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Genetic Diversity of *Borrelia burgdorferi* and Detection of *B. bissettii*-Like DNA in Serum of North-Coastal California Residents[▽]

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In North America, Lyme borreliosis (LB) is a tick-borne disease caused by infection with the spirochete *Borrelia burgdorferi*. We studied the genetic diversity of LB spirochetes in north-coastal California residents. Spirochete DNA was detected in 23.7% (27/114) of the study subjects using a PCR protocol optimized for increased sensitivity in human sera. Californians were most commonly infected with *B. burgdorferi ospC* genotype A, a globally widespread spirochete associated with high virulence in LB patients. Sequence analysis of *rrf-rrl* and *p66* loci in 11% (3/27) of the PCR-positive study subjects revealed evidence of infection with an organism closely related to *B. bissettii*. This spirochete, heretofore associated with LB only in Europe, is widely distributed among ticks and wildlife in North America. Further molecular testing of sera from residents in areas where LB is endemic is warranted to enhance our understanding of the geographic distribution and frequency of occurrence of *B. bissettii*-like infections.

Lyme borreliosis (LB) is a tick-borne disease of humans living in temperate regions of North America and Eurasia and is caused by infection with a spirochetal bacterium within the *Borrelia burgdorferi* sensu lato complex. Worldwide, 16 named genospecies have been described within the complex (22, 28, 29). Of these, six genospecies have been identified in North American ticks, i.e., *B. americana*, *B. andersonii*, *B. bissettii*, *B. burgdorferi* sensu stricto, *B. californiensis*, and *B. carolinensis* (see references 28 and 29), but only *B. burgdorferi* sensu stricto (hereinafter referred to as *B. burgdorferi*) has been isolated from North American LB patients. The characteristic sign of early-stage LB infection is an erythematous rash called erythema migrans (EM). Untreated infections may disseminate hematogenously to other parts of the body, leading to oligoarticular arthritis and cardiac and neurological abnormalities, including meningitis, facial palsy, and peripheral radiculoneuropathy (24). The highest rates of LB in the United States occur in the Northeast, upper Midwest, and northern California (2), but the assemblages of vectors, host species, spirochetes, and habitats that make up LB transmission cycles differ markedly among these regions (3, 24).

Mendocino County, located in north-coastal California, is a large, climatically and ecologically diverse county containing diverse dense woodland types. In this region, the western black-legged tick, *Ixodes pacificus*, is the primary vector of *B. burgdorferi* transmission to humans. Human exposure to vector ticks is thought to occur primarily in dense woodlands having leaf or fir needle litter ground cover where the density of *B. burgdorferi*-infected nymphs is particularly high (7). Mendocino County typically has one of the highest reported rates of LB in the state (5). The *B. burgdorferi* infection prevalence in *I. pacificus* nymphs collected countywide in 2004 was 4.9% (range, 0 to 22% across 78 sites) (12), and isolated collections of nymphs in small residential communities have yielded infection prevalences of 13.6% in 1992 (7) and 12.4% (range, 3.9 to 41.3%) in 1998 (38).

The population structure of *B. burgdorferi* in *I. pacificus* ticks from Mendocino County differs significantly from that found in *I. scapularis* ticks from the northeastern United States. Nucleotide sequence analysis of the *B. burgdorferi ospC* gene that encodes a major immunoreactive surface lipoprotein, OspC, revealed that only 62% of the genotypes in infected *I. scapularis* nymphs also exist in *I. pacificus* nymphs in Mendocino County (13). A testimony to the divergence of LB spirochetes in California from those in the Northeast is the fact that the most common *ospC* genotype of the Mendocino County nymphs collected in 2004, accounting for 24% of the alleles, was the previously undescribed *ospC* genotype H3 (13).

Increasing evidence suggests that the virulence phenotype of LB spirochetes in humans is associated with genotypic variation within the *ospC* locus. Using data from multiple northeastern studies, Dykhuizen and colleagues (8) ranked the relative invasiveness, or likelihood of hematogenous dissemination, of spirochetes possessing various *ospC* genotypes in the following descending order: A, I, K, B, N, H, C, and M. Although almost 100 cases of LB are reported in Californians each year (5), *ospC* genotypes have only been defined in seven human-derived samples to date: genotypes I, H, and M ($n = 1$ each) in skin biopsy specimens (22, 34) and genotype A in isolates obtained from skin ($n = 1$), blood ($n = 2$), and cerebrospinal fluid ($n = 1$) (34). The diversity of *ospC* genotypes infecting and causing disease in residents of California has not been well established. In particular, we have little understanding of the relationship between spirochete transmission, or dissemination, in humans and the population structure of *ospC* genotypes in local tick vectors.

The goal of our study was to describe the genetic diversity of

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B. burgdorferi sensu lato spirochetes infecting residents of a rural Mendocino County community at high risk for LB (heretofore referred to as CHR). We relate our findings to what is known about the spirochete sequence diversity in tick vectors from the same county and to available clinical and serological data obtained from study subjects (17, 18). A major challenge was overcoming the low sensitivity of most PCR protocols for *B. burgdorferi* sensu lato DNA detection in blood or blood products (1). In addition, we expected lower levels of spirochete DNA in human serum than in ticks and mammalian tissues processed regularly in our laboratory (13, 20, 32). Therefore, we optimized DNA extraction and PCR protocols to better isolate and amplify small quantities of intracellular and cell-free *B. burgdorferi* DNA in CHR sera. Sequence analysis of PCR-positive sera revealed that disseminated *B. burgdorferi* infections in this community typically involved a single, highly virulent *ospC* genotype. Notably, three CHR residents had evidence of infection with a *B. bissettii*-like spirochete, suggesting that *B. bissettii* should be evaluated more closely as a potential human pathogen in the United States.

