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EVIDENCE SUPPORTING THE PRESENCE OF *BORRELIA BURGDORFERI* IN MISSOURI

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Abstract. Although Lyme disease is commonly seen in the southcentral United States, the epidemiology of the disease is poorly defined there. The purpose of this study was to document the presence of *Borrelia burgdorferi* in ticks collected in southeastern Missouri and around the city of St. Louis. Spirochetes were detected and identified as *B. burgdorferi* by immunofluorescent antibody (IFA) tests using the monoclonal antibody H5332 in 1.9% of *Amblyomma americanum* and 2.0% of *Dermacentor variabilis* ticks collected. The identity of IFA-positive organisms was verified by polymerase chain reactions (PCRs) with two different sets of *B. burgdorferi*–specific primers followed by Southern blotting. The DNA sequences of amplified 371-basepair PCR products from two positive Missouri ticks showed 97–98% identity with that obtained by the same method for the B31 strain of *B. burgdorferi*. These results confirm that *B. burgdorferi* is present in questing *D. variabilis* and *A. americanum* ticks in areas of Missouri where Lyme disease occurs. Additional studies are needed to determine the role of these ticks in the epidemiology of Lyme disease in Missouri and neighboring states.

Lyme disease (Lyme borreliosis) is a tick-borne illness caused by *Borrelia burgdorferi* in the United States. The disease has been identified on six continents and the suggested vectors have been principally ticks of the *Ixodes ricinus* complex. Clinically diagnosed Lyme disease is relatively common in Missouri. Satalowich reported 643 cases in Missouri from 1989 to 1992.[1] All these cases met criteria of the Centers for Disease Control and Prevention (Atlanta, GA), and all known tick exposures were in Missouri. Missouri was listed as ninth in incidence of Lyme disease by state in 1990 and eighth in number of cases for the period 1989–1990 (Lyme Disease Surveillance Summary, Centers for Disease Control, Fort Collins, CO, unpublished data)[2]. The rashes on most Missouri patients are consistent with and virtually identical to published erythema migrans rashes.[3] Spirochetes have been identified in silver-stained sections of biopsies from several of the rashes (Masters E, Weil G, unpublished data). Other clinical manifestations of Lyme disease in Missouri patients are similar to signs and symptoms reported nationally.[4]

Borrelia burgdorferi has not been isolated from humans in Missouri or its neighboring states, in spite of many attempts. It has been isolated and grown in culture from ticks in these states only a few times (lone star tick, *Amblyomma americanum* in Texas, black-legged tick, *I. scapularis* in Texas, Georgia, and Oklahoma, and the winter tick, *Dermacentor albipictus* in Oklahoma).[5–7] *Borrelia burgdorferi* has been isolated from the cotton mouse and the white-footed mouse in Georgia and Oklahoma, respectively.[5,8] Because of the small number of isolates, some people have viewed the reports of Lyme disease in Missouri and neighboring states with skepticism. This paper reports evidence for the identification of *B. burgdorferi* in Missouri ticks by indirect fluorescent antibody (IFA), DNA, and 16S rRNA studies.

MATERIALS AND METHODS

Tick collection. The tick collection sites were in rural locations in St. Louis County and in several counties around Cape Girardeau (southeastern Missouri) where Lyme disease patients lived or were exposed to ticks. Free-living ticks were collected from April to September, 1989 by dragging a 1-m^2 flannel cloth through the vegetation. The purpose of the collections was to obtain ticks for examination for the presence of *B. burgdorferi*. Since this was not an ecologic study, the collections were not made on specific grids or repeatedly in the same collection area.

Indirect fluorescent antibody test. The IFA test was used to identify *B. burgdorferi* in collected ticks. The ticks were dissected with sterile forceps and a small portion of the gut was smeared in one well of a sterile, teflon-coated, 12-well microscope slide (Cell-Line Assoc., Newfield, NJ). The smears were air-dried, fixed in acetone for 10 min, and stored at −80°C until they were tested.

