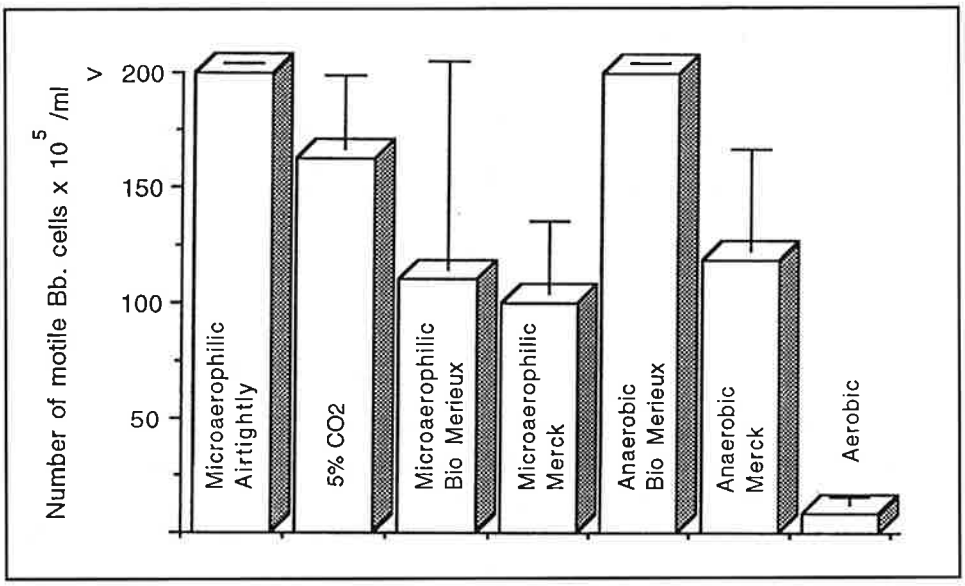

Fig: Evaluation of optimal environmental conditions for growth of B. burgdorferi *strain B31 in 24-well tissue culture plates at 35°C for 4 days. The inoculum size was 5×10^5 actively growing cells/mL.*

Evaluation of optimal growth conditions for *B. burgdorferi* in 24-well tissue culture plates

Before use, the 24-well tissue culture plates (Costar, Cambridge, Mass) were equilibrated with sterile neopeptone solution (5% in water, Difco, Detroit, Mich). Two mL of BSK II medium,[2] inoculated with actively growing borreliae to a final concentration of 5×10^5 cells/mL as determined with a Petroff-Hausser counting chamber and darkfield microscopy, were dispensed into the wells. Anaerobic and microaerophilic conditions were produced by gas packs (Anaerocult A and Anaerocult C, Merck, Darmstadt, Germany; Generbag Anaer and Microaer, Bio Merieux, France). To obtain microaerophilic conditions without gas packs, the plates were taped and sealed in an airtight nylon bag. Five percent CO_2-enriched milieu was obtained in a CO_2 incubator (Heraeus, Cytoperm 8080, Hanau, Germany). Aerobic conditions were obtained with untaped plates. After 4 days of incubation at 35°C, the number of motile borreliae/mL was determined by a Petroff-Hausser counting chamber and dark-field microscopy (Fig). The experiments were performed three times in duplicate.

Antibiotics

Freshly prepared stock solutions of penicillin G, ceftriaxone (Sigma, Munich, Germany), cefotaxime, cefodizime, roxithromycin (A. Roussel, Vienna, Austria), ciprofloxacin (Miles Davis, Kankakee, Ill), and mecillinam (Hoffmann-La Roche, Vienna, Austria) were serially twofold diluted to final antibiotic concentrations of penicillin G, 0.015 to 32 μg/mL; ceftriaxone, 0.008 to 2 μg/mL; cefotaxime, 0.015 to 4 μg/mL; cefodizime, 0.03 to 4 μg/mL; roxithromycin, 0.008 to 1 μg/mL; ciprofloxacin, 0.25 to 4 μg/mL; and mecillinam, 0.25 to 4 μg/mL.

24-well method (broth macrodilution method using 24-well tissue culture plates)

BSK II medium, serial twofold dilutions of antibiotics, and a culture of actively growing borreliae (5×10^5 cells/mL) were dispensed into the wells to a total volume of 2 mL. Positive control wells contained borreliae without antibiotics; negative control wells contained BSK II medium only on each plate. The plates were taped and sealed in an airtight nylon bag and incubated for 4 days at 35°C. MIC was defined as the lowest antimicrobial concentration with $\leq5\times10^5$ motile borreliae/mL as determined with a Petroff-Hausser counting chamber and darkfield microscopy. A color change of BSK II medium from pink to yellow, caused by metabolic activities of the spirochetes, was helpful to limit microscopic examination of the cultures to only a few wells of the plate. Of all wells exhibiting inhibition of growth, 100 μL were subcultured into the wells of another 24-well culture plate containing 1900 μL of fresh BSK II medium without antibiotics. The plates were taped and sealed as described, incubated for 3 weeks at 35°C, and examined for the presence of spirochetes by darkfield microscopy (400×). MBC was defined as the lowest antimicrobial concentration at which no spirochetes could be subcultured. The 24-well method was used to determine MIC and MBC of antimicrobials alone or in combination. To assess the antimicrobial activity of mecillinam combined with penicillin or ceftriaxone by checkerboard testing, four 24-well tissue culture plates were arranged to form a square consisting of 96 wells.

Tube method (broth macrodilution method using tubes)

BSK II medium, serial twofold dilutions of antibiotics, and a culture of actively growing borreliae were dis-

Table 1

MICs and MBCs for **Borrelia burgdorferi** *Strain B31* Obtained by the 24-well Method, the Tube Method, and the Microdilution Method*

Antibiotic	Method	MIC in μg/mL		MBC in μg/mL	
		Modal	(range)	Modal	(range)
Penicillin G	Tube	0.06	(0.06)	8	(4 to 16)
	24-well	0.06	(0.06)	8	(4 to 8)
	Microdilution	0.06	(0.06)	-	
Ceftriaxone	Tube	0.03	(0.03)	0.5	(0.25 to 0.5)
	24-well	0.03	(0.03)	0.5	(0.25 to 0.5)
	Microdilution	0.03	(0.015 to 0.06)	-	

**Results are based on three determinations in duplicate.*
MIC=minimum inhibitory concentration; MBC=minimum bactericidal concentration.

pensed into tubes (100×13 mm, Falcon, Becton Dickinson, NJ) as described for the 24-well method to a total volume of 6.5 mL/tube. The tubes were capped tightly and incubated for 4 days at 35°C. MIC was determined as described for the 24-well method. Of all cultures showing inhibition of growth, 300 μL were subcultured into 6.2 mL of fresh BSK II medium without antibiotics and incubated for 3 weeks at 35°C. MBC was determined as described for the 24-well method.

Microdilution method (broth microdilution method using 96-well microtiter plates)

BSK II medium, serial twofold dilutions of antibiotics, and a culture of actively growing borreliae were dispensed into U-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark) to a total volume of 200 μL/well. The plates were sealed with sterile adhesive plastic and incubated for 4 days at 35°C. MIC was determined as described for the 24-well method. Of all cultures showing inhibition of growth, 10 μL were subcultured into 190 μL of fresh BSK II medium without antibiotics. The plates were sealed as described and incubated for 3 weeks at 35°C. MBC was determined as described for the 24-well method.

RESULTS

Optimal growth conditions for *B. burgdorferi* in 24-well tissue culture plates were observed when the plates were taped and sealed in an airtight nylon bag without using gas packs (Fig). The 24-well method for susceptibility testing of *B. burgdorferi* was based on this procedure.

The Table shows the MICs and MBCs of penicillin G and ceftriaxone for *B. burgdorferi* strain B31 obtained by the 24-well method, the tube method, and the macrodilution method. The MIC and MBC values of the 24-well method were equal to those of the tube method and the macrodilution method and in close agreement with the values reported in the literature. The MBCs obtained by the microdilution method were not reproducible because evaporation, especially in the wells on the edges of the plates, affected the results. Experiments to develop a reliable MBC microdilution method were not successful.

To assess the reproducibility of the 24-well method, MICs and MBCs of penicillin and ceftriaxone for *B. burgdorferi* strain B31 were determined on at least six different occasions. Both MIC and MBC results were within a range of 3 $\log_2$ dilution units, ie, within the anticipated precision of a twofold dilution test.[18]

When the 24-well method was used for checkerboard testing of mecillinam combined with penicillin or ceftriaxone for *B. burgdorferi* strain B31, the MICs of the antimicrobials alone were 1 μg/mL for mecillinam, 0.06 μg/mL for penicillin, and 0.03 μg/mL for ceftriaxone. In combination, mecillinam and penicillin exhibited synergism at concentrations of one fourth the respective MICs. When mecillinam was combined with ceftriaxone, synergism was demonstrated at concentrations of one eighth the respective MICs. As the corresponding MIC results of repeated checkerboard tests ranged within 3 $\log_2$ dilution units, the precision of the test was well anticipated.[18]

DISCUSSION

The 24-well macrodilution method, developed for in vitro susceptibility testing of *B. burgdorferi*, is hoped to prove as simple and economical as the microdilution MIC method,[15] and can yield both MIC and MBC values.

B. burgdorferi is a slow-growing bacterium with a generation time of about 11 hours in BSK II medium at 35°C.[2,21] For broth-susceptibility testing of *B. burgdorferi,* incubation periods of 3 to 4 days for MIC and up to 3 weeks for MBC determination have been reported.[4-11] In screw-capped tubes, long incubation periods are not problematic. In microtiter plates, although sealed, modest amounts of evaporation may increase the concentration of the antibiotics being tested significantly, and thus produce

artifactually low MICs or MBCs.[20] To validate the 24-well method, MICs and MBCs obtained by the 24-well method were compared with those obtained by the tube method and the microdilution MIC method. Identical results for MICs and MBCs from the tube method, the 24-well method, and the microdilution MIC method confirm that the 24-well method can be used for in vitro MIC and MBC determination of *B. burgdorferi*.

For MBC determination of antimicrobials against *B. burgdorferi,* different incubation periods have been reported. The authors found that borreliae could recover from the antibiotic effect for up to 3 weeks. Based on these observations, MBC was defined as the lowest antimicrobial concentration at which no spirochetes could be subcultured within 3 weeks of incubation. This MBC procedure requires a 100% killing of the original inoculum. According to the National Committee for Clinical Laboratory Standards, a 99.9% killing of the original inoculum,[19] which can be determined by the subsurface plating method, is recommended.[15] A unique method for both MICs and MBCs, however, might be preferable to a combination of two methods with different growth conditions for the fastidious Lyme spirochetes.

The color change of the BSK II medium from pink to yellow gave a valuable hint for growth of *B. burgdorferi*. In cultures of high passage strains, the color of BSK II medium did not change from pink to yellow up to concentrations of about 10^6 cells/mL. In cultures of low passage strains, no color change was observed up to concentrations of about 10^7 cells/mL.