MATERIALS AND METHODS

Sample and epidemiologic data collection. During an earlier prospective epidemiologic study carried out in 1988 and 1989, serum specimens were collected from current ($n = 99$) or former ($n = 20$) residents of the CHR (population, ~150), which is located several kilometers northwest of Ukiah in Mendocino County, CA (17, 18). Consent forms and questionnaires eliciting demographic and potential LB risk factors were filled out at entry. Detailed descriptions of the study area and the questionnaire were described previously by Lane et al. (18). Immediately following collection in 1988 and 1989, sera were split three ways, stored at −74°C, and promptly tested for the presence of anti-*B. burgdorferi* antibodies (17, 18). No further serological testing was performed during the present study. Extraction and sequence analysis of serum-derived DNA took place in 2009 and 2010. To our knowledge, sera did not undergo a thawing incident between 1988/1989 and 2009. Permission to perform this study was granted by the University of California, Berkeley, Committee for the Protection of Human Subjects.

Statistical analyses were performed in Stata v. 11 (35).

DNA extraction. DNA extraction from serum was carried out using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) spin protocol for blood or body fluids, with several modifications, including an increase in the starting serum volume (600 μl), the addition of carrier RNA (Qiagen; final concentration, 0.05 μg/μl) and proteinase K (Qiagen), and a lengthened 56°C incubation time of 30 min. In 23.9% (26/109) of the 1988 samples, <600 μl of serum was available for DNA extraction and reagent volumes were adjusted according to the amount of serum available according to the manufacturer's recommendations. The modified DNA extraction protocol increased the sensitivity of *B. burgdorferi* sensu lato detection in CHR sera. Using an unmodified QIAamp protocol, only 53% (9/17) of the CHR test sera were *B. burgdorferi* sensu lato *rrf-rrl* PCR positive, compared to 100% (17/17) using the modified protocol (data not shown).

DNA was extracted from the California *B. bissettii* and *Borrelia* sp. culture isolates chosen from our collection (CA55, CA135, CA370, CA372, CA376, CA390, CA402, CA393, and CA400) using the DNeasy Blood and Tissue Kit (Qiagen) protocol for Gram-negative bacteria.

PCR. Sera were screened first for the presence of *B. burgdorferi* sensu lato DNA using a nested PCR targeting the 5S-23S (*rrf-rrl*) rRNA intergenic spacer (IGS) region (20). *B. burgdorferi* DNA-positive samples underwent nested PCR for the spirochete *ospC* gene (4, 13) and the *p66* gene using previously published primers (6). The partial *p66* gene also was amplified in putative *B. bissettii*-positive serum specimens, as well as in the aforementioned *B. bissettii* and *Borrelia* sp. culture isolates. Samples that generated amplicons at at least three loci (*rrf-rrl*, *p66*, and *ospC*) were tested by 16S-23S (*rrs-rrlA*) IGS1 PCR (4, 13). Because of variability in PCR results between loci, we established the criterion that study subjects had to have a PCR-positive result in at least two loci in order to be considered *B. burgdorferi* sensu lato DNA positive.

We compared PCR results for the *rrf-rrl* locus at six melting temperatures, 94, 93, 91, 88, 84, and 82°C, and found that only denaturation at 91°C and 88°C produced a visualizable PCR product in all three dilutions of our *B. burgdorferi* test sample and that products were most abundant following 88°C denaturation (data not shown). Modification of published cycling parameters for *rrf-rrl*, *ospC*, and *p66* amplification performed in the present study included a 3-min template denaturation step at 88°C, followed by 40 cycles of 88°C for 30 s, annealing for 2 min at 55°C for *rrf-rrl*, 50°C for *ospC*, and 45°C for *p66*, and 72°C for 45 s (1 min for *ospC* and *p66*), followed by a final extension at 72°C for 7 min. The annealing step for internal primers lasted 30 s at 58°C for *rrf-rrl*, 1 min at 53°C for *ospC*, and 1 min at 55°C for *p66*. Modification of IGS1 PCR only included an 88°C template denaturation step. *B. burgdorferi* isolate CA4, which belongs to *ospC* genotype A and 16S-23S IGS1 sequence type 10 (22, 39), was used as a positive control. Products from positive samples were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced at the University of California, Berkeley, DNA Sequencing Facility using internal PCR primers.

Extensive efforts were made to reduce the possibility of cross-contamination as recommended by Kwok and Higuchi (16), including the use of aerosol barrier pipette tips, separate UV-irradiated workstations for the setup of master mix preparation and PCRs (including dedicated pipettes), treatment of all environmental surfaces and tube racks with 10% bleach after each use, frequent glove changing, and performing DNA extraction, PCR setup, and PCR product analysis in different rooms. No *Borrelia* culture work was conducted in the laboratory during human sample DNA extraction. PCRs were set up inside an AirClean Systems Combination PCR Workstation (AirClean Systems, Raleigh, NC). One negative control (UV-treated, deionized water) was used for every test sample, and these controls never gave a positive result during the study.

Sequence analysis. The *rrf-rrl*, *p66*, *ospC*, and IGS1 sequences were assembled and manually edited using Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI). Genotype assignments for 525- to 528-bp *ospC* contigs were based on direct comparison to sequences available in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) as described previously (13). *rrf-rrl* and *p66* contigs were aligned with sequences available in GenBank using ClustalX (v1.83.1) and trimmed to 207 bp (*p66*) and 204 bp (*rrf-rrl*) using Mesquite v2.6 (http://mesquiteproject.org). Sequences from putative *B. bissettii*-positive samples were examined by Bayesian analysis of *rrf-rrl* and *p66* sequences using MrBayes v3.1. The best-fit nucleotide substitution model for the *rrf-rrl* locus was HKY + Γ, as selected by MrModelTest2 v2.3 (http://www.abc.se/~nylander/). For the protein-coding *p66* locus, we applied the codon-based model of nucleotide substitution, chosen for its ability to consider both nucleotide and amino acid level information of synonymous and nonsynonymous nucleotide substitutions. When *rrf-rrl* and *p66* sequence alignments were concatenated, each partition was assigned its respective model. Convergence of each run was confirmed using the Are We There Yet software online (http://ceb.csit.fsu.edu/awty). Pairwise genetic distances among *rrf-rrl* sequences were determined using PAUP* version 4.0 (37).