The monoclonal antibody H5332 (kindly provided by Dr. A. Barbour, University of Texas Health Science Center, San Antonio, TX) was used in these studies. It binds to the 31-kD outer surface protein A (*osp* A) of *B. burgdorferi* and is specific for that species. Ten microliters of a 1:8 dilution of H5332 in phosphate-buffered saline (PBS), pH 7.2, was placed on each well and incubated for 30 min in a 37°C humidified chamber. The slides were washed by dipping each one vertically 10 times in each of a series of three 250-ml beakers of sterile PBS, pH 7.2. After 10 slides were washed, all solutions were changed. The slides were air-dried. The secondary antibody was fluorescein-labeled goat antibody to mouse IgG and IgM (Kirkegaard and Perry, Gaithersburg, MD). Ten microliters of a 1:20 dilution of the secondary antibody in PBS, pH 7.2, was placed on each well, incubated as above, washed, air-dried, and cover-slipped with sterile 70% glycerol in PBS, pH 7.2. The slides were examined with a Zeiss (Thornwood, NY) Standard 16 epifluorescence microscope equipped with a 40/0.75 Neofluor objective and an exciter-barrier filter set BP 450-490/LP520. A separate positive control slide made with B31 strain spirochetes was always included as a check on the procedure. Smears were considered positive if they had at least five clearly stained spirochetes. If there were fewer than five spirochetes, the smears were considered questionably positive.

Polymerase chain reaction (PCR). The PCR amplification procedure was used to substantiate the IFA identification of the spirochetes as *B. burgdorferi*. It was not feasible to test all midgut smears by PCR. Most midgut smears that were positive or questionably positive by IFA were tested by PCR. The PCR was also performed with a representative sampling of IFA-negative smears from ticks from areas with positive smears and from areas that never yielded positive smears. The source of tick materials for the PCR procedures was the gut smears that were stained by the IFA method. Five microliters of Tris-EDTA buffer, pH 8.0, were placed on the smear, which was vigorously scraped with a plastic pipette tip, and the loosened material was then picked up with the pipette. Samples were boiled for 5 min and processed as described below. Positive controls were *B. burgdorferi* strain B31 and negative controls were midgut smears from laboratory-reared *I. dammini* provided by Dr. Richard Pollack (Harvard School of Public Health, Boston, MA).

Two different PCR methods were used. Method A (used by B-WL, C-SX, DF, and GW) was modified from that described by Rosa and Schwan.[9] The 371-basepair (bp) chromosomal target sequence they described is unique to *B. burgdorferi* and it is reported to be highly conserved in North American isolates.[10] The primers and target sequence are shown in Figure 1. Specimens were amplified with the reagents supplied with the Geneamp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, CT) and primers A and C for 30 cycles (1 min at 94°C, 30 sec at 37°C, and 1 min at 60°C). Pipette tips with filters were used to minimize the possibility of cross-contamination of specimens. The sensitivity of the method was evaluated with *B. burgdorferi* strain B31 (#35210; American Type Culture Collection, Rockville, MD). As few as 1,000 organisms were detectable by agarose gel electrophoresis of the A-C PCR product (Figure 2). Sensitivity was improved to 100 organisms by Southern hybridization of the A-C PCR product with a probe that was itself produced by a PCR of *B. burgdorferi* DNA with primers B and H (Figure 1). The B-H probe was labeled with horseradish peroxidase, and binding of the probe under high stringency conditions to target DNA in Southern blots was detected by chemiluminescence with standard X-ray film (ECL Kit: Amersham Laboratories, Arlington Heights, IL).

In PCR method B (used by RM), 5 μl of sterile distilled water was used to remove the material from two IFA-positive smears of American dog ticks and one smear from a lone star tick. The lone star tick had fewer than five apparent spirochetes. The material was sent to RM at the Rocky Mountain Laboratory in Montana. The samples and negative controls were treated with proteinase K for 30 min at 37°C in PK buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml of gelatin, 120 μg/ml of proteinase K, 0.45% Nonidet P40, and 0.45%