The checkerboard method is the technique used most frequently to assess antimicrobial combinations in vitro.[22] Standard checkerboard testing with *B. burgdorferi* using tubes is cumbersome and requires large amounts of antimicrobials and media.[20] A microdilution checkerboard technique[23] for *B. burgdorferi* has not been reported previously. The use of 24-well tissue culture plates offers procedural and economical advantages. In our experiments, the combinations of mecillinam with penicillin or ceftriaxone showed synergism against *B. burgdorferi*. The β-lactams act on the bacterial cell wall synthesis by inhibition of penicillin-binding proteins. Mecillinam may potentiate the activities of penicillin or ceftriaxone by acting at complementary target sites.[20]

The 24-well macrodilution method combines the advantages of both the macrodilution tube method (determination of MIC and MBC of antimicrobials alone and in combination) and the microdilution well method (economical and easy handling) for in vitro susceptibility testing of *B. burgdorferi*.

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Use of PCR Assays to Monitor the Clearance of *Borrelia burgdorferi* DNA From Blood Following Antibiotic Therapy

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ABSTRACT

A polymerase chain reaction (PCR) test was used to follow the persistence of *Borrelia burgdorferi* in the blood of patients with early- and late-stage Lyme disease and to monitor the effectiveness of antibiotic therapy.

Blood samples were analyzed by PCR using primers specific for the outer surface protein (Osp) A gene of *B. burgdorferi*. In control experiments using known amounts of cultivated *B. burgdorferi* spiked into blood cultures, as few as 10 copies of the spirochete DNA could be detected by Southern hybridization. Patients with early- and late-stage Lyme disease were enrolled in the study and followed up by serology, PCR, and symptoms at regular intervals for up to 2 years.

Early Lyme disease patients were defined as those who reported a tick bite and exhibited erythema migrans, the skin rash characteristic of infection with *B. burgdorferi*. Late Lyme disease patients were defined as those presenting with at least two late-stage symptoms (neurologic, cardiac, or arthritic) and confirmed by positive serology (Lyme Western Blot).

Seven of 21 (33%) early Lyme disease patients and 10 of 20 (50%) late-stage patients not on antibiotic therapy at the time of enrollment were PCR positive. All seven PCR-positive early Lyme disease patients and 8 of the 10 PCR-positive late-stage patients became PCR negative within 2 weeks of antibiotic treatment. Of an additional eight late-stage patients who had been on therapy for more than 2 months at the time of enrollment, three were PCR positive. All five late-stage patients who remained PCR positive while under therapy (two from the newly treated group and three from the previously treated group) became PCR negative when alternative therapy was administered.

These observations demonstrate a significant correlation between PCR results and application of antibiotic therapy, providing further support for the usefulness of PCR in identifying periods of active infection and monitoring the effectiveness of antibiotic therapy in patients with Lyme disease.

Key words: Lyme disease, PCR, blood, antibiotic therapy

INTRODUCTION

Lyme disease, the most common tick-borne disease in the United States, is caused by infection with *Borrelia burgdorferi*, a spirochete transmitted by tick bite.[1,2] Early infection is characterized by the appearance of a skin rash called erythema migrans (EM), which spreads from the initial site of the tick bite to form a characteristic bull's-eye-like appearance. Other early symptoms may include flu-like symptoms, headache, fever, muscle aches, and fatigue. Treatment of early-stage Lyme disease patients with oral antibiotics such as doxycycline, amoxicillin, and erythromycin usually prevents the subsequent reappearance of more serious symptoms.[3] In many cases, however, early symptoms of Lyme disease are not noticed or infection is not diagnosed properly. If left untreated, more serious late-stage symptoms may develop. In addition to the generalized symptoms seen in early disease, late-stage infections may involve dermatologic, neurologic, cardiac, and arthritic manifestations.

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More aggressive therapy, often involving several courses of intravenous antibiotics, may be required to treat patients at this stage.

A major problem with managing Lyme disease is difficulty identifying periods of active infection, thus assessing the effectiveness of antibiotic therapy. Commonly used laboratory tests are inadequate for monitoring the state of current infection. Antibody tests for Lyme disease are useful for confirming probable exposure to the infecting agent, but do not distinguish between active and inactive infection. The absence of specific antiborrelial antibody response may result from insufficient antibody titer, immune suppression, or the transient nature of the immune response, which may give rise to false-negative results. Conversely, a positive antibody response may persist long after the infecting organism has been cleared from the body, and have no relationship to current infection.[4] In addition, false-positive results may result from crossreactivity with other infections, such as syphilis or mononucleosis. These limitations of the immune response itself, coupled with complex criteria for interpretation of serological results, hinder the usefulness of ELISA and immunoblot assays in monitoring the effectiveness of therapy.[5] Because of such complications, misinterpretation of serologic tests is common and may lead to misdiagnosis and improper treatment of Lyme disease, particularly in many endemic areas.[6,7] Furthermore, as symptoms may be variable or difficult to measure objectively, end points of therapy are not well defined. In the absence of well-defined measures of therapy regimens' efficacy, the optimum dosage and duration of therapy or the adequacy of inexpensive oral antibiotics versus the requirement for more expensive, potentially toxic intravenous regimens cannot be evaluated rigorously.[8]

A need exists for more direct tests for Lyme disease that can be used in concert with measurement of symptoms and the host's immune response to help monitor the status of actual infection. Direct detection of *B. burgdorferi* may involve direct culture of the spirochete or detection of its antigens or DNA in biologic specimens. The difficulty in growing this organism from clinical material and the uncertainty of its location in the human host make culture a cumbersome, time-consuming, and not easily reproducible test that is not well suited for routine monitoring.[9-11] Direct antigen tests for *B. burgdorferi* are not readily available for routine use or are insufficiently sensitive to detect the minute quantities of this organism that may exist in tissues or body fluids. The application of the polymerase chain reaction (PCR) for the detection of *B. burgdorferi* DNA in blood, cerebrospinal fluid, urine, skin, and synovial fluid of patients with early- and late-stage Lyme disease, on the other hand, can provide significant advantages over other direct methods.[12-16] Blood and urine specimens from patients with active infection have been shown to contain low levels of *Borrelia*. Such low levels may be difficult to detect by culture, but may be detectable with the use of the PCR assay, which is capable of detecting as little as the equivalent of fewer than 10 organisms per milliliter of fluid. In fact, PCR may be able to detect the presence of *B. burgdorferi* DNA in the blood of patients with definite clinical features of Lyme disease prior to the appearance of IgG and IgM antibody.[17]

Although the use of PCR in diagnosis of Lyme disease is under active investigation, studies from many laboratories indicate that this method can be of significant value, particularly in verifying active *Borrelia* infection.[18] Skin biopsies in from 60% to 70% of EM or acrodermatitis chronica atrophicans lesions were found to be positive by PCR.[19-22] In contrast, all biopsies examined from the same sites of these patients posttherapy were negative. In addition, the finding of *Borrelia* DNA in cerebrospinal fluid correlates quite well with neuroborreliosis.[13,23-25] Goodman et al[26] reported that PCR detection is at least three times more sensitive than culture for identifying spirochetemia in early Lyme disease and correlated with clinical evidence of disseminated disease. Additional studies have shown a correlation of results of PCR assays with response to antibiotic therapy of treated patients.[27,28] Schmidt et al[18] reported the presence of *B. burgdorferi* DNA in the urine of 88 of 97 (91%) patients with Lyme borreliosis before treatment. All but seven of these became nonreactive following treatment. *Borrelia* DNA had been present in the urine of 50% of late-stage patients, increased to 90% in the first few days posttherapy, and remained negative thereafter.[14]

In this study, we extended previous findings of the correlation of PCR in identifying active infection in early- and late-stage Lyme disease and monitoring the effectiveness of therapy. Blood specimens from early- and late-stage patients were collected at regular intervals to examine the presence of *Borrelia*-specific antibody and DNA levels under various treatment regimens. Our results demonstrate that PCR may be a useful marker of response to therapy when used in conjunction with serologic measurements and clinical observations.

METHODS

In the course of this study, we followed early- and late-stage Lyme disease patients in the New England, New York, and New Jersey areas at various times after administration of therapy. Only patients who met the Centers for Disease Control and Prevention (CDC) criteria for early and late Lyme disease were enrolled. Blood specimens were collected from these patients at regular intervals over a 2-year period and examined for the presence of Lyme-specific antibody by ELISA, Western blot, and IgM capture

assays and DNA sequences by PCR. The therapy used, if any, was determined by the participating physicians, who varied widely in choice of antibiotic and duration of therapy. Decisions for administration of therapy were made largely on the basis of symptoms. All samples were coded; serology and PCR testing were done blindly in a clinical reference laboratory by technicians with no knowledge of patient history or status. The specimens were decoded and compiled into the analyzed study as reported here.

Patients

Blood specimens were collected from early-stage Lyme disease patients at the time of the appearance of EM and from serologically confirmed late-stage Lyme disease patients at the time of presentation with dermatologic, neurologic, cardiac, or arthritic symptoms characteristic of late-stage Lyme disease. Data collected included symptoms observed and type, dosage, and duration of therapy. A variety of oral and intravenous therapy options used by participating physicians included amoxicillin, tetracycline, clarithromycin, penicillin G, doxycycline, minocycline, ceftriaxone, cefixime, vancomycin, and azithromycin. Follow-up specimens were collected at 1 week after initiation of therapy and at approximately 4- to 6-week intervals thereafter.

Specimen collection and processing

Whole blood (5 mL) was collected in EDTA tubes (lavender top) and shipped out the same day at room temperature. The blood was mixed by inversion and centrifuged at 1500 rpm for 20 minutes. Plasma was collected from the top of the tube and the thin white buffy coat on top of the red cells was collected separately. The plasma was mixed with a total genomic extract from 1000 cultured human lymphocytes (H9 cells) and the DNA extracted using QiaAmp columns (QIAGEN Tissue DNA isolation kit, QIAGEN Inc, Santa Clarita, Calif) according to the manufacturer's instructions. The cells (buffy coat) were resuspended in 1.5 mL phosphate buffered saline (PBS), centrifuged at 2000 rpm for 5 minutes, and resuspended in 100 μL of DNA lysis buffer (1% sodium dodecyl sulfate [SDS], 10 mM Tris HCl, 1 mM EDTA, pH 7.8) per 10^6 cells. Proteinase K was added to a final concentration of 50 μg/mL and the samples were incubated for 1 hour at 55°C. DNA was extracted with phenol/chloroform and the aqueous layer was precipitated with an equal volume of isopropanol. The DNA pellet was rinsed with 75% ethanol and resuspended in water. One μg of purified DNA as quantitated by A_{260} was used per PCR reaction. In earlier studies, we also examined urine specimens from matched patients. Two mL of urine were centrifuged at 12 000 × g for 30 minutes. The pellet was resuspended in PBS and processed as described for plasma.

Selection of oligonucleotide primers and labeling

The primers used for amplification were selected specifically to target a 448 bp region of the OspA gene of *B. burgdorferi* (accession no. gene bank database L23138). These primers demonstrate complete homology with *Borrelia burgdorferi sensu stricto* and the closely related species *Borrelia garinii* and *Borrelia afzelii,* but do not crossreact with the more distantly related *Borrelia hermsii*, which do not cause Lyme disease. An oligonucleotide corresponding to an internal sequence between the primers was modified with NH_2 at the 5′ end and subsequently labeled with alkaline phosphatase for use as a probe. The sequences of the primers and probe used in this study are:

Sense: OspA-L5′-TGGATCTGGAGTACTTGAAG-GCGT-3′

Antisense: OspA-R5′-AGTGCCTGAATTCCAAGCT-GCAGT-3′

Probe: -5′-NH_2-TAA-CAA-GAG-CAG-ACG-GAA-CCA-GAC-3′

PCR analysis

The PCR reaction consists of a buffer mixture containing 60 mM Tris-HC1, 15 mM ammonium sulfate, 2 mM $MgCl_2$ pH 9.0, 50 pMol each OspA primer, 200 μm (each) deoxynucleotide triphosphates (dATP, dGTP, dCTP, and dTTP), water, and 2.5 units of taq polymerase in a final volume of 100 μL. One μg of DNA extract from buffy coats or extract from 200 μL of plasma was used per PCR reaction. The PCR reaction was carried out in a Perkin-Elmer Cetus Thermocycler System 9600 using the following conditions: a denaturation step at 94°C for 2 minutes followed by three-step 35 cycles at 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds. A final elongation step at 72°C for 10 minutes was included. The reaction was terminated at 4°C until the samples were removed.