Nucleotide sequence accession numbers. New sequences obtained during this study were deposited in GenBank under accession numbers GU994142 to GU994165 and are shown in bold in phylogenetic trees (see Fig. 1 to 3). GenBank accession numbers for previously published isolates are shown in phylogenetic trees but are in regular type. Genospecies designations for isolates with previously published sequences are based on either restriction fragment length polymorphism (3) or multilocus sequence analysis (25) as assigned by other authors.

RESULTS

***B. burgdorferi* infection prevalence and *ospC* genotypes in CHR sera.** We analyzed the diversity of *B. burgdorferi* sensu lato genospecies and *B. burgdorferi ospC* genotypes in sera collected in 1988 and 1989 from CHR residents who were part of a prospective epidemiologic study aimed at identifying risk factors for LB in the community (17, 18). Sera from 114 of the 119 original study participants were available, 109 specimens from 1988, 80 from 1989, and 75 from both years. In previous studies, 15 to 20% of the 1988 samples and ≥24% of the 1989 samples tested positive for antibodies against *B. burgdorferi* (17, 18).

In serum from CHR study subjects, we detected *B. burgdorferi* sensu lato DNA in 27/114 (23.7%) in both years, 19/109 (17.4%) in 1988, and 12/80 (15%) in 1989 ($P > 0.05$) (Table 1). Sequence analysis revealed that among the *B. burgdorferi* sensu

TABLE 1. *B. burgdorferi* sensu lato genospecies and *B. burgdorferi ospC* genotypes identified in sera of CHR residents in 1988 and 1989 in relation to study questionnaire and physician diagnostic data[a]

CHR ID	1988		1989		No. of tick bites (1986-1988)	Self-reported LB signs, symptoms, treatment, and PD prior to 1988							1988 PD
	GS (GT)[b]	LB Ab[c]	GS (GT)	LB Ab		PD	EM	Flu-like	Joint pain	Neurological	Cardiac	Antibiotic	
2	*B. burgdorferi* (A)	±	*B. burgdorferi*	−	3	−	−	−	−	−	−	−	−
3	*B. bissettii*-like/*B. burgdorferi*	−	*B. burgdorferi*[d]	NA	NA	NA	NA	NA	NA	NA	NA	−	NA
8	*B. burgdorferi* (A/1)	±		−	3	−	−	+	−	−	−	−	NA
13		−	*B. burgdorferi* mixture (A/10)	−	12	−	−	−	−	−	−	−	−
14		Pos	*B. burgdorferi* (A)	Pos	20	+	+	+	+	−	−	+	NA
18		±	*B. burgdorferi*	Pos	3	−	+	+	+	−	−	−	+
23	*B. burgdorferi* (A)	±		−	5	+	−	NA	+	−	−	−	−
31	*B. burgdorferi* (A/1)	±		−	4	−	−	+	+	+	−	−	+
32		Pos	*B. burgdorferi* (A)	Pos	0	+	−	−	+	−	−	+	+
33		−	*B. burgdorferi* (A)	−	8	+	+	−	+	−	−	+	+
39	*B. burgdorferi* (A)	±		−	0	−	−	−	−	−	−	−	−
40	*B. burgdorferi*	±		−	1	+	−	−	−	+	−	+	+
42	*B. burgdorferi*	−	−	−	3	−	−	−	−	−	−	+	−
45	*B. burgdorferi* (A)	Pos	*B. burgdorferi* (A)	Pos	1	+	−	+	NA	+	+	+	+
53	*B. burgdorferi* (A)	±		−	NA	−	−	−	+	−	−	−	+
54	*B. burgdorferi* (A)	−	NA	NA	NA	NA	NA	NA	NA	NA	NA	−	NA
61	*B. burgdorferi* (F)	−		−	0	−	−	−	−	−	−	−	NA
62	*B. burgdorferi* (A/1)	−	NA	−	5	−	+	+	−	+	NA	−	+
66	*B. bissettii*-like/*B. burgdorferi*	−	*B. burgdorferi*	−	2	−	+	−	+	−	−	−	−
70	*B. burgdorferi* (A)	Pos	NA	NA	NA	NA	NA	NA	NA	NA	NA	−	NA
75	*B. burgdorferi* (A)	Pos	*B. burgdorferi* (A)	Pos	6	−	−	−	−	−	−	−	+
77	NA	Pos	*B. burgdorferi* (A/1)	Pos	10	+	+	NA	+	+	−	−	+
79	*B. burgdorferi* (A)	±		NA	NA	NA	NA	NA	NA	NA	NA	−	NA
91	*B. burgdorferi* (A/1)	−		−	3	+	+	+	+	NA	NA	−	+
96		±	*B. burgdorferi*	−	2	+	−	+	+	−	−	−	−
101		Pos	*B. burgdorferi* (A)	Pos	3	+	+	+	+	−	−	+	+
105	*B. bissettii*-like	−	NA	NA	2	−	−	−	−	−	−	+	NA

[a] Questionnaire responses, physician diagnostic (PD) data, and LB antibody test results were gathered during earlier studies (17, 18). LB Ab, serological test for LB antibodies; NA, data not available; +, positive for LB symptoms or diagnosis; −, negative for LB symptoms, diagnosis, and antibody test results.

[b] Where genotypes (GT) are listed as (*x*/*y*), *x* refers to the *ospC* genotype and *y* refers to the IGS1 sequence type. *ospC* genotypes are not reported for all CHR residents due to negative *ospC* PCR results or poor sequencing data.

[c] Samples are designated "Pos" if they were test positive for LB antibodies in two or more laboratories; "±" (borderline) samples were test positive for LB antibodies in only one laboratory. Borderline results were not available in 1989.

[d] CHR3-89 was PCR positive only for the *p66* locus and was not included in prevalence calculations. All other CHR samples with genospecies (GS) data were PCR positive at two or more loci.