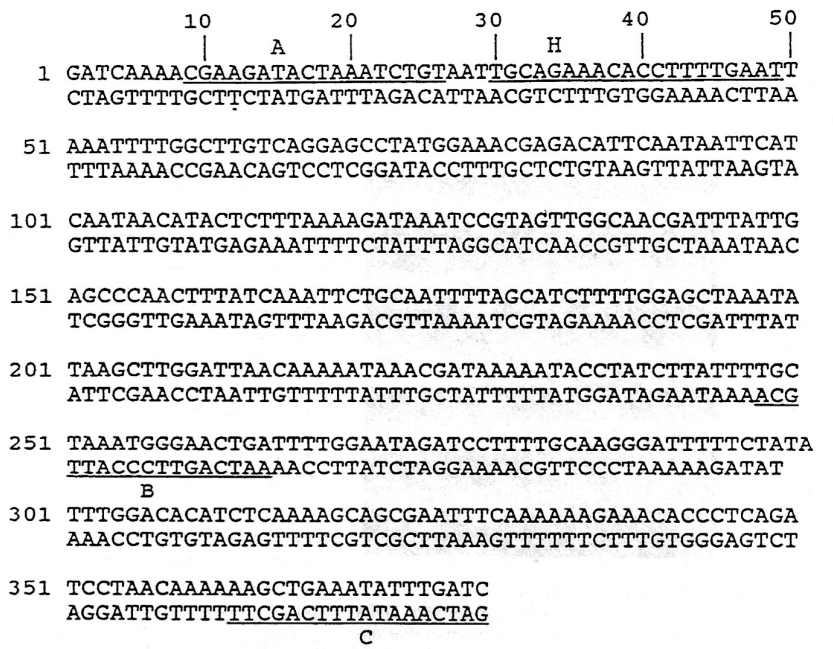

FIGURE 1. Nucleotide sequence of target DNA in the polymerase chain reaction (PCR) assay specific for *Borrelia burgdorferi* (adopted from Rosa and Schwan[10]). Single-stranded oligonucleotide corresponding to the sequences designated A and C represent PCR primers. Oligonucleotides B and H represent the internal primers used to amplify a DNA probe that was used to confirm the identity of the product of the PCR with primers A and C by Southern blot.

Tween 20). The proteinase K was inactivated by boiling for 20 min. The entire sample was overlaid with mineral oil and used in the PCR. The primers targeted 16S rRNA gene sequences specific for Lyme disease isolates and have been previously described.[10] All reagents were from the Geneamp kit and were used as instructed by the manufacturer (Perkin-Elmer-Cetus). Thirty-five amplification cycles were performed as follows: 40 sec at 94°C, 20 sec at 50°C, and 1 min at 72°C. After amplification, the mineral oil overlay was removed, the reactants were extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol. Amplification products were resuspended in tracking dye (15% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol) and electrophoresed in a 1% agarose gel in standard Tris-acetate-EDTA buffer. Following visualization of the amplification products by staining the gel with ethidium bromide, the DNA was transferred onto Genescreen (Dupont-New England Nuclear, Boston, MA) membranes for Southern blot analysis as previously described.[11] Southern blots were probed with the 16S-B oligonucleotide probe (5′-GGGGAATAATTATCTCTAAC), which complements bases 1009–1028 of the *B. burgdorferi* 16S rRNA sequence. Hybridizations and washes were conducted as previously described.[11] The blots were exposed to XAR5 film (Eastman Kodak, Rochester, NY) with intensifying screens at −70°C.

Sequence analysis of *B. burgdorferi* DNA. The 371-bp DNA fragment obtained by amplification of material from infected ticks or from bacterial culture with primers A and C was gel purified, subcloned into the TA cloning vector (pCR1000; Invitrogen, San Diego, CA), and subsequently transferred into pBluescript (Stratagene, La Jolla, CA) for double-stranded sequencing with T7 and T5 primers. Two TA clones from each specimen were sequenced (both strands). Sequence comparisons were performed with PC-Gene software (Intelligenetics, Mount View, CA).

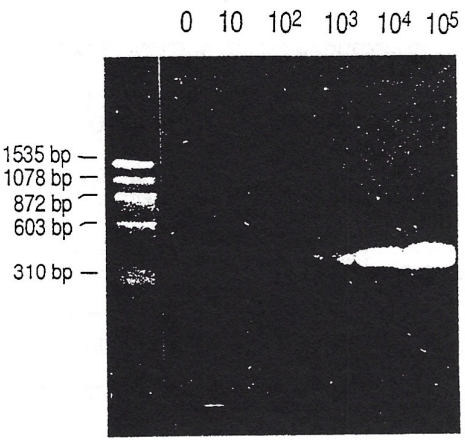

FIGURE 2. Sensitivity of the polymerase chain reaction (PCR) assay for *Borrelia burgdorferi* DNA (method A). One-tenth of the reaction product was analyzed by agarose gel electrophoresis. A stock sample of boiled *B. burgdorferi* isolate (B31) was serially diluted in water. The number of *B. burgdorferi* cells in the stock sample before boiling (1.4×10^8/ml) was estimated by counting in a Petroff-Hausser Chamber with a darkfield microscope. The numbers in the figure refer to the estimated number of spirochetes in each PCR assay. The sensitivity of the assay was improved by a factor of 10 by Southern blot with labeled probe B-H. bp = basepairs.