Controls

Stringent quality control practices were observed to assure the validity of the PCR system. Sample processing and preparation of PCR reagents were carried out in separate areas within a laboratory dedicated exclusively to preamplification steps. All reagents were pretested and prealiquoted for one-time usage. All postamplification steps (eg, detection) were carried out in a different laboratory.

Carefully selected negative, positive, and sensitivity controls were included for all stages of the testing procedure and were included in each run. These controls included a serial dilution of *B. burgdorferi* DNA ranging from 8 to 2000 copies. A positive signal for at least the 30-copy sample and correspondingly stronger signals for the higher-copy samples must be observed for the run to be accepted.

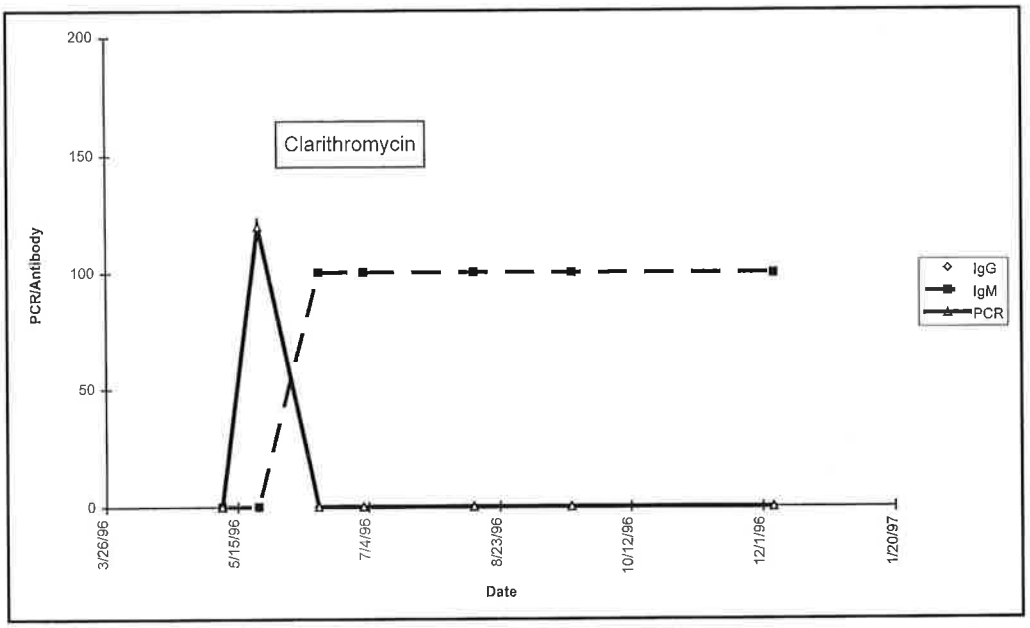

Fig 1: Early-stage Lyme disease patient no. 53. At the time of initial visit, this patient presented with erythema migrans and flu-like symptoms. He was IgG and IgM negative but PCR positive. Patient was treated with clarithromycin, became PCR negative within 1 week, and remained PCR negative at all subsequent visits. IgM positivity appeared shortly following onset of therapy and persisted throughout the period of observation. IgG remained negative.

Every run also contained extracts from at least one normal donor (negative control) and from blood samples from normal donors spiked with 400 copies of *B. burgdorferi* DNA (positive control), which were taken through the entire extraction, amplification, and detection procedure. Additional negative controls included extracts from H9 cells (2 ng) and buffer only (5 μL) controls to ensure that no previously generated amplicons had contaminated a run.

To rule out false-positive reactions, all clinical specimens were split in two so that one aliquot could be amplified with the OspA primers while the duplicate aliquot was tested for the presence of PCR inhibitors. For the inhibitor test, each plasma specimen was spiked with 1000 copies of human genomic DNA (from H9 cells) and amplified with primers for HLADQα, a single copy gene present in all human cells.[29] The buffy coat DNA already contained human sequences and did not need to be spiked. PCR products were fractionated on agarose gels, and the HLADQα products was examined by ethidium bromide staining. Any specimen that gave a negative or low specific signal for HLADQα was reextracted and reamplified. If the result remained low or negative, the specimen was considered uninterpretable.

Detection of PCR products by Southern Blot hybridization

Twenty μL of PCR products were mixed with 5 μL of 5× loading dye (xylene cyanol and bromphenol blue), loaded on a 2.0% agarose gel, and electrophoresed at 100 volts for 1 hour. The gel was stained with ethidium bromide and the DNA was visualized by photography using an ultraviolet (UV) transilluminator. The gel was treated with 0.4 N NaOH and the DNA transferred to nylon membrane overnight (12 to 15 hrs) at room temperature by Southern blotting. After transfer, the membrane was air dried, UV crosslinked at 1200 μJoules, and rinsed in water. The detection of PCR products was achieved by hybridization of the membrane with an alkaline phosphatase-labeled oligoprobe and detected with a chemiluminescent substrate. The alkaline phosphatase-labeled probes were synthesized with an amino group that was incorporated at the 5′-end of the oligonucleotide for further labeling with the alkaline phosphatase probe. Probe labeling was carried out using the Quantum Yield Hybridization and Detection kit (Promega) following manufacturer's instructions.

Antibody tests

The plasma fractions were tested for the presence of *B. burgdorferi* antibodies using inhouse IgM and IgG ELISA, IgM and IgG Western blots, and an IgM capture assay.

RESULTS

The PCR methods used in these studies provide a highly sensitive and specific measure of *B. burgdorferi* in clinical specimens. The primers selected for OspA amplification of the *Borrelia* genome were selected from conserved regions of the OspA gene of *B. burgdorferi sensu lato.* In validating the performance of these primers, we consistently obtained a positive signal with extracts of 50 copies of cultures of several different isolates of *B. burgdorferi sensu stricto*, *B. afzelii,* and *B. garinii.* In contrast, extracts with up to 10^6 copies of *B. hermsii* were negative. No signal was detected with plasma or buffy coat extracts

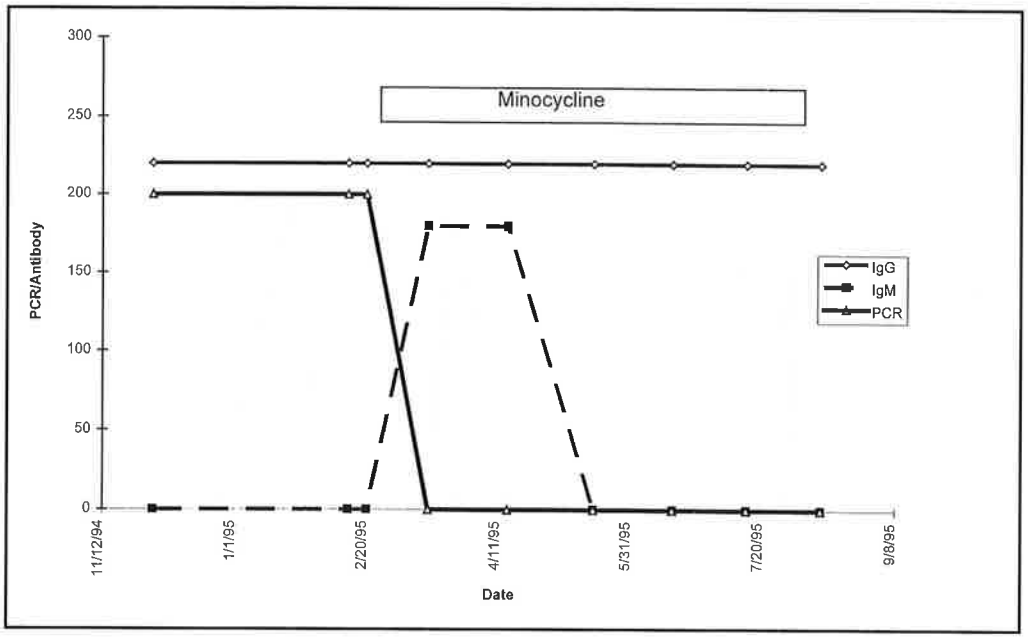

Fig 2: Late-stage Lyme disease patient no. 16. This patient had Lyme disease of over 5 years' duration and had presented with several neurologic manifestations of Lyme disease. Patient was IgG and PCR positive but IgM negative at the time of enrollment. Following administration of minocycline therapy, the patient became PCR negative and IgM positive. IgM positivity declined with time, whereas IgG positivity persisted.

of 40 normal blood donors. Reconstitution studies in which blood samples were spiked with serial dilutions of *B. burgdorferi* DNA or intact organisms showed that as few as 10 copies of the purified spirochetal genome per specimen could be detected by this method.

In initial experiments, we compared the yield of *B. burgdorferi* DNA from plasma and buffy coat fractions of blood. In previous studies with more than 2000 blood specimens submitted for Lyme PCR testing, we found that buffy coat fractions were about four- to fivefold more likely to yield a positive PCR result than plasma samples from the same patient (7% versus 1.5% positivity rate for buffy coat and plasma, respectively). In those preliminary studies, very few urine samples from the same patients were found to be PCR positive (<1%).

Analysis of PCR data as a function of therapy revealed specific correlations that could be interpreted as demonstrating clearance of infection following therapy. Some representative results are shown in Figs 1-4 for patients in early- or late-stage disease who were not under antibiotic treatment or for chronic late-stage disease patients who had been under therapy for extended periods.

Fig 1 shows a typical pattern of a PCR-positive early-stage patient. This patient had initially presented with EM and experienced flu-like symptoms. The initial blood specimen was PCR positive, but IgM and IgG negative. Within 2 weeks of treatment with clarithromycin, this patient became PCR negative. The patient also became IgM seropositive and remained positive throughout a 7-month follow-up period. Although the average period for IgM positivity is 2 to 3 months, a prolonged IgM positivity could be indicative of chronic infection or that the infection is reactivated. The IgG titer failed to come up within this period.

Table 1 shows the serology and PCR results of all early Lyme disease patients examined. These patients presented with EM at the time of the first visit and were enrolled in the study. Of 21 of these patients, seven (33%) were PCR positive and 12 (57%) were IgM positive at the time of the first visit. Following antibiotic therapy, all patients, including all seven of the initially PCR-positive patients, were PCR negative. Following therapy, 12 of 16 (75%) of these patients remained or became IgM positive. In contrast, only 2 of 21 (10%) early-stage patients were IgG positive, and 3 of 16 remained IgG positive after antibiotic therapy.

