Lone Star Tick-Infecting Borreliae Are Most Closely Related to the Agent of Bovine Borreliosis

STEPHEN M. RICH,[1] PHILIP M. ARMSTRONG,[2,3] RONALD D. SMITH,[4] AND SAM R. TELFORD III[2]*

Division of Infectious Disease, Tufts University School of Veterinary Medicine, North Grafton,[1] and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston,[2] Massachusetts; Southwest Foundation for Biomedical Research, San Antonio, Texas[3]; and Department of Veterinary Pathobiology, University of Illinois School of Veterinary Medicine, Urbana, Illinois[4]

Although *Borrelia theileri*, the agent of bovine borreliosis, was described at the turn of the century (in 1903), its relationship with borreliae causing Lyme disease or relapsing fever remains undescribed. We tested the previously published hypothesis that spirochetes infecting Lone Star ticks (*Amblyomma americanum*) may comprise *B. theileri* by analyzing the 16S ribosomal DNAs (rDNAs) and flagellin genes of these spirochetes. *B. theileri*, the *Amblyomma* agent, and *B. miyamotoi* formed a natural group or clade distinct from but most closely related to that of the relapsing fever spirochetes. *B. theileri* and the *Amblyomma* agent were 97 and 98% similar at the nucleotide level within the analyzed portions of the 16S rDNA and the flagellin gene respectively, suggesting a recent divergence. The agent of bovine borreliosis might be explored as a surrogate antigen for the as-yet-uncultivable *Amblyomma* agent in studies designed to explore the etiology of a Lyme disease-like infection associated with Lone Star ticks.

Ticks comprise two major natural groups, or clades: the Ixodidae (hard ticks) and the more primitive mite-like Argasidae (soft ticks) (4, 14). The spirochetes associated with these morphologically and biologically divergent kinds of ticks have generally been referred to as either hard or soft tick-borne borreliae (16). Interestingly, of the speciose hard ticks, solely the Lyme disease-like spirochetes (*Borrelia burgdorferi* sensu lato), transmitted by *Ixodes* spp. (subfamily Prostriata), have been extensively studied. Although *B. theileri*, associated with *Rhipicephalus* and *Boophilus* (both classified within the ixodid subfamily Metastriata), was described during the early part of the 20th century (17), its relationship to other borreliae remains poorly described. Spirochetes have been detected within the metastriate Lone Star ticks (*Amblyomma americanum*) (1, 2, 10, 18, 24), but only recently has their taxonomic status been analyzed. Their identity has assumed prominence because Lone Star ticks are associated with a syndrome of undescribed etiology (referred to as southern tick-associated rash infection or Masters' disease) that is confused with Lyme disease (3, 8, 22; P. M. Armstrong, L. Rosa Brunet, A. Spielman, and S. R. Telford III, submitted for publication). Because deer served as reservoir hosts for *Boophilus* spp. in the south central United States during the 19th and early 20th centuries, we previously suggested that spirochetes infecting *Amblyomma*, a deer-dependent tick, may represent a host shift of *B. theileri* (26). We tested this hypothesis by analyzing the nucleotide sequences of the 16S ribosomal DNAs (rDNAs) and flagellin (*fla*) genes of *B. theileri* and the *Amblyomma* agent, and we determined the genetic relationship of these previously uncharacterized spirochetes to others of the genus *Borrelia*.

MATERIALS AND METHODS

Sources of borreliae. *B. theileri* was derived from naturally infected *Boophilus microplus* originally collected from a Mexican site, as described previously and under an existing U.S. Department of Agriculture permit (25). A colony of infected *Boophilus* ticks was produced by allowing nymphs to engorge on infected cattle. The resulting adult ticks were examined by the hemolymph test (5) to determine whether they were infected, allowed to engorge and oviposit to maintain the infected colony, and stored frozen at −70°C until they were analyzed.

Nymphal and adult *A. americanum* ticks were collected by sweeping vegetation on Gibson Island, Md., during 1994 and nondestructively assayed for spirochetal infection by dark-field microscopy of hemolymph samples taken from the amputated stump of a leg (5). Of 388 ticks, 2 contained spirochetes. Their gut contents were removed and homogenized in 100 μl of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl), and total DNA was extracted by a standard phenol-chloroform extraction protocol. Attempts to cultivate the agent in BSK II and Kelly's medium failed.