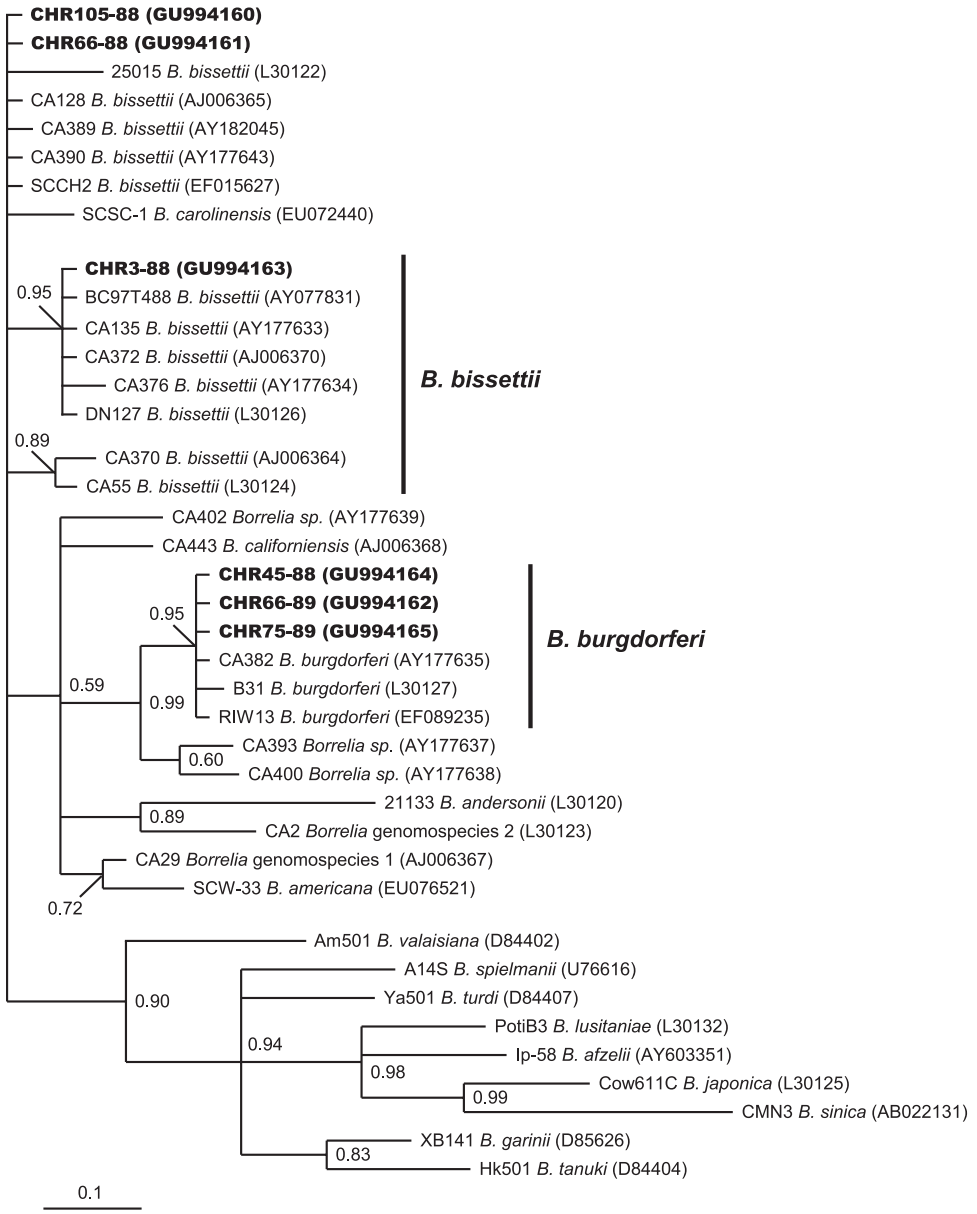

FIG. 1. Unrooted 50% majority rule consensus tree for *B. burgdorferi* sensu lato *rrf-rrl* alignment (204 bp) determined by Bayesian analysis using the nucleotide substitution model HKY + Γ (5,000,000 generations; burn-in, 0.5; sample frequency, 1,000; four chains). Values at nodes refer to Bayesian posterior probabilities (the proportion of sampled trees containing the taxon bipartition). The scale bar corresponds to branch length, expressed as the number of substitutions per site. Taxon names given to CHR serum specimens are in bold. GenBank accession numbers for sequences generated during this study also are in bold.

lato-positive individuals analyzed, 16/19 (84.2%) in 1988 (not including putative mixed infections, CHR3 and CHR66) and 12/12 (100%) in 1989 ($P > 0.05$) were infected with *B. burgdorferi*, the North American agent of LB (Table 1). The remaining three PCR-positive samples contained DNA closely related to *B. bissettii* (see below). *rrf-rrl* and *p66* sequences from two individuals infected with *B. burgdorferi* (CHR45-88 and CHR75-89) were analyzed phylogenetically to demonstrate their relatedness to published *B. burgdorferi* isolates, including the B31 type strain, and their distance from other *Borrelia* genospecies (Fig. 1 to 3).

The 206-bp *rrf-rrl* sequences were identical to each other, with the exception of CHR61-88, which contained one nucleotide change (T→G) at position 65 corresponding to B31 (GenBank accession no. L30127), and CHR67-88, which had a mixture at position 51. CHR13-89 was a clear mixture throughout the sequence, so we did not perform cloning to further examine individual genospecies or genotypes. Amplification of *B. burgdorferi ospC* genotype A from the DNA sample allowed us to conclude that *B. burgdorferi* was at least one of the genospecies found in this serum sample (Table 1).

In total, *ospC* sequences were successfully obtained in 87.5% (14/16) of the *B. burgdorferi*-positive sera from 1988. Thirteen (92.9%) of the typeable samples were *ospC* genotype A, and