TABLE 1

Analysis of tick gut smears for the presence of Borrelia burgdorferi *by IFA using monoclonal antibody H5332*

	Males	Females	Nymphs	Larvae	Total
Amblyomma americanum					
No. tested	202	249	1,283	18	1,752
No. positive	5	6	22	0	33
% positive	2.5	2.4	1.7	0	1.9
Dermacentor variabilis					
No. tested	274	166	3	0	443
No. positive	4	5	0	0	9
% positive	1.5	3.0	0	0	2.0

RESULTS

Indirect fluorescent antibody test. Table 1 shows that 1.9% of the 1,752 lone star ticks and 2% of the 443 American dog ticks examined were positive for the presence of *B. burgdorferi*. Figure 3 shows IFA-positive spirochetes in a midgut smear from an adult female *A. americanum* from Bollinger County, Missouri. Positive ticks were found throughout the study period although the peak numbers were observed in June and July. According to the Missouri Department of Health, the peak incidence of Lyme disease in Missouri is in May, June, and July. Positive ticks were found in seven counties in eastern Missouri: Bollinger, Cape Girardeau, Green, Jefferson, St. Louis, Scott, and Stoddard. Some of the positive ticks were collected from sites where people with Lyme disease had reported tick exposures. Ticks from 11 other sampled counties were all negative by IFA but samples from some counties were quite small.

Detection of DNA. Polymerase chain reaction method A detected *B. burgdorferi* DNA in 38 (70%) of 54 tick smears that were positive or questionably positive by IFA with the monoclonal antibody H5332 (Table 2). These positive ticks included adult *D. variabilis* and nymphs and adults of *A. americanum*. The IFA-negative ticks from areas of diagnosed Lyme disease were sometimes positive by PCR but no positive PCR test results were obtained from smears of IFA-negative ticks from areas believed to be free of Lyme disease or from smears of laboratory-reared *I. dammini*. Sequences amplified from two Missouri ticks (one lone star tick and one American dog tick) were compared with the sequence obtained with the B31 reference strain (Shelter Island, NY) in Figure 4. The Missouri sequences were 97% and 98% identical with that of the reference strain.

Three tick smears were tested for *B. burgdorferi* DNA by PCR method B (16S rRNA target

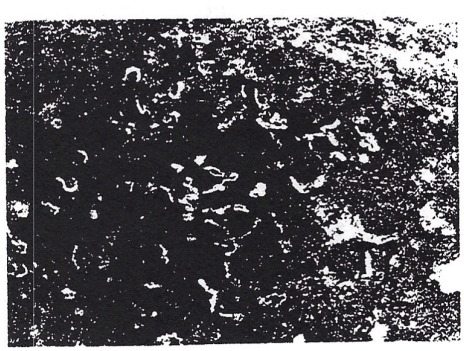

FIGURE 3. Fluorescence photomicrograph showing spirochetes labeled with monoclonal antibody H5332 in a midgut smear from an adult female *Amblyomma americanum* tick from Bollinger County, Missouri (original magnification × 400).

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B31       CGAAGATACTAAATCTGTAATTGCAGAAACACCTTTTGAATTAAATTTTG    50
MO27      CGAAGATACTAAATCTGTAATTGCAGAAACACCTTTTGAATTAAATTTTG    50
MO510     CGAAGATACTAAATCTGTAATTGCAGAAACACCTTTTGAATTAAATTTTG    50
          **************************************************

B31       GCTTGTCAGGAGCCTATGGAAACGAGACATTCAATAATTCATCAATAACA   100
MO27      GCTTGTCAGGAGCCTATGGAAACGAGACATTCAATAATTCATCAATAACA   100
MO510     GCTTGTCAGGAGCCTATGGGAACGAGACATTCAATAATTCATCAATAACA   100
          ******************.*******************************