DNA amplification and sequencing of borrelial genes. PCR was used to amplify an approximately 350-bp portion of the *fla* gene (primers FLA-1 [5′-GAT GATGCTGCTGGCATGGGAGTTGCGGG-3′] and FLAG-5 [5′-CCTGAAA GTGATGCTGGTG-3′]), as well as an 886-bp fragment of the 16S rDNA (primers BOR-16SA [5′-TGTTAATTGATGAAAGGAAGCC-3′] and BOR-16SE [5′-TTTACATGCTGGTAACAGAC-3′]). Each 50-μl reaction mixture contained 5 μl of EXPAND 10× PCR buffer (Boehringer Mannheim), 0.2 μl of each deoxynucleoside triphosphate (10 μM), 0.2 μl of EXPAND *Taq* polymerase (Boehringer Mannheim), 1 μl of each primer (25 μM), and about 10 ng of template. Each of the 35 cycles consisted of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min in a Perkin-Elmer (model 480) thermal cycler.

Double-stranded amplification products were electrophoresed in a 1.5% agarose gel in 1× Tris-borate-EDTA buffer and were extracted from excised gel bands using the GeneClean kit (Bio 101). Cycle sequencing was performed using the Applied Biosystems dye-labeled dideoxy termination kit, and the products were purified and analyzed on a 6% polyacrylamide gel in an ABi 373A DNA sequencer (Applied Biosystems), as recommended by the manufacturer.

Sequences were edited using the SeqEd 675 DNA sequence editor program (Applied Biosystems). Each sequence was verified in duplicate by analyzing both strands of the amplification product.

Phylogenetic analysis. *Borrelia* gene sequences were initially aligned with the CLUSTALW program (28). Corrections to alignments were made manually, particularly in the case of the *fla* gene, where the algorithm failed to preserve the codon reading frame. The sequences that we derived from *B. theileri* and the *Amblyomma* agent were compared with representative *Borrelia* 16S rDNA and *fla* gene sequences accessioned in GenBank (Table 1). When the Harvard lab-

* Corresponding author. Mailing address: Dept. of Immunology and Infectious Diseases, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. Phone: (617) 432-4079. Fax: (617) 432-1796. E-mail: stelford@hsph.harvard.edu.

TABLE 1. Spirochete species used for phylogenetic analysis

Species	Strain	GenBank accession no.[a]	
		fla	16S rDNA
B. afzelii	DK4		X85194
	HT61	D63366	
B. barbouri	NA[b]	**U42432**	**U38374**
B. burgdorferi	B31	X15661	U03396
B. crocidurae	NA	X75204	
	UESV/MER		U42291
B. duttonii	NA	U28497	
	UESV/117DUT		U42288
B. garinii	20047	D82846	D67018
B. hermsii	HS1	X53940	U42292
	HS1	M86838	
B. hispanica	NA	U28498	
	UESV/246		U42294
B. japonica	IKA2	D82853	L40598
B. lonestari	Texas20	U26704	U23211
B. miyamotoi	HT31	D43777	D45192
B. parkeri	NA	B82863	U42296
B. recurrentis	NA	D86618	U42300
B. theileri	NA	**U42431**	**U38375**
B. turicatae	M2007	D82862	U42299
Cristispira sp.	CP1		U42638

[a] New GenBank accession numbers are in boldface.
[b] NA, not applicable or not designated.

oratory submitted the sequences for the *Amblyomma* agent to GenBank in October 1995 (1), those for *B. lonestari* (2) were under embargo and unavailable for comparison. At the time we had provisionally named this spirochete *B. barbouri*, in honor of Alan Barbour's many contributions to the study of *Borrelia* biology. The sequences accessioned under the names *B. lonestari* and *B. barbouri*

are identical and derive from the same entity; pending a formal designation in *Bergey's Manual*, we here use the term *Amblyomma* agent to refer to it.

Two 50% majority rule consensus phylograms, one for each of the two genes examined, were constructed by performing 1000 bootstrap replications of the PAUP 4.0 (27) program's "Fast" stepwise addition heuristic tree search method. In the 16S rDNA-based analysis, *Cristispira* sp. served as the outgroup, whereas the *fla*-based phylogeny is an unrooted network

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers U38374, U38375, U42431, and U42432.