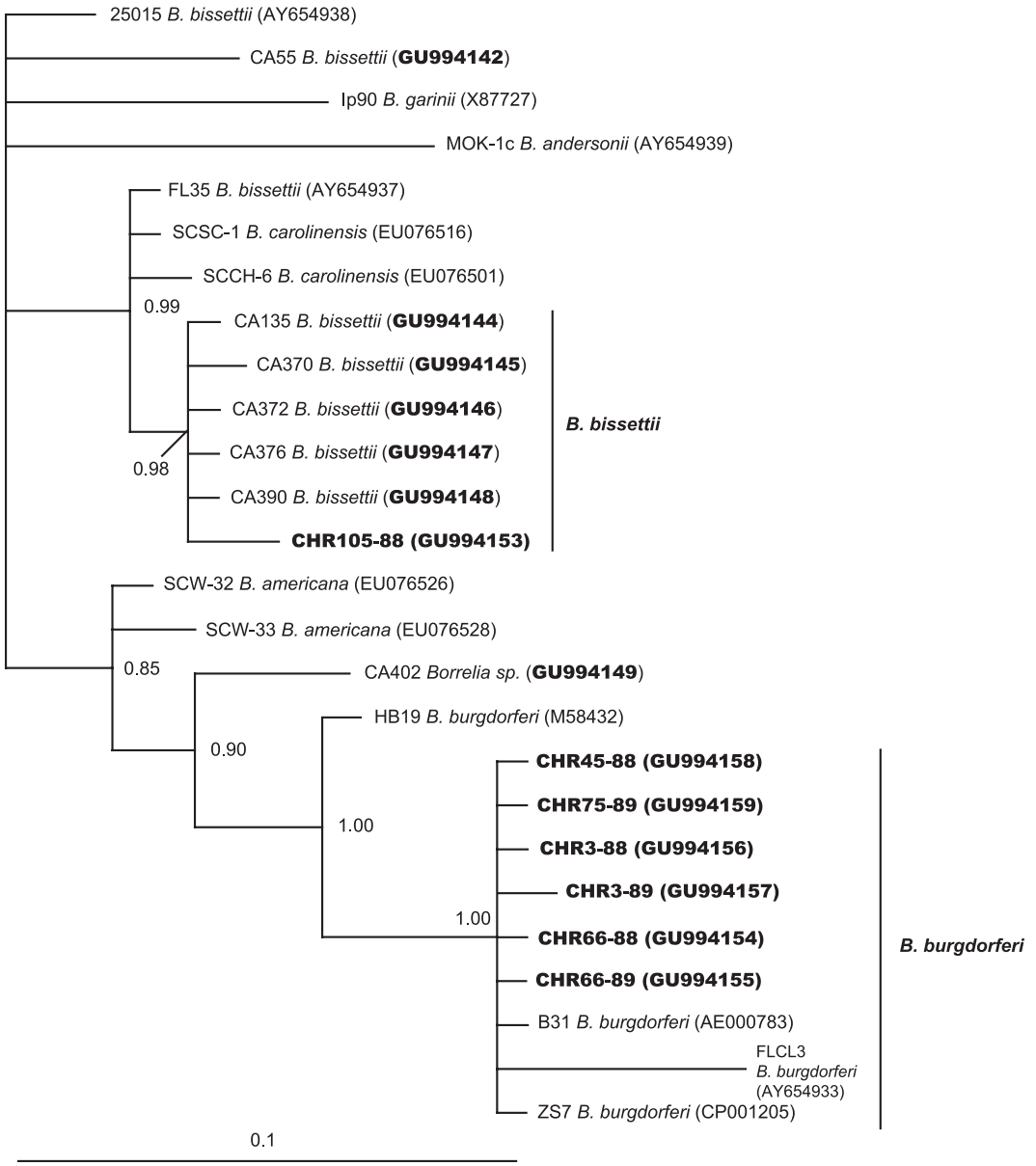

FIG. 2. Unrooted 50% majority rule consensus tree for *B. burgdorferi* sensu lato *p66* alignment (207 bp) determined by Bayesian analysis using a codon-based model of nucleotide substitution (1,000,000 generations; burn-in, 0.5; sample frequency, 1,000; four chains). Taxon names given to CHR serum specimens are in bold. GenBank accession numbers for sequences generated during this study also are in bold. Values at nodes and branch lengths are defined in the legend to Fig. 1.

one (CHR61-88) was *ospC* genotype F. In 1989, 100% of the eight *ospC* PCR-positive samples were genotype A (Tables 1 and 2). *ospC* genotype A sequences (525 to 528 bp) were identical to each other, with the exception of CHR13-89 and CHR91-88, which each contained mixtures at a single nucleotide position and were identical to the *ospC* genotype A samples found in the local *I. pacificus* population (e.g., GenBank accession no. AY275213). *ospC* F was also identical to *ospC* F sequences found in Mendocino County *I. pacificus* ticks (e.g., GenBank accession no. L42896).

In the six *ospC* genotype A serum samples for which the IGS1 locus could be successfully amplified and sequenced, five contained IGS1 sequence type 1 and one contained IGS1 sequence type 10 (Table 1). Interestingly, the only IGS1 type 10 sample, CHR13-89, was also identified as a mixture in the *rrf-rrl* sequence. *ospC* genotype A/IGS1 type 1 was found in *B. burgdorferi* type strain B31 isolated from *I. scapularis* collected on Shelter Island, NY. *ospC* A/IGS1 type 10 was found in strains CA4 and CA6, both isolated from *I. pacificus* collected in Sonoma County, CA, in 1987 (39).

Clinical and serological attributes of *B. burgdorferi*-positive individuals. Clinical and serological data previously gathered from CHR residents and analyzed (17, 18) were reevaluated in the light of our new, PCR-based evidence of *B. burgdorferi* infection among the study subjects. Three out of 16 (18.8%) and 7/12 (58.3%) ($P = 0.039$) of the *B. burgdorferi* PCR-posi-