Fig 2 shows a typical pattern for a patient (no. 16) who had presented with late-stage symptoms, but was not initially under therapy. This patient presented with several neurologic manifestations of Lyme disease, including peripheral neuropathy and burning paresthesias. Although this patient had been treated repeatedly for a long duration in the past, he had been antibiotic-free for a 4-month period prior to enrollment in this study. He was IgM negative but IgG positive, and remained IgG positive throughout the 11 months that he was followed. Significantly, this patient exhibited positive PCR results on three sequential visits. When placed on minocycline therapy, he became PCR negative, and remained negative throughout the follow-up period. A corresponding abatement of symptoms occurred at this time. Although this patient originally was IgM negative, he became positive for the first two visits after application of therapy, and was negative thereafter.

Fig 3 shows a patient who recently had entered late-stage Lyme disease (no. 3). Six months prior to entry into

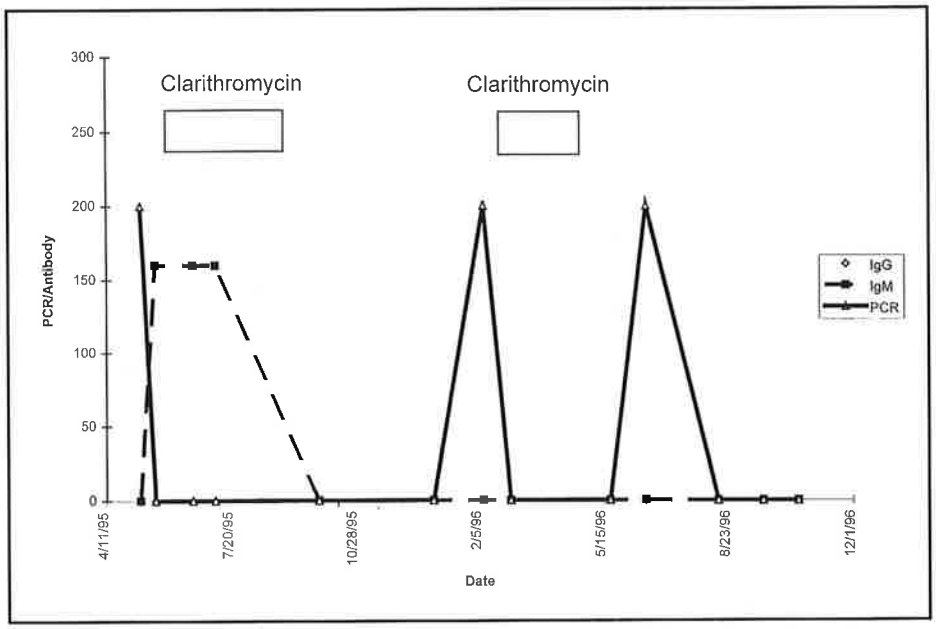

Fig 3: Late-stage Lyme disease patient no. 3. This patient had been infected with Borrelia burgdorferi *only 6 months prior to entry into the study, but already had exhibited arthritic symptoms in various joints. The patient originally was seronegative but PCR positive. He responded well to antibiotic therapy and became PCR negative but IgM positive. Within 4 months of cessation of therapy, he again became PCR positive, and again was cleared with antibiotic. This pattern represents an excellent demonstration of the inverse nature of PCR positivity and application of antibiotic therapy.*

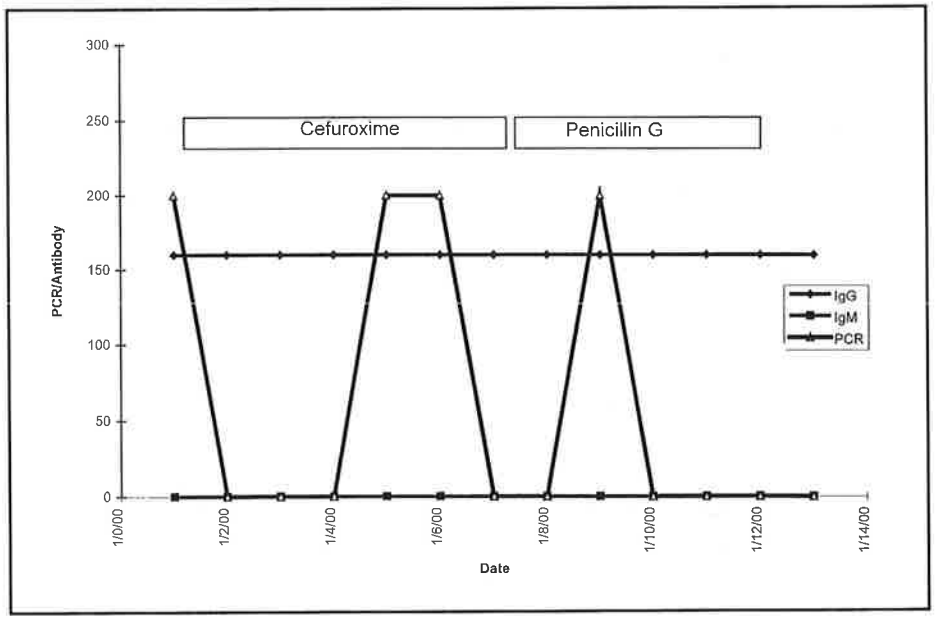

Fig 4: Late-stage Lyme disease patient no. 27. This chronic late-stage Lyme disease patient had severe neurologic symptoms and had undergone extensive periods of antibiotic therapy in the past. He was IgG positive but IgM negative throughout the study. The initial period of PCR positivity appeared to respond well to antibiotic therapy, but sporadic PCR-positive samples were observed on subsequent follow up. The patient showed some response to a change in therapy in that the PCR positivity disappeared and remained negative thereafter. His symptoms never fully resolved and remained intermittent throughout this period. This patient represents a poor responder to antibiotic therapy.

the study, this patient had exhibited EM and other early symptoms, including fever, nausea, and severe headache. At the time of this first visit, he was PCR positive and IgM positive, and complained of arthritis in the knees, toes, shoulders, fingers, and neck, which are characteristic late-stage symptoms. Following administration of clarithromycin therapy, he became PCR negative and noted improvement in his arthritic symptoms. Within 4 months of cessation of therapy, however, he became PCR positive, and again became PCR negative following a second round of antibiotic treatment. Interestingly, yet another transient period of PCR positivity was observed within 3 weeks of discontinuation of that round of therapy. Although the vast majority of patients observed remained PCR negative when therapy was discontinued, 4 of 18 (22%) patients showed reactivation of infection as demonstrated by PCR. This pattern of inverse response to the administration of antibiotic or cessation of therapy is particularly well demonstrated in this patient.

Fig 4 demonstrates another pattern observed in a late-stage chronic patient (no. 14) under therapy. This patient had chronic Lyme disease for a period of more than 8 years, including several bouts of antibiotic therapy in and out of the hospital. His symptoms included meningitis, spastic paraparesis, and meningoencepahalitis. Although the initial treatment resulted in an apparent loss of PCR

Table 1
Early Lyme Disease

Patient no.	Before therapy			After therapy			End of therapy		
	Serology			Serology			Serology		
	IgM	IgG	PCR	IgM	IgG	PCR	IgM	IgG	PCR
4	N	N	N	N	N	N	N	N	N
7	R	N	N	R	N	N			
8	R	R	N	R	R	N	R	R	N
9	R	N	N						
20	R	N	N	R	R	N	R	N	N
24	N	N	P	R	N	N			
25	R	N	N	R	N	N			
26	R	N	P	R	N	N			
31	R	R	P						
32	R	N	P	R	N	N			
34	R	N	P	R	N	N	R	N	N
39	R	N	N	R	N	N			
40	N	N	N						
41	R	N	N	R	N	N	R	N	N
42	R	N	N	R	N	N	R	N	N
43	N	N	N	N	R	N			
45	N	N	N	N	N	N			
49	N	N	P	N	N	N	N	N	P
50	N	N	N						
51	N	N	N						
53	N	N	P	R	N	N	R	N	N

PCR=polymerase chain reaction; N=negative (Western blot—no specific IgG or IgM bands, or pattern insufficient to satisfy Centers for Disease Control and Prevention [CDC] criteria for positive. PCR—no specific band on Southern blot); R=reactive (Western blot pattern satisfies CDC criteria for positive IgG or IgM reactivity); P=positive by PCR (specific band on Southern blot)

positivity, symptoms did not improve, and in fact, the patient tested PCR positive at three of the six subsequent time points examined. When the therapy was changed to another antibiotic, the patient became PCR negative and remained negative under this drug for the following 5-month period. The symptoms remained variable during this time, however, and although some periods of apparent improvement were observed, no consistent abatement of symptoms took place.

In examining the relationship of PCR and therapy in late-stage Lyme disease patients, we divided these patients into two categories: those who were on long-term antibiotic therapy at the beginning of the study and those who were antibiotic free. Although all these patients presented with late Lyme disease symptoms, the majority of the patients in the former group were chronic patients with persistent, rather than intermittent, symptoms.

Table 2 shows the results of the late Lyme disease patients with intermittent symptoms who were not under therapy at the outset of this study. Of these 20 patients, 10 (50%) were PCR positive at the time of enrollment. Following administration of antibiotic therapy, 8 of the 10 PCR-positive patients became PCR negative. Table 3 shows the results of late Lyme disease patients who were undergoing therapy at the outset of this study. As pointed out, most of these were patients with chronic Lyme disease, and under continuous therapy for 2 to 10 months prior to the start of this study. In this group, three of the eight (37.5%) patients were PCR positive despite the fact that they were under antibiotic therapy. All three of these patients and both of the patients in Table 2 who had remained PCR positive despite administration of therapy became PCR negative when a different antibiotic was substituted.

In contrast to the significant difference in frequency of PCR-positive blood samples prior to and following therapy (17 of 41 [41%] versus 2 of 41 [5%] for PCR), the incidence of IgG and IgM seropositivity was similar (17 of 41 [41%] versus 22 of 43 [51%] for IgM; 26 of 36 [72%] versus 22 of 43 [51%] for IgG) for the before- and after-therapy groups. Thus, PCR appears to provide a meaningful indicator of response to therapy, whereas antibody positivity does not.

Table 2
Late Lyme Disease Before Therapy

	Before therapy			After therapy			Change of therapy			End of therapy		
	Serology			Serology			Serology			Serology		
Patient no.	IgM	IgG	PCR	IgM	IgG	PCR	IgM	IgG	PCR	IgM	IgG	PCR
1	N	R	P	N	R	N						
2	N	R	N	R	N	P	R	R	N			
3	R	R	P	N	N	N				N	N	P
6	N	R	P	N	N	N				N	R	N
11	N	R	P	N	N	N				N	N	N
12	N	R	N	N	R	N				N	R	N
13	N	R	N	N	R	N						
14	R	R	N	R	N	N				R	R	N
16	N	R	P	N	R	N						
17	N	R	P	N	R	N						
19	R	R	P	R	R	N						
21	R	R	P	R	R	N						
23	N	R	N	N	R	N						
28	N	R	N	N	R	N						
35	N	N	P	N	N	P	N	N	N			
36	N	R	N									
44	N	R	P	N	R	N				N	R	N
47	R	R	N	R	R	N						
52	N	R	N	N	N	N						
55	N	N	N	N	N	N				N	R	N

PCR=polymerase chain reaction; N=negative (Western blot—no specific IgG or IgM bands, or pattern insufficient to satisfy Centers for Disease Control and Prevention [CDC] criteria for positive. PCR—no specific band on Southern blot); R=reactive (Western blot pattern satisfies CDC criteria for positive IgG or IgM reactivity); P=positive by PCR (specific band on Southern blot)

DISCUSSION

The PCR assays described in this paper provide a very sensitive direct detection of *B. burgdorferi* in blood. The extensive precautions and multiple controls at the various steps in this study ensure reliable results. A significant difference between our study and those previously reported is the use of buffy coat samples as source of DNA for the PCR assays. In matched samples from a single individual, the buffy coat signal is significantly higher than the corresponding plasma or urine specimens.