B31       TACTCTTTAAAAGATAAATCCGTAGTTGGCAACGATTTATTGAGCCCAAC   150
MO27      TACTCTTTAAAAGATAAATCCGTAGTTGGCAACGATTTATTAAGTCCAAC   150
MO510     TACTCTTTAAAAGATAAATCCGTAGTTGGCAACGATTTATTAAGTCCAAC   150
          ****************************************.** *****

B31       TTTATCAAATTCTGCAATTTTAGCATCTTTTGGAGCTAAATATAAGCTTG   200
MO27      TTTATCAAATTCTGCAATTTTAGCATCTTTTGGAGCTAAATATAAGCTTG   200
MO510     TTTATCAAATTCTGCAATTTTAGCATCTTTTGGAGCTAAATATAAGCTTG   200
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B31       GATTAACAAAAATAAACGATAAAAATACCTATCTTATTTTGCAAATGGGA   250
MO27      GATTAACAAAAGTAAACGATAAAAATACCTATCTTATTTTGCAAATGGGA   250
MO510     GATTAACAAAAATAAACGATAAAAATACCTATCTTATTTTGCAAATGGGA   250
          ***********.**********************************

B31       ACTGATTTTGGAATAGATCCTTTTGCAAGGGATTTTTCTATATTTGGACA   300
MO27      ACTGATTTTGGAATAGATCCTTTTGCAAGCGATTTTTCTGTATTTGGACA   300
MO510     ACTGATTTTGGAATAGATCCTTTTGCAAGCGATTTTTCTGTATTTGGACA   300
          ***************************** *********.**********

B31       CATCTCAAAAGCAGCGAATTTCAAAAAAGAAACACCCTCAGATCCTAACA   350
MO27      CATCTCAAAAGCAGCGAATTTCAAAAAAGAAACACCCTCAGATCCTAACA   350
MO51      CATCTCAAAAGCAGCGAATTTCAAAAAAGAAACACCCTCAGATCCTAACA   350
          **************************************************

B31       AAAAAGCTGAAATATTTGATC   371
MO27      AAAAAGCTGAAATATTTGATC   371 Dermacentor variabilis female
MO51      AAAAAGCTGAAATATTTGATC   371 Amblyomma americanum female
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FIGURE 4. Alignment of the amplified DNA sequences obtained by the polymerase chain reaction with primers A and C with a reference strain of *Borrelia burgdorferi* (B-31) and two indirect fluorescent antibody–positive ticks (M027 and M0510). Identity of nucleotides is shown by the asterisks. Differences are shown by the dots or spaces.

sequence). The two IFA-positive smears from American dog ticks had amplification products of the appropriate size after PCR (Figure 5). The questionable smear from the lone star tick was PCR negative. Southern blot analysis of the amplification products from the tick gut smears using the 16S-B hybridization probe demonstrated that the amplification products were derived from the 16S rRNA gene and were not due to spurious amplification. Amplification product was not detected in the *A. americanum* sample or in the negative control.

DISCUSSION

Erythema migrans rashes associated with tick exposure and other clinical manifestations of Lyme disease are relatively common in southeastern Missouri. Although biopsy specimens from individuals with these rashes have shown spirochetes after silver staining, no spirochetes have been cultured from those with rashes in spite of many attempts.

In this report, spirochetes from the lone star ticks and the American dog ticks have been

TABLE 2

Results of polymerase chain reaction (PCR) analysis of ticks collected in Missouri and tested for the presence of Borrelia burgdorferi *by an indirect fluorescent antibody (IFA) test*

IFA result	No. positive/ no. tested*	% positive
Positive	27/39	69
Questionably positive	10/14	71
Negative†	14/36	39
Negative‡	0/20	0

* The difference in PCR positivity observed between the combined IFA-positive and questionably positive ticks versus IFA-negative ticks from areas with questionably positive ticks was statistically significant (Yates' corrected $\chi^2 = 7.16$, $P = 0.007$).
† From areas with ticks positive by IFA.
‡ From areas with no ticks positive by IFA.