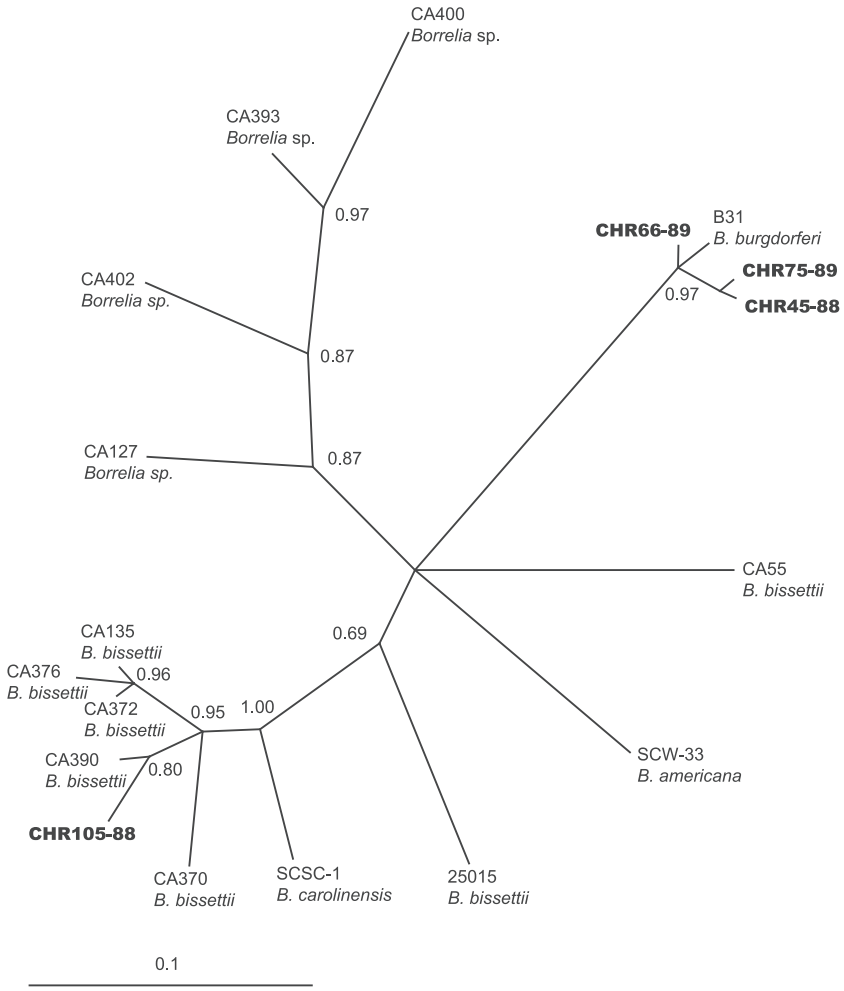

FIG. 3. Unrooted 50% majority rule consensus tree for concatenated alignments of *B. burgdorferi* sensu lato *rrf-rrl* and *p66* loci (395 bp) determined by Bayesian analysis using partitioned nucleotide substitution models: HKY + Γ and codon based (1,000,000 generations; burn-in, 0.5; sample frequency, 1,000; four chains). Taxon names given to CHR serum specimens are in bold. Values at nodes and branch lengths are defined in the legend to Fig. 1.

tive sera collected in 1988 and 1989, respectively, were test positive for LB antibodies using the most stringent criteria (Tables 1 and 2). Greater concordance between PCR and serology was found when borderline samples were considered (Table 2). Most *B. burgdorferi* PCR-positive individuals reported one or more LB clinical manifestations or had physician-diagnosed LB (Tables 1 and 2). Of the 24 subjects who were *B. burgdorferi* PCR positive in at least 1 year, 79.1% reported a history of one or more tick bites. In the same 24 individuals, joint pain was the most common symptom, followed by EM and flu-like illness, neurological manifestations, and cardiac abnormalities (Tables 1 and 2).

For 1988 sera, *B. burgdorferi* PCR-positive findings were associated with self-reported EM ($P = 0.033$) and joint pain ($P = 0.022$). For 1989 sera, *B. burgdorferi* PCR positivity was associated with seropositivity ($P = 0.001$) and the occurrence of one or more tick bites ($P = 0.043$). An association was found between samples that were *B. burgdorferi* PCR positive ($P = 0.002$) and seropositive ($P < 0.001$) in both 1988 and 1989. Unlike *ospC* genotype A subjects, the individual infected with *ospC* genotype F did not recall a tick bite, clinical LB manifestations, or a physician diagnosis.

Seven (29.2%) of the 24 individuals who were PCR positive for *B. burgdorferi* reported previous treatment with antibiotics on the 1988 questionnaire (Table 1). Study subject CHR105, who tested positive for a *B. bissettii*-like agent (see below), also reported antibiotic treatment prior to 1988. Curiously, that individual did not report either a history of a physician diagnosis of LB or clinical manifestations compatible with the disease.

Sequence analysis of *B. bissettii*-like DNA in human sera. Analysis of *rrf-rrl* sequences of serum specimens collected from three CHR residents in 1988 revealed the presence of DNA from a spirochete closely related to *B. bissettii* (Table 1). To characterize the samples further, we attempted to amplify the *p66*, *fla*, *ospC*, and 16S rRNA (*rrs*) genes, but only *p66* amplicons could be consistently obtained from all three samples. To further assist our analysis, we sequenced the partial *p66* gene in six previously characterized *B. bissettii* culture isolates from California, CA55, CA135, CA370, CA372, CA376, and CA390,

TABLE 2. Prevalence of *ospC* genotypes and history of LB antibody detection, signs, symptoms, and physician diagnosis in *B. burgdorferi*-positive CHR residents, 1988 to 1989

Serum/DNA attribute	No. of samples (% of total)[a]		1988 vs 1989 *P* value	No. of samples (% of total), either 1988 or 1989 (24 subjects)[a]
	1988 (16 subjects)	1989 (12 subjects)		
Seropositive				
Positive only	3 (18.8)	7 (58.3)	0.05	8 (33.3)
Positive + borderline	11 (68.8)	NA (NA)	NA	NA (NA)
≥1 LB sign or symptom and/or PD[b]	9 (56.3)	9 (75.0)	>0.05	16 (66.6)
History of tick bite	10 (76.9)	10 (83.3)	>0.05	19 (79.1)
ospC-typeable samples[c]	14 (87.5)	8 (66.7)	>0.05	20 (83.3)
ospC genotype A	13 (92.9)	8 (100)	>0.05	19 (95.0)
ospC genotype F	1 (7.1)	0 (0)	>0.05	1 (5.0)
Nontypeable samples	2 (12.5)	4 (33.3)	>0.05	4 (16.7)

[a] Does not include data from individuals with *B. bissettii*-like infections. Attribute totals are calculated from Table 1. NA, data not available.
[b] LB symptoms are listed in Table 1.
[c] *ospC* genotype frequency calculations used the total number of *ospC*-typeable samples as the denominator.

and three *Borrelia* sp. isolates, CA402, CA393, and CA400 (3, 25, 26).