We currently have no adequate explanation for this observation. We considered the possibility that the differences in yield may be related to processing or stability differences. Experiments in which the plasma samples from patients whose buffy coats showed a positive PCR reaction, however, consistently showed lower probability of obtaining a positive signal than the buffy coats from the same patient. Studies in which these plasma samples were processed directly by the same Proteinase K/phenol extraction method used for buffy coats, or premixed with lymphocytes from uninfected donors prior to processing, failed to show an increase in *B. burgdorferi* DNA recovery.

An alternative explanation for this observation may be that the spirochetes associate with lymphocytes, or are phagocytosed in the process of host immune response. In fact, at a recent Lyme Disease Conference (Ninth Annual Scientific Conference on Lyme Borreliosis, Boston, Mass, 1996), David Dorward presented a video showing *B. burgdorferi* actually attaching to B lymphocytes and becoming engulfed by them. Alternatively, *Borrelia* may exist in aggregates or immunocomplexes that simply copurify with the buffy coats. In any case, our studies suggest that these fractions yield more meaningful PCR results and are worthy of further study.

The correlations presented in this paper provide further support to the utility of PCR assays in monitoring therapy. First, late Lyme patients under therapy have a much lower rate of PCR positivity (2 of 41 [5%]) than those not under therapy (17 of 41 [41%]). Secondly, when individual PCR-positive Lyme patients are followed longitudinally from prior to therapy to posttherapy, 15 of 17 [88%], become PCR negative. A small but significant subset of patients (5 of 38 [13%]) remain PCR positive even when undergoing therapy suggestive of antibiotic resistance.

Table 3
Late Lyme Disease, Long-term Therapy

Patient no.	After therapy			Change of therapy			End of therapy		
	Serology			Serology			Serology		
	IgM	IgG	PCR	IgM	IgG	PCR	IgM	IgG	PCR
18	N	R	N						
22	N	R	P	N	R	N			
27	N	R	P	N	R	N	N	R	P
33	R	R	P	R	R	N			
37	R	R	N				N	R	N
38	R	R	N						
48	N	R	N				N	R	P
54	N	R	N						

PCR=polymerase chain reaction; N=negative (Western blot—no specific IgG or IgM bands, or pattern insufficient to satisfy Centers for Disease Control and Prevention [CDC] criteria for positive. PCR—no specific band on Southern blot); R=reactive (Western blot pattern satisfies CDC criteria for positive IgG or IgM reactivity); P=positive by PCR (specific band on Southern blot)

Even here, however, five of five (100%) patients became PCR negative following a switch in therapy. Finally, within several months of cessation of therapy, 4 of 18 (22%) patients became PCR positive. Moreover, a temporal relationship between PCR results and onset of therapy was observed in that patients became PCR negative within 2 weeks of administration of therapy (Figs 1-4). Taken together, these trends show an inverse relationship between therapy and PCR-positive blood samples.

Only 7 of 21 (33%) early Lyme disease patients and 10 of 20 (50%) late Lyme disease patients not on antibiotic therapy were PCR positive. Whether the PCR-negative patients were not at a spirochetemic stage or whether the sensitivity of the PCR assay is insufficient to detect all positive patients is unclear. Sensitivity may be an issue, as in many cases, only a weak PCR signal is observed on the Southern blots, representing fewer than 30 copies of the spirochete in the sample. For such weak samples, duplicate PCR measurements may not give consistent results, so some false-negative results are likely to occur. We believe, however, that the positive results observed are true indications of the presence of *Borrelia* DNA in the specimen. The positive interpretation is dependent on the presence of a specific hybridization with internal sequences to provide a band of defined size. The inclusion of multiple negative controls in each run helped ensure against the possibility of false-positive signals due to amplicon contamination. Positive PCR reactions are seen most frequently in patients not on antibiotic therapy, and in many cases are followed by the appearance of an IgM-specific response. Such a pattern of seroconversion to IgM positivity (Figs 1-3) is expected to follow periods of active spirochetemia, further corroborating the validity of the PCR results. Where PCR positivity was seen in patients under antibiotic therapy, these patients became negative immediately after change to another antibiotic.

In contrast to the high correlations of PCR results with therapy, serologic results are completely independent of therapy, and in the case of IgG, tend to remain constant or gradually decrease with time; IgM titers tend to be of relatively short duration and are found infrequently in late-stage patients. Thus, antibody levels were not found to be informative concerning the status of response to therapy, although in some cases, IgM levels were found to rise within 2 to 3 weeks following a positive PCR reaction, providing indirect evidence of prior active infection.

The correlation of PCR results with the time of administration of therapy is consistent with the interpretation that PCR positivity reflects periods of active replication in the host. As such, these assays should be very valuable in diagnosing current infections, and thereby provide a useful tool to monitor the responsiveness of therapy.

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Effects of Two Commonly Used Anesthetics on the in vitro Growth of *Borrelia burgdorferi* and *Borrelia garinii*

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ABSTRACT

The effects of the anesthetics ketamine HCL and xylazine on the growth and survival of *Borrelia burgdorferi* and *Borrelia garinii* were tested in vitro. These compounds often are used by researchers working with wild mammals, including those attempting isolation of *Borrelia* species.

Ketamine HCl and xylazine with ketamine HCl (xylazine/ketamine HCl, 1:10) have similar minimal inhibitory concentrations and minimal bactericidal concentrations for *B. burgdorferi* and *B. garinii*. Xylazine used alone adversely affects growth and is bactericidal to both species at lower concentrations than ketamine HCl or xylazine/ketamine HCl.

For investigations concerned with isolation of *Borrelia* species from wild mammals, the minimum amount of ketamine HCl or xylazine/ketamine HCl necessary to anesthetize the animal is recommended; use of xylazine alone should be avoided.

Key words: *Borrelia burgdorferi,* antimicrobial, anesthetics

INTRODUCTION

Most studies of the interaction of drugs with *Borrelia burgdorferi sensu lato* are concerned with the minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of antibiotics. *B. burgdorferi* has been found susceptible, with varying degrees, to a number of antibiotics in vitro, including ampicillin, ceftriaxone, cefuroxime, doxycycline, erythromycin, minocycline, penicillin G, and tetracycline-HCL.[1-4] Variation in antibiotic susceptibility can differ among genospecies of *B. burgdorferi sensu lato.*[4] Test results also depend on whether the spirochetes are exposed in vitro or in vivo[5,6] and incubation temperature.[7]

No studies documenting the interactions of Ketaset (ketamine-HCl) and Rompun (xylazine sulfate) with *Borrelia* species have been published. These drugs are commonly used as anesthetics for studies of pathogens and parasites of wild mammals, including *B. burgdorferi.*[8-16] Ketamine HCl is a nonnarcotic, nonbarbiturate anesthetic; xylazine is a nonnarcotic sedative that functions as an analgesic and muscle relaxant.[17]

Ketamine HCl used alone or in tandem with xylazine (xylazine/ketamine HCl) can affect the outcome of experiments with microorganisms such as gram-negative bacteria,[18] *Pseudomonas,*[19] eukaryotic organisms such as *Trypanosoma cruzi* (Chagas disease),[20] and microfilariae (lymphatic filariasis).[21] Ketamine HCl and xylazine also are used to anesthetize wild mammals to attempt isolation of *B. burgdorferi sensu lato.* We investigated the effects of these two anesthetics on the in vitro growth of *B. burgdorferi* and *Borrelia garinii.*

METHODS

The B-31 strain of *B. burgdorferi* and the IP-90 strain of *B. garinii* were tested in BSK[22] and MKP[23] media, respectively, and incubated at 33°C. These media were tested because of their use in attempts to isolate ticks from mammals. Duplicate tubes containing concentrations of 0.2 to 2 mg/mL of ketamine HCl alone, 0.2 to 2 mg/mL of xylazine/ketamine HCl (1:10), and 0.1 to 1 mg/mL of xylazine alone were added to each tube except control tubes and mixed thoroughly. A Petroff-Hauser

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Table 1

MICs and MBCs of Ketamine HCl, Xylazine/Ketamine HCl (1:10), and Xylazine for* Borrelia burgdorferi *(BSK Media) and* Borrelia garinii *(MKP Media)

	B. burgdorferi		*B. garinii*	
	MIC (μg/mL)	**MBC (μg/mL)**	**MIC (μg/mL)**	**MBC (μg/mL)**
Ketamine HCl	600	800	1200	1600
Xylazine/ketamine HCl	600	800	1000	1600
Xylazine	100	300	300	400

MIC=minimal inhibitory concentration; MBC=minimal bactericidal concentration.

Table 2

Dosages of Ketamine HCl and Xylazine Given to Wild Mammals[8,9,16] Based on Common Weights of These Species[24]

Species (weight)	**Ketamine HCl (μg)**	**Xylazine (μg)**
White-footed mouse (20 g) *(Peromyscus leucopus)*	500 to 800	5 to 80
Prairie vole (40 g) *(Microtus ochrogaster)*	1000 to 1600	10 to 160
Raccoon *(Procyon lotor)*	1.5×10^5 to 2.4×10^5	400 to 2.5×10^4

counting chamber and dark-field microscopy were used to count *Borrelia* throughout the study. *Borrelia* were added to media for a final concentration of 5×10^5 cells/mL as recommended by the National Committee for Clinical Laboratory Standards.[24] Spirochetes were counted on days 4, 8, and 15. MICs were determined on day 4 by comparing the minimal dilution in which cell growth differed from the controls. MBCs were determined on days 4, 8, and 15 by recording the dilution/tubes showing no spirochetes. Effects of ketamine HCl, xylazine/ketamine HCl, and xylazine on each species were compared using chi-square analysis.

RESULTS AND DISCUSSION

The growths of *B. burgdorferi* and *B. garinii* were affected similarly by ketamine HCl and xylazine/ketamine HCl (Table 1). Xylazine adversely affects growth of both spirochete genospecies at a much lower concentration. No comparisons between MBCs and MICs of *B. burgdorferi* and *B. garinii* were made because of different growth conditions of each species.

Ketamine HCl can have a minor suppressive action on septicemia in pigs.[19] In *T. cruzi,* ketamine HCl can block in vivo conversion of L-arginine.[20] Ketamine HCl alone, or administered in tandem with xylazine, can increase the number of microfilariae in the peripheral blood of *Meriones unguiculatus* (jirds or Mongolian gerbils).[21] This can cause parasite-induced mortality in the vector mosquito or can be used to increase microfilaremias in jirds with low numbers of circulating microfilariae.

Typical dosages of ketamine HCl and xylazine given to wild mammals[8,9,16] are based on weights of white-footed mice *(Peromyscus leucopus),* prairie voles *(Microtus ochrogaster),* and raccoons *(Procyon rotor),*[24] usually in mg/kg (Table 2). Doses of ketamine HCl and xylazine above the MICs and MBCs for both *Borrelia* species commonly are used and may preclude isolation if injected near sequelae (Table 1). Our study shows that these two anesthetics affect the in vitro growth of *B. burgdorferi* and *B. garinii.* Additional investigations of the effects of these anesthetics on *Borrelia* in vivo should be conducted. For investigations concerned with isolation of *Borrelia* species from wild manuals, the minimum amount of ketamine HCl or xylazine/ketamine HCl (1:10) necessary to anesthetize the animal is recommended; the use of xylazine alone is discouraged.