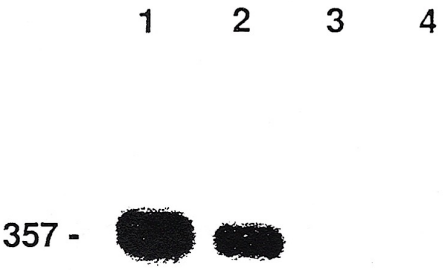

FIGURE 5. Southern blot analysis of *Borrelia burgdorferi* specific products amplified by method B of the polymerase chain reaction (PCR) used. The PCR products were fractionated by agarose gel electrophoresis, blotted, and tested with the 16S-B probe. Lanes 1 and 2, *Dermacentor variabilis;* lane 3, *Amblyomma americanum;* lane 4, negative control (water). The value on the left is in basepairs.

identified as *B. burgdorferi* by IFA tests with the monoclonal antibody H5332 and molecular methods. The results of the PCR amplification of the material scraped from the IFA slides did not agree completely with the IFA results. Some negative IFA readings were positive by PCR. We were very conservative in our IFA readings and small spirochetes or fragments may have been scored as negative although DNA from these fragments might have been amplified. The IFA-positive and PCR-negative results may be due to incomplete removal of material from the smear. Therefore, the IFA results in Table 1 may underestimate the incidence of *B. burgdorferi* in Missouri ticks. The high level of homology of the 371-bp chromosomal fragment amplified from Missouri spirochetes with the B31 strain of *B. burgdorferi* may be due to the highly conserved nature of the chromosomal DNA amplified with primers A and C.[9] Some *B. burgdorferi* strains from two different sources have shown 99.9% homology.[12] The 16S rRNA signature nucleotide sequences used in this study are species-specific for spirochetes associated with Lyme disease, and PCR primers directed against them differentiate *B. burgdorferi* from *B. hermsii, B. garinii,* and other *Borrelia* species.[13] These procedures showed that the spirochetes from *D. variabilis* were *B. burgdorferi.* Therefore, the results of these three tests strongly suggest that the spirochetes in Missouri ticks are *B. burgdorferi.*

Some mention must be made of the possibility of cross-contamination of *B. burgdorferi* bacteria or DNA during the IFA procedure. We took numerous precautions in the handling of specimens and in the washing procedures. Approximately 190 12-well slides were tested by IFA in this study and only three had IFA-positive smears in adjacent wells. The vast majority of the 12-well slides were completely negative by IFA. Therefore, even if some cross-contamination did occur, we believe that it could not account for all the positive PCR results presented here. Although PCR results often differed from the IFA, positive PCR results were significantly associated with IFA positivity.

Successful culturing of *B. burgdorferi* from ticks from Missouri and other southcentral and southeastern states has been rather rare, in spite of numerous attempts. It is possible that the spirochetes from the lone star and American dog ticks have special nutritional requirements that are not provided by Barbour, Stoener, Kelley II media. The reservoir for Missouri spirochetes is not known, and it is possible that it may impose some special nutritional requirements for growth of the spirochetes. Further studies on culturing need to be done.

Although the results of transmission studies with the lone star and American dog ticks have all been negative, these ticks appear to be the best candidates for vectors of *B. burgdorferi* in Missouri and other southern states at this time.[6, 14–16] They readily bite humans. Adult lone

star ticks have been removed from several people who later developed physician-diagnosed erythema migrans rashes at the site of the bite and who were successfully treated with antibiotics (Masters E, Craig G, Notre Dame University, Notre Dame, IN, unpublished data). Schulze and others have suggested that the lone star tick might be a vector of *B. burgdorferi* in New Jersey.[17] Although the southern strain of *I. scapularis* is present in Missouri, it is rather rare in the questing state and we have seldom found any in the summer months (May–September) in contrast to the summer abundance of the northern strain of *I. scapularis* (formerly *I. dammini*) in the northeastern and northcentral states.[18, 19] *Ixodes scapularis* is usually collected on deer and it is seldom a pest on people in Missouri (Feir D, unpublished data). In addition, none of the 287 adult *I. scapularis* collected near St. Louis were positive by IFA with monoclonal antibody H5332 (Xie C-S, Feir D, unpublished data). Therefore, it does not appear to be a good candidate as a vector in Missouri at this time. Much more data need to be obtained to explain all aspects of Lyme disease in the southern half of the United States and to relate these findings to Lyme disease in the northeastern, northcentral, and western United States and other parts of the world.

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