The *rrf-rrl* sequence of CHR3 collected in 1988 (CHR3-88) clustered in a strongly supported clade with *B. bissettii* isolates, including the DN127 type strain (26) (Fig. 1). In contrast, the *p66* sequences from both CHR3-88 and CHR3-89 (the latter serum sample was collected from the same individual in 1989) clustered with *B. burgdorferi* isolates, including the B31 type strain (Fig. 2). Although multiple peaks were not observed in sequencing chromatograms (data not shown), our findings suggest that subject CHR3 was coinfected with *B. burgdorferi* and a *B. bissettii*-like agent.

The *rrf-rrl* sequences of DNA isolated from serum samples collected in 1988 from subjects CHR66 (CHR66-88) and CHR105 (CHR105-88) were identical (Table 3) and formed a polytomy along with five *B. bissettii* isolates and *B. carolinensis* isolate SCSC-1 (Fig. 1). Compared to CHR3-88 and other members of the DN127 clade shown in Fig. 1, the *rrf-rrl* sequences for CHR66-88 and CHR105-88 possessed a 4-bp deletion corresponding to nucleotide positions 84 to 87 of DN127 (GenBank accession no. L30126) and four nucleotide substitutions (T→G at position 95 and G→A at positions 105, 107, and 181). Similar to the *p66* sequence of CHR3, that of CHR66-88 clustered with *B. burgdorferi* isolates, not *B. bissettii* isolates (Fig. 2). In 1989, both the *p66* and *rrf-rrl* sequences of CHR66 clustered with *B. burgdorferi* (see concatenated sequences in Fig. 3). We suspect that, like subject CHR3, the CHR66 individual was coinfected with *B. burgdorferi* and a *B. bissettii*-like agent. The taxonomic relationship between CHR105-88 and known *B. burgdorferi* genospecies was better resolved at the *p66* locus and remained apparent when *rrf-rrl* and *p66* were concatenated. CHR105-88 *p66* formed a clade exclusively with *B. bissettii* isolates (Fig. 2). When the two loci were concatenated, CHR105-88 clustered with *B. bissettii* isolate CA390 (Fig. 3).

Questionnaire data were not available for study subject CHR3. CHR66 had a history of EM and joint pain. Both CHR66 and CHR105 had a history of tick bites. Subject CHR105 had no history of LB signs, symptoms, or diagnosis. All individuals with evidence of single infections or coinfections with a *B. bissettii*-like organism were seronegative in assays performed in 1988 and 1989 (Table 1).

Comparison of European and Californian human-derived *B. bissettii* and *B. bissettii*-like DNAs. We compared the nucleotide sequences of *B. bissettii*-like DNA extracted from CHR105-88, CHR3-88, and CHR66-88 to *B. bissettii* DNA extracted from the serum of European patients with suspected LB (31) (Table 3). Using a pairwise distance matrix of a 195-bp region of the *rrf-rrl* locus, we found that DNA from CHR105-88, CHR3-88, and CHR66-88 was 97 to 100% similar to that of *B. bissettii* strains 25015 and DN127 and 96 to 98% similar to *B. bissettii* DNA derived from Czech Republic patient sera. In contrast, the same three CHR sequences were only 93% similar to *B. burgdorferi* type strain B31. The analyzed *rrf-rrl* regions of *B. bissettii* strains 25015 and DN127 were 92% and 93% similar, respectively, to that of *B. burgdorferi* B31 (Table 3).

DISCUSSION

***B. burgdorferi* sensu lato DNA detection and amplification in human serum.** Molecular analysis of *B. burgdorferi* sensu lato spirochetes infecting humans is a modern and potentially powerful way to improve our understanding of LB disease, ecology, and epidemiology. This tool can be particularly useful when spirochete cultivation from patient serum is unsuccessful (31) or when antibodies are difficult to detect (14). There are inherent challenges to *B. burgdorferi* DNA amplification in serum, including low or transient spirochetemia and PCR inhibition by host DNA (1). The modified QIAamp method and accompanying low-temperature denaturation PCR used herein allowed a higher rate of *B. burgdorferi* sensu lato detection in CHR study subjects than otherwise would have been possible. The high detection rate may have been due, at least in part, to the large volume of serum used in DNA extraction and our ability to amplify both intra- and extraorganismal (cell-free) *B.*

TABLE 3. Pairwise distance matrix of *rrf-rrl* nucleotide sequences derived from European *B. bissettii* infections versus California *B. bissettii*-like infections[a]

Pairwise distance[b]	Similarity[c]											
	B31	25015	DN127	*p1c8*	*p2e4*	*p3h10*	*p9b3*	*p11a8*	*p13b1*	*p16a2*	**CHR105**	**CHR3**	**CHR66**
B. burgdorferi B31 L30127		0.92	0.93	0.92	0.92	0.92	0.92	0.92	0.92	0.93	0.93	0.93	0.93
B. bissettii 25015 L30122	0.08		0.97	0.95	0.96	0.95	0.95	0.96	0.96	0.95	0.98	0.97	0.98
B. bissettii DN127 L30126	0.07	0.03		0.96	0.97	0.96	0.96	0.97	0.97	0.96	0.98	1.00	0.98
B. bissettii p1c8 FJ431136	0.08	0.05	0.04		0.99	0.99	0.99	0.99	0.99	0.99	0.97	0.96	0.97
B. bissettii p2e4 FJ431137	0.08	0.04	0.03	0.01		0.99	0.99	1.00	1.00	0.99	0.98	0.97	0.98
B. bissettii p3h10 FJ431138	0.08	0.05	0.04	0.01	0.01		0.99	0.99	0.99	0.99	0.97	0.96	0.97
B. bissettii p9b3 FJ431139	0.08	0.05	0.04	0.01	0.01	0.01		0.99	0.99	0.99	0.97	0.96	0.97
B. bissettii p11a8 FJ431140	0.08	0.04	0.03	0.01	0.00	0.01	0.01		1.00	0.99	0.98	0.97	0.98
B. bissettii p13b1 FJ431141	0.08	0.04	0.03	0.01	0.00	0.01	0.01	0.00		0.99	0.98	0.97	0.98
B. bissettii p16a2 FJ431142	0.07	0.05	0.04	0.01	0.01	0.01	0.01	0.01	0.01		0.97	0.96	0.97
CHR105-88	0.07	0.02	0.02	0.03	0.02	0.03	0.03	0.02	0.02	0.03		0.98	1.00
CHR3-88	0.07	0.03	0.00	0.04	0.03	0.04	0.04	0.03	0.03	0.04	0.02		0.98
CHR66-88	0.07	0.02	0.02	0.03	0.02	0.03	0.03	0.02	0.02	0.03	0.00	0.02	