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Two Lessons From the Canine Model of Lyme Disease: Migration of *Borrelia burgdorferi* in Tissues and Persistence After Antibiotic Treatment

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ABSTRACT

Nymphal or adult Ixodid ticks transmit *Borrelia burgdorferi*, the causative agent of Lyme disease or Lyme borreliosis, to a wide variety of species. Although infection as indicated by seropositivity is common, under natural conditions, clinical disease presents only in a few species, particularly in humans and dogs. The clinical picture (especially the development of acute arthritis) observed in the field and in the laboratory suggests that Lyme disease in dogs and humans is very similar.

Although *B. burgdorferi* is assumed to spread through the body via the hematogenous route, we have evidence that the spirochetes disseminate from the site of the tick inoculation by migration. Between days 55 and 166 postinfection, 500 tissue samples were cultured from 20 tick-infected beagles. Tissues closest to the infection site harbored spirochetes more frequently than did more distant tissues. Of all tested tissues taken from the front quadrant (synovium, lymph node, fascia, and muscle) that contained the site of inoculation, 75% were culture positive. In the opposite front quarter, 60% of the tissues were positive for *B. burgdorferi;* tissues from the hind quarters showed 26% and 16% culture positivity when they originated from the side of exposure or from the opposite side, respectively.

The development of severe arthritis with clinically evident lameness was associated with the side of infection. Of 70 tick-infected and lame dogs, 80% developed the first episode of acute arthritis in joints closest to the tick bites after a median incubation of 68 days. Ten percent of the dogs showed clinically evident lameness in the limb of the opposite front quadrant after 121 days, 8.6% in the ipsilateral hind quadrant after 103 days, and 1.4% in the opposite hind quadrant after 123 days posttick exposure.

We have shown that in untreated dogs, *B. burgdorferi* can persist in connective tissue for at least a year and perhaps for life. In two studies, antibiotic treatment with amoxicillin or doxycycline for 30 days failed to eliminate persistent infection in 11 dogs. Immediately after treatment, borreliae could not be demonstrated, antibody levels declined, and joint lesions were prevented or cured. Live spirochetes, however, persisted in the tissue of at least three dogs as *B. burgdorferi* DNA was detected in all 11 treated dogs for up to 6 months after treatment, at which time antibody levels again began to rise.

Additional therapeutic studies using intravenously administered ceftriaxone or oral azithromycin are underway in an attempt to identify a successful treatment regime.

Key words: *Borrelia burgdorferi*, Lyme arthritis

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INTRODUCTION

Under natural conditions, Lyme disease or Lyme borreliosis occurs in humans, dogs, cats, horses, and cows,[1-4] with the highest incidence in humans and dogs. Although a number of mammalian species become infected with *Borrelia burgdorferi* after tick exposure, the infection often remains subclinical.[5-8] Under experimental conditions, Lyme disease has been induced in a variety of laboratory animals.[9]

Experimentally induced Lyme arthritis in dogs recapitulates that seen in dogs infected in the field. Tick-infected specific-pathogen-free (SPF) beagles developed acute mono- or oligoarthritis after a median incubation time of approximately 66 days,[10] and showed nonsuppurative

polyarthritis when examined during a later phase of infection.[1] As in man, infected dogs produce specific antibodies against *B. burgdorferi,* demonstrated with Western blots and enzyme-linked immunosorbent assays (ELISA). Their sera, however, lack borreliacidal activity and antibodies against the borrelia-specific outer surface protein A (OspA).[11] In experimentally infected dogs, *B. burgdorferi* persisted in tissues for at least 1 year. Spirochetes were isolated mainly from skin, fascia covering muscles, muscle tissue, joint capsules, pericardium, peritoneum, meninges, and lymph nodes draining infected tissues.[12]

In the mammalian host, borreliae can use specific host enzymes. Plasminogen and urokinase-type plasminogen activators bind to the surface of *B. burgdorferi.*[13-15] Plasminogen activators convert plasminogen into active surface-bound plasmin, which in turn can digest high molecular-weight glycoproteins of the extracellular matrix enzymatically. This ability to digest host connective tissue matrices would facilitate in vivo locomotion of the organisms, which are known to be motile in vitro. In this report, we suggest that *B. burgdorferi* migrates actively through tissue and probably finds niches where the organism can survive therapeutic levels of a 30-day antibiotic treatment.

MIGRATION OF *BORRELIA BURGDORFERI* IN TISSUES

Current literature describes Lyme borreliosis as a disease that is initiated with the deposition of *B. burgdorferi* in the host's skin. The bacterium is believed to multiply in the dermis initially, then adjusts to the new environment, migrates to blood vessels, and eventually enters the circulation to colonize distant tissues.[16] As *B. burgdorferi* adheres to and penetrates through endothelial cells in vitro,[17,18] the bacterium was assumed to gain access to the circulatory system in vivo, from which it might be transported to new sites of the body. Although *B. burgdorferi* often has been isolated from the blood, the primary importance of hematogenous dissemination has been assumed rather than proven.

Contrary to the current concept that *B. burgdorferi is* disseminated throughout the body via the hematogenous route, in our studies, *B. burgdorferi* appeared to reach its target organs of dogs by migration through tissues from the site of the tick bite. When 500 tissue samples from 20 tick-infected dogs (25 tissue samples from each dog) were cultured for the presence of live *B. burgdorferi* between 55 and 166 days after tick exposure, the infection rate of the tissue was inversely related to its distance from the tick bite. Tissues closest to the tick bite contained live spirochetes more frequently than did tissues further removed. Seventy-five percent of all cultured tissues (synovial membranes, fascia, muscle, and lymph node) from

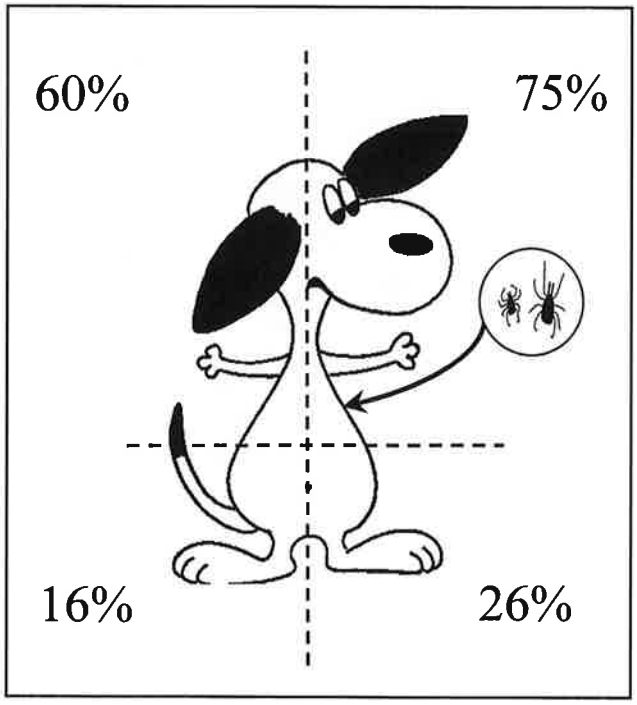

Fig 1: Distribution of B. burgdorferi *in the tissue of 20 dogs cultured 55 to 166 days after tick exposure. The proportions of culture-positive tissues per quadrant are shown.*

the front quadrant ipsilateral to the tick bite were positive for *B. burgdorferi*, as were 60% of the tissues from the opposite front quadrant (Fig 1). In contrast, of all tissues collected from the hind quadrants, 26% from the side of the tick bite and only 16% from the opposite side contained live spirochetes.[10] This pattern occurred up to 5 months postinfection, allowing ample time for bloodborne spread.

Clinical signs seemed to reflect the infection with *B. burgdorferi* in joint tissues. Of 70 dogs that showed severe clinical lameness, 56 had acute arthritis in the limb closest to the tick bite (Fig 2) after a median incubation time of 68 days (42 to 153 days). Seven dogs developed severe clinical arthritis in the joints of the opposite front quadrant, and another six dogs developed arthritis in the limb of the ipsilateral hind quadrant. Only one dog had arthritis in the opposite hind quadrant. Interestingly, the onset of lameness was delayed by a median of 35 days (onset of lameness after 52 to 154 days) in joints further removed from the site of *Borrelia* inoculation (Fig 2).

Histologic examination of tissue from 100 dogs revealed pronounced (joint capsules, tributary lymph nodes) to virtually absent (central and peripheral nervous system) inflammation. In infected dogs with severe lameness, inflamed joints contained increased volumes of synovial fluid containing polymorphonuclear leukocytes (suppurative arthritis), and in some cases, fibrin clots

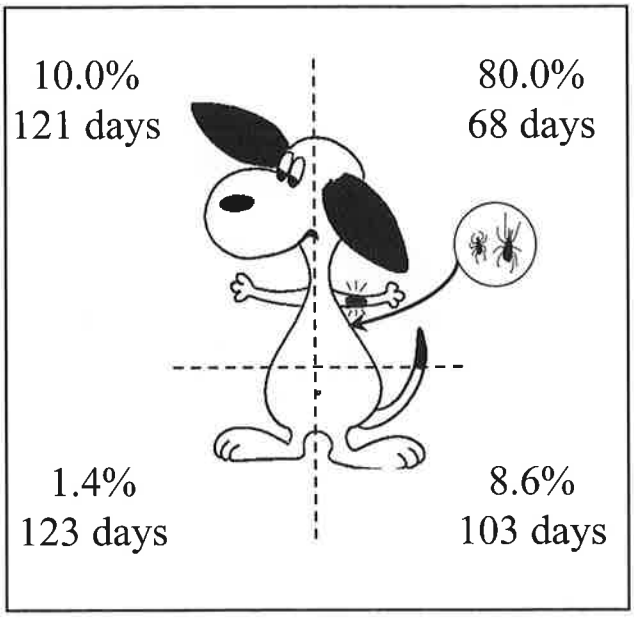

Fig 2: Frequency of lameness and median incubation time in relation to the side of tick exposure. Most dogs (80%) developed acute arthritis in the limb closest to the tick bites after a median incubation period of 68 days. In total, 70 dogs showed acute arthritis with severe lameness in one limb 42 to 154 days after tick exposure.

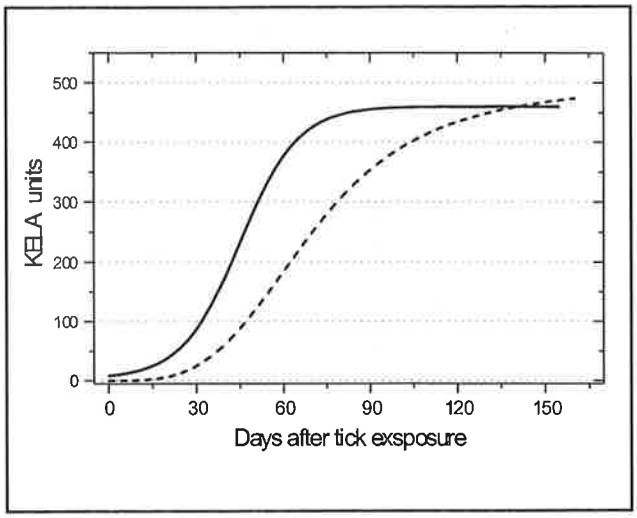

Fig 3: KELA antibody titers to B. burgdorferi *in dogs experimentally infected with ticks. The solid line shows the mean antibody response of dogs exhibiting severe lameness in one or multiple joints. Infected dogs that failed to develop clinical signs responded more slowly (dashed line) and reached maximal antibody titers approximately 140 days after the infection with* B. burgdorferi.

were noted in the joint cavity (fibrinopurulent arthritis). Joints of other limbs showed inflammation of lesser degree (nonsuppurative arthritis) with plasma cells, lymphocytes, and monocyte infiltration in synovial membranes. In dogs with acute forelimb arthritis, axillary lymph nodes in close proximity to inflamed joints showed moderate-to-severe cortical hyperplasia with follicular proliferation and paracortical expansion.