RESULTS

Relationships among spirochetes. Maximum-parsimony analysis was used to determine the relatedness of *B. theileri*, the *Amblyomma* agent, and *B. recurrentis* to several other *Borrelia* species, based on the 16S rDNAs and *fla* genes. The low levels of nucleotide polymorphism in either the flagellin gene or 16S rDNA limits the information for resolving terminal taxa. However, reliable inferences can be drawn from the two trees for the more broadly defined groupings. Consistent with many other previous reports (9, 15, 20, 21, 23), the group of *B. burgdorferi* sensu lato spirochetes (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) group as a diverse monophyly (Fig. 1). Our analysis also places *B. japonica* within the clade comprised of *B. burgdorferi* sensu lato.

Phylograms based on both *fla* and 16S rDNA data sets (Fig. 1) demonstrate that *B. theileri*, the *Amblyomma* agent, and *B. miyamotoi* form a monophyletic group most closely related to the two soft tick-transmitted spirochete clades. Based on the
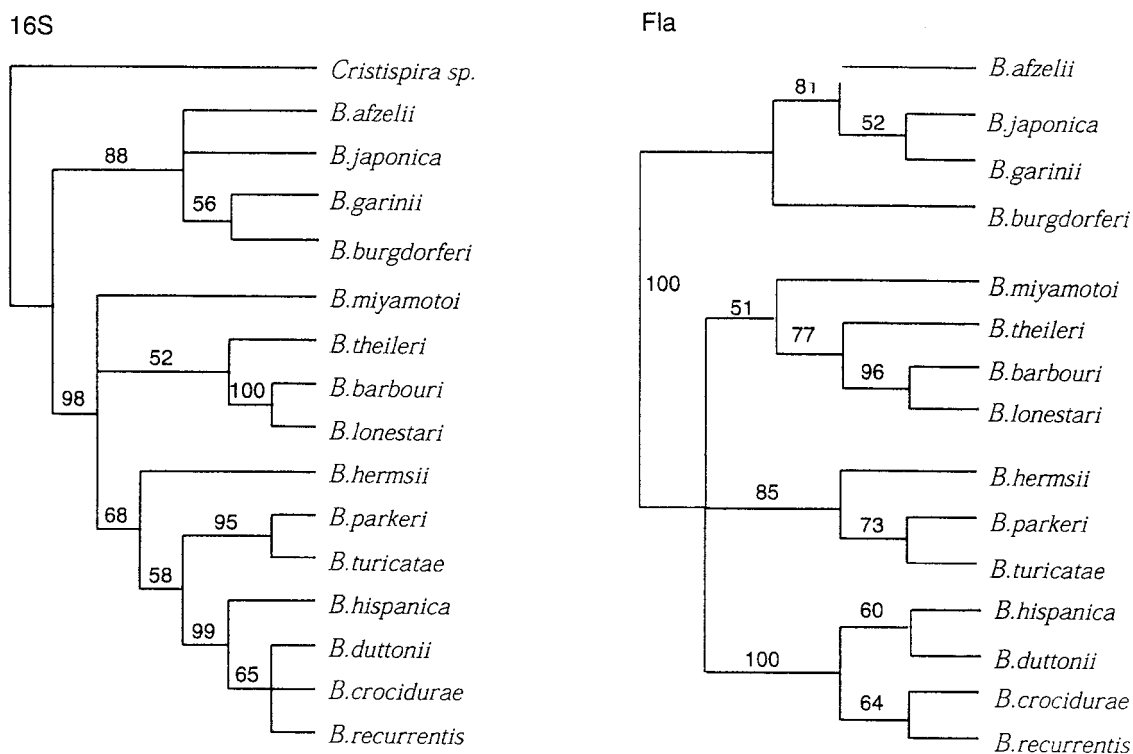

FIG. 1. Bootstrap consensus (1,000 times) of neighbor-joining trees of *Borrelia* spirochetes using both 16S rDNA (left) and flagellin (right) loci. The oral spirochete *Cristispira* sp. was used as an outgroup. Distances were derived from the Tamura-Nei algorithm (27a). Numbers indicate the bootstrap support for each branch.

fla DNA sequence, these *Borrelia* spp. form a monophyletic sister group to