[a] European *B. bissettii* sequences are in italics, and California *B. bissettii*-like sequences are in bold. Underlining indicates distance/similarity between human-derived European and California sequences.
[b] Average number of changes per base pair for each pair of sequences compared.
[c] A value of 1.00 indicates 100% similarity, i.e., an average of 0.00 nucleotide changes per base pair between sequences. Sequence names are abbreviated versions of those found on the pairwise distance axis.

burgdorferi DNA by not pelleting the spirochetes. Experimentation with low denaturation temperature PCR has been successful in increasing the detection of "minority alleles" containing clinically relevant microdeletions (21) and in targeting the amplification of short DNA segments (42). Similarly, low-temperature template denaturation techniques may be useful to other researchers interested in amplifying small segments of microorganismal DNA from blood products.

Dominance of *ospC* genotype A in Mendocino County human sera. We found a dominance of infection with a single *B. burgdorferi* genotype among CHR residents. Of the 20 *B. burgdorferi* samples from study subjects whose DNA could be typed, 19 (95%) were of *ospC* genotype A. This proportion is significantly higher than in some northeastern United States communities, in which *ospC* A was detected in 23.5% of blood cultures ($P < 0.001$) (41) and 36.2% of skin isolates ($P = 0.012$) (15). The *B. burgdorferi* clonal complex defined by *ospC* genotype A is ecologically highly invasive and established in a broad range of host species in Europe and North America (27). In the northeastern United States, where the frequency of *ospC* A alleles is high in *I. scapularis* populations (40, 41), this genotype is often one of the most, if not the most, commonly found in organisms that cause disseminated *B. burgdorferi* infections (8, 9, 15, 34, 41). Its predominance in this small Californian population is a further testament to its virulence and to its ability to disseminate from the initial site of infection, particularly considering that the prevalence of *ospC* A alleles in host-seeking *I. pacificus* nymphs collected within CHR and throughout Mendocino County is only 10% (unpublished data) and 10.9%, respectively (13). However, ticks assayed in previous genotyping efforts were collected in 2004 (13) and thus did not overlap temporally with the time of human serum collection for the present study. Alternatively, the high prevalence of a single genotype may reflect the fact that all 114 study subjects resided within a small geographic area, namely, an ~5,300-acre former working cattle ranch that had been divided into 40-acre parcels during the 1970s. Further sampling of human sera beyond CHR, in other areas where LB is endemic, is necessary to capture the true diversity of *B. burgdorferi* sensu lato spirochetes infecting humans in the Far West. Analysis of more recently collected human serum specimens in California also is needed to better understand the relationship between *ospC* allelic frequency in vector ticks and the current risk of human exposure to highly virulent *B. burgdorferi* genotypes.

Detection of *B. bissettii*-like spirochetes in human sera. We present compelling PCR- and DNA sequence-based evidence of disseminated infection with a *B. bissettii*-like spirochete in 3 (2.6%) of 114 CHR residents tested. Heretofore, only *B. burgdorferi* had been identified in human-derived samples in North America. It is not clear from our results whether the agent is associated with disease or specific clinical manifestations because of the simultaneous detection of *B. burgdorferi* DNA or the lack of available data. Recent descriptions of *B. bissettii* DNA with genetic similarity to strains DN127 and 25015 in patients with LB symptoms in the Czech Republic and Slovenia, respectively, highlight the growing recognition of *B. bissettii* as an agent of LB in central and southern Europe (23, 30, 31, 36). In European studies, infection with *B. bissettii* alone is associated with clinical signs of LB, including flu-like symptoms, arthralgia, weakness, and myalgia, among others (31,

36), or, in a singular case, endocarditis and aortic valve stenosis (30). Laboratory data also support the pathogenic potential of *B. bissettii* isolates originating in Colorado in CH3/HeJ mice (33).

Enzootic *B. burgdorferi* is maintained in northern California oak woodlands in a cycle involving *I. pacificus* ticks and reservoir host western gray squirrels (*Sciurus griseus*) (11, 19, 32). There, *I. pacificus* nymphs are implicated in LB transmission to humans (7). *B. bissettii* is also found in *I. pacificus* ticks collected in oak woodlands, especially those that abut chaparral, where dusky-footed wood rats (*Neotoma fuscipes*) and *Peromyscus* sp. mice maintain *B. bissettii* in a transmission cycle involving non-human-biting *I. spinipalpis* ticks (3, 11, 19). In the laboratory, *I. pacificus* ticks are competent vectors of *B. bissettii*, but in nature, they may not feed as readily on *B. bissettii* reservoirs as on *B. burgdorferi* reservoirs (10). Taken together, it seems plausible that residents of CHR, inhabiting lands covered predominantly by woodland-grassland, dense woodland, and grassland with some areas of chaparral, could be exposed to both genospecies via the attachment of *I. pacificus* ticks in the vicinity of their homes.

Conclusions. In summary, we report laboratory techniques for enhancing the detection of *B. burgdorferi* sensu lato DNA in human sera. We also describe what *Borrelia* genospecies, as well as what genotypes of *B. burgdorferi*, infect humans in an area in the far western United States where LB is highly endemic. In so doing, we present the first molecular evidence that a *B. bissettii*-like spirochete occasionally infects people in North America. These findings significantly expand our knowledge of the molecular epidemiology of LB in this region and will, it is hoped, serve as a stimulus for other researchers to consider *B. bissettii* as a potential human pathogen throughout the geographic range of this broadly distributed spirochete.

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