We also investigated infected dogs that failed to develop severe clinical arthritis, but exhibited stiffness and occasional slight lameness (approximately 30% of 100 tick-exposed beagles). Histopathologic examination of these cases revealed nonsuppurative arthritis in multiple joints and mild-to-moderate cortical hyperplasia in the lymph nodes. Inflammation was noted sporadically in other parts of the body in addition to synovial and lymphatic tissues. Meninges and peripheral nerves were found with accumulations of leukocytes. In one case, the pericardium showed lymphocyte infiltration. In general and regardless of clinical signs, in all tick-infected dogs, the severity of joint and lymph node inflammation was related to the site of infection.

Few reports suggest that high concentrations of spirochetes in tissue favor inflammation and disease.[19] We believe that data from these canine studies are in accord with the hypothesis that the number of infecting organisms is important. During the phase of acute arthritis, more tissue samples were culture positive when compared with infected dogs with slight or no lameness examined at a later time period. In lame dogs, on average, 10 tissue samples contained live spirochetes; only two tissue samples were culture positive in dogs without severe clinical signs (25 tissues per dog). The antibody response in infected dogs showing severe, slight, or no lameness supports this theory (Fig 3). Dogs with severe lameness responded quickly to *B. burgdorferi* antigen and reached maximal antibody titers within 50 to 90 days after infection. In contrast, dogs with minor or no clinical signs responded more slowly, reaching maximal antibody titers more than 90 days after the infection with *B. burgdorferi.*

These results suggest that colonization of tissues by *B. burgdorferi* is by centrifugal, active migration and not via the hematogenous route. Bloodborne spread should result in an even distribution of spirochetes throughout the body and the incidence of arthritis should be similar in all joints. Not infrequently, viable spirochetes or borrelia DNA have been found in blood samples from patients,[20] and *B. burgdorferi* can survive in packed blood cells and blood products for several weeks.[21-23] The presence of borreliae in the blood, however, does not necessarily imply that spirochetes use this medium as a vehicle for tissue colonization. Interestingly, a good relationship between the onset of clinical signs and the presence of *B. burgdorferi* in the blood has been reported.[24] During active disease characterized by arthritis or neurologic symptoms, the probability of detecting the spirochete in the blood was the highest. Therefore, the appearance of *B. burgdorferi* in the bloodstream could be a coincidental event.

Spirochetes on their journey through tissues might penetrate blood vessels and enter the circulation. Subsequently, they could be removed by polymorphonuclear neutrophils in the blood or fixed macrophages in the liver and spleen. Under these circumstances, the circulatory system of the host would represent a dead end in the life cycle of the organism. This questions the reliability of blood-based and, in general, of any body fluid-based diagnostic test for intact spirochetes or spirochetal DNA.

Certainly, these tests are of value when positive results are obtained. On the other hand, negative results obtained from body fluids may reflect a low level of infection. In the dog model, we detected *B. burgdorferi* reliably in skin but infrequently in blood by culture and polymerase chain reaction (PCR). We found the organism in the synovium of joints but not in synovial fluids, and in meninges but not in cerebrospinal fluid.[12] We found that tests on tissue samples (especially skin biopsy samples) could confirm or rule out infection more accurately.

A similar observation was made by Bosler et al, who demonstrated that biopsies from canine ear tissue but not blood reliably contained *B. burgdorferi* after *Ixodes scapularis* ticks had fed upon dogs.[25] Infected blood donors still pose some risk for recipients, and this issue should receive some attention in future studies, although to date, no case of infection by blood transfusion has been reported.[26]

The hypothesis of tissue migration is challenged by the fact that about 20% of patients show multiple skin rashes after tick exposure,[27] implying a rapid bloodborne dissemination of *B. burgdorferi.* In natural disease, however, the possibility of other concurrent disorders or multiple tick-transmitted agents' (eg, *Babesia, Ehrlichia)* causing a rash exists. In addition, people living in endemic areas could have been exposed to *B. burgdorferi* previously. Many people do not recall tick bites,[27] and only a portion of the infected population exhibits clinical symptoms.[28] Multiple exposures to *Borrelia* may induce widespread rashes, or the specific humoral response may be involved. Further, skin rash may result indirectly after infection from thrombocytopenia or vascular injury.

The migration of *B. burgdorferi* through tissue is very important in terms of the pathogenesis and treatment of Lyme disease. An unfavorable outcome in Lyme disease is related to late diagnosis and late treatment.[29] Years of untreated and persistent infection may have allowed the invasion of tissues throughout the body. As we have shown with our dog model, the pericardium and the meninges may become infected within months after tick exposure.[12]

Is there a way to stop the spread of the organism and influence the outcome of the disease? Studies investigating the effects of immediate antibiotic treatment shortly after tick exposure (meta-analysis of published data) have shown that among 600 patients with *Ixodes* bites, no individual in the antibiotic-treated group but 1.4% of those in the untreated placebo group contracted infection.[30] Although these studies were statistically inconclusive, a trend suggesting protection by early treatment was evident. The possibility that inoculated spirochetes are eliminated during the early phase of infection by antibiotic treatment exists.

With the mouse model, Shih et al showed that infection was prevented when a 6-mm disk of skin was removed from the site where the tick bite occurred within 48 hours after tick detachment.[31] Alternatively, antibiotic treatment may reduce the number of organisms, prevent further dissemination, and confine the organisms to a small tissue area around the site of inoculation.

A low level of infection may represent a manageable condition that allows the coexistence of the host and the infectious agent without the development of clinical signs typical for Lyme disease. On the other hand, long-term infection may result in a condition where *B. burgdorferi* can be found throughout the body. As we have shown in a previous study, the presence of *B. burgdorferi* in the tissue can be the cause of the upregulation of certain factors, especially of the chemokine interleukin-8 in the synovium.[10] Multiple sites of infection represent a large number of organisms. Perhaps a widespread infection causes the upregulation of these factors at multiple sites of the body and produces a more severe clinical condition.

PERSISTENCE OF *BORRELIA BURGDORFERI* AFTER ANTIBIOTIC TREATMENT

Untreated *B. burgdorferi* infection in humans and animals generally persists for months or years, and perhaps for life, despite a strong humoral immune response.[1,9,32,33] *B. burgdorferi* tends to invade poorly vascularized connective tissues,[34,35] which may protect them from the immune response. In general, *B. burgdorferi* can be found in tissue rather than in body fluids. We have found *B. burgdorferi to* persist in synovial membranes but not in synovial fluid, in lymph nodes but not in blood, in meninges but not in cerebrospinal fluid, and in pericardium and peritoneum.[12] Another possible explanation for persistence is invasion of cells and intracellular survival.[36-39]

The most commonly used antibiotics for the treatment of acute Lyme disease in humans and dogs are orally administered doxycycline and amoxicillin.[40-43] Doxycycline, a tetracycline, has bacteriostatic activity; amoxicillin, a β-lactam antibiotic, is bacteriocidal. Tetracyclines have intracellular effects but β-lactam antibiotics do not.[44] In addition to the bacteriostatic effect of doxycycline,

tetracyclines recently have been shown to have protective and antiinflammatory effects in joints of animals with inflammatory arthritis.[45-48] Promising results have been reported by using minocycline, another tetracycline.[49] In addition, the macrolides azithromycin and roxithromycin in combination with co-trimoxalone, and clarithromycin have been used for the treatment of early and late symptoms of Lyme disease. The relapse rates varied between 0% in cases with early symptoms and 24% in cases with late symptoms of the disease.[49-51] In the more chronic forms of human Lyme arthritis, intravenous delivery of ceftriaxone, a third-generation cephalosporin, is the treatment of choice.[52-55]

Contradictory reports of the in vivo efficacy of antibiotics in the treatment of Lyme disease exist.[40,43,49,52,56-65] Treatment of early and acute Lyme disease, in most cases, has beneficial effects on clinical signs; treatment of chronic Lyme diseases is less successful. Whether antibiotic treatment eliminates persistent infection remains uncertain. Isolation of *B. burgdorferi;* detection of spirochetal antigen or PCR from skin biopsies, synovial fluids, or urine[66-70]; or the persistence of serum antibodies to *B. burgdorferi*[71,72] have been used as criteria of ongoing infection or recovery frequently. The answer obtained with these techniques may be incomplete, as a low-level persistent infection cannot be ruled out.

As in humans, contradictory results have been obtained in experimental animals.[73-77] Interestingly, in one study in mice,[73] evidence of infection by isolation or PCR was not found by 20 and 30 days after ceftriaxone treatment. Sixty days after treatment, however, some animals again became positive, suggesting that ample time between treatment and testing allowed the organism to recover and replicate to detectable numbers.

We completed two studies of doxycycline or amoxicillin treatment of Lyme disease in SPF dogs.[78] Treatment was initiated 50 and 70 days after dogs were exposed to adult ticks *(I. scapularis)* infected with *B. burgdorferi* in the first and second group, respectively. At this time, dogs were seropositive, cultures from skin biopsy samples were positive, and two dogs in the second trial had shown severe lameness in the limbs closest to the site of infection. The ticks were collected in Westchester County, NY, and had a 60% infectivity rate. Antibiotic treatment was maintained for 30 days. Six infected dogs received approximately 10 mg/kg of body weight of doxycycline orally twice a day. Five infected dogs received approximately 20 mg/kg of body weight of amoxicillin orally (three dogs 3 times a day and two dogs twice a day). Six infected dogs remained untreated. Antibiotic levels, measured in plasma, were at therapeutic levels. After the antibiotic treatment was completed, the dogs were kept for an additional 2 to 6 months before euthanasia was performed.

For isolation of *B. burgdorferi,* skin punch biopsy samples (4-mm disks) were collected from all dogs at monthly intervals. In addition, samples from 23 different tissues were collected from each dog at necropsy, with care to avoid cross-contamination. The tissues included synovial membranes from joints, limb muscle and fascia, lymph nodes, pericardium, peritoneum, and meninges in addition to skin biopsies.

Before the initiation of treatment, cultures from skin biopsy samples were positive in dogs subsequently subjected to antibiotic treatment and in untreated infected (control) dogs. Within 2 weeks of antibiotic treatment, skin punch biopsy samples from all treated dogs became culture negative. The skin biopsy samples remained culture negative in all treated dogs throughout the experiment, with the exception of one sample taken from a dog 6 months after doxycycline treatment, and remained positive in the control dogs.

Isolation attempts from postmortem tissues gave similar results. Two to six months after cessation of treatment, *B. burgdorferi* was isolated from multiple tissues of all six untreated infected control dogs but only from one axillary lymph node from a doxycycline-treated dog and from one axillary lymph node from an amoxicillin-treated dog.

PCR with primers for the OspA gene, which is located on a plasmid, and for the chromosomal 23 rRNA gene of *B. burgdorferi* performed on the same tissues gave different results. PCR was positive for skin punch biopsy samples from all dogs before antibiotic treatment was initiated and remained positive for samples taken from untreated infected control dogs. Within 2 weeks of initiation of the antibiotic treatment, skin punch biopsy samples from two of six doxycycline-treated and two of five amoxicillin-treated dogs became PCR negative; the remaining antibiotic-treated dogs showed weak positive reaction at that time. Between 51 and 134 days after treatment was completed, however, skin punch biopsy samples from all infected and antibiotic-treated dogs again were positive by PCR. In addition, multiple tissues from three of four doxycycline-treated and two of three amoxicillin-treated dogs that were tested had specific DNA bands by PCR. Multiple tissues from untreated infected control dogs also were PCR positive. The question remains whether detected *Borrelia* DNA originates from live spirochetes or is a remnant from infections prior to treatment. Evidence that DNA can be detected in tissue only when live *B. burgdorferi* are present, however, exists.[73,79]

Antibody responses of untreated and treated *Borrelia*-infected dogs were tested by kinetic ELISA (KELA) and by Western blots. Positive KELA levels (>100 units) appeared in all dogs between 6 and 8 weeks after infection, and reached high levels (up to 450 KELA units) at the time when antibiotic treatment was initiated.

Thereafter, titers in control dogs (infected but untreated) increased further and remained high until the dogs were killed 5 to 9 months after infection. Antibody levels declined in all antibiotic-treated dogs by 4 weeks after initiation of treatment and reached negative levels in four of six doxycycline-treated and four of five amoxicillin-treated dogs. In dogs that were kept for 6 months after antibiotic treatment was completed, however, antibody levels began to rise again. Three doxycycline-treated and two amoxicillin-treated dogs responded with increasing titers at that time.

Western blots from one doxycycline-treated, one amoxicillin-treated, and one untreated infected control dog showed similar results. Multiple bands specific for *B. burgdorferi,* such as the 23 kDa band (OspC), the 29 kDa band (OspD), the 39 kDa band, and the 41 kDa band (flagellin), were present in all three dogs at the time of initiation of treatment and remained strong in the untreated control dog thereafter. These bands became weaker within 4 weeks after the initiation of antibiotic treatment (except the band at 23 kDa in the doxycycline-treated dog), but 6 months after antibiotic treatment was completed, they became stronger again (Fig 4).

Joint histopathology of treated and untreated control dogs revealed the following picture: joint lesions characterized by mild-to-severe synovitis in one or more joints with plasma cell and lymphocyte infiltration were seen in four of six control dogs. No significant lesions were seen in any of the amoxicillin-treated dogs and only one of the doxycycline-treated dogs had mild joint lesions by 9 months after infection.

In conclusion, we have shown that treatment with high doses of amoxicillin or doxycycline for a 30-day period was not sufficient to eliminate persistent infection in *B. burgdorferi*-infected dogs. Improvements were noted after the treatment: initially, the spirochete population was reduced below detectable levels, joint lesions were prevented or cured, and antibody levels declined. Persistent borreliae, however, stimulated antibody response again by 6 months after cessation of treatment and the possibility of clinical and pathologic relapses remained. Because of the similarity of Lyme disease in dogs and humans, human Lyme disease probably behaves in a similar way.

The persistence of *B. burgdorferi* in mammalian hosts after antibiotic treatment is reminiscent of other members of the order Spirochaetales. Humans and animals with acute symptoms of leptospirosis respond well to penicillin treatment. *Leptospira*, however, may persist after treatment, and kidney failure with uremia is a frequent consequence several months later.[80,81] Antibiotic treatment of humans with syphilis often fails to eliminate treponemae from the central nervous system during later stages of syphilis.

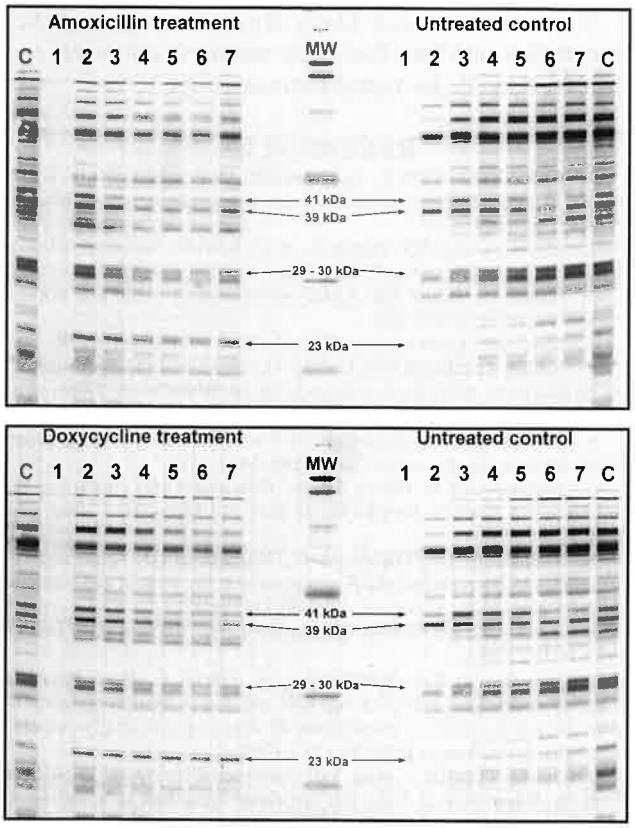

Fig 4: Western blots of dogs infected with B. burgdorferi *and treated 30 days with amoxicillin (top) or doxycycline (bottom), and of a control dog (top and bottom, right panel). In both treated dogs, the intensity of the bands increased until treatment was initiated (lanes 1 and 2; 0 and 64 days after tick exposure, respectively). During and after treatment, most bands faded (lanes 3 to 6; 92, 120, 176, and 260 days after exposure). When the experiment ended, in both treated dogs, KELA antibody titers again increased and Western blot bands appeared more intense (lane 7, 273 days after tick exposure). MW=molecular weight marker; C=control serum—tick-induced infection with* B. burgdorferi.

Where do the spirochetes persist and how do they evade the antibiotic treatment? The migration of spirochetes into poorly vascularized connective tissues may be one explanation. Antibiotic effects may be less effective in metabolically dormant borreliae. Intracellular persistence is a possibility. Perhaps persistent spirochetes develop resistance to antibiotics or new variants emerge.[82] This could be tested by isolation of treatment-resistant borreliae or with antibiotic sensitivity tests in vitro. Once these questions are resolved, designing a treatment that eliminates persistent *B. burgdorferi* infection in humans and animals may be possible.

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Three active life stages of *Ixodes scapularis* photographed with three poppy seeds to indicate size. A male is at the 10 o'clock position; A female is at the 2 o'clock position; and three nymphs are at the 6 o'clock position.

These specimens were collected by flagging in Watchung Reservation, Union County, NJ.

James L. Occi
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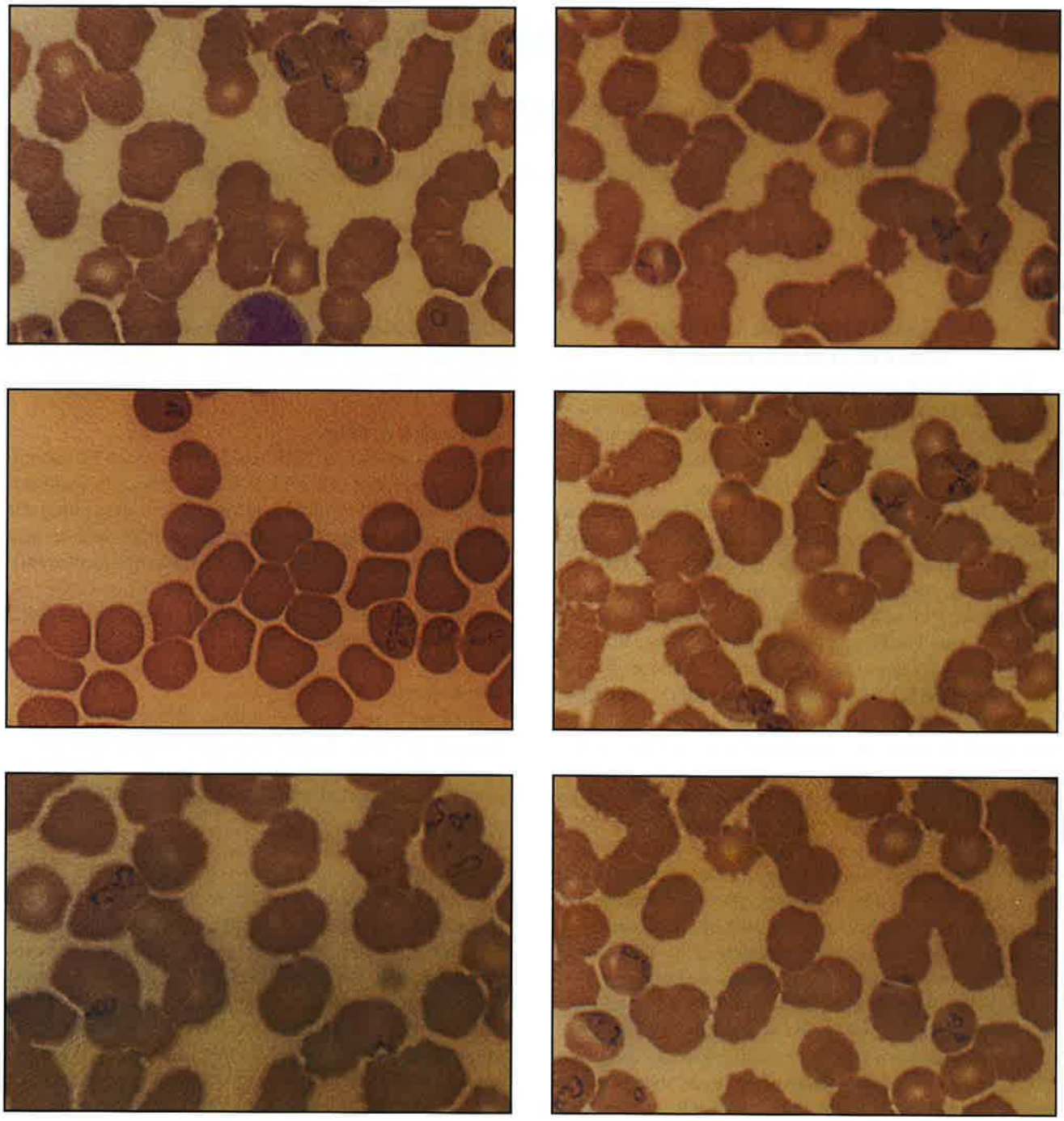

Giemsa-stained blood smears from a fatal case of babesiosis in Missouri demonstrating various stages of the babesial organism (designated MO1) in erythrocytes. Description of the case and laboratory studies can be found in Herwaldt BL, Persing DH, Precigout EA, et al. A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. *Ann Intern Med.* 1996;124:643-650.

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Journal of Spirochetal and Tick-borne Diseases

Dedicated to science and art in spirochetal and tick-borne diseases

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The *Journal of Spirochetal and Tick-borne Diseases* publishes quarterly reviews and original work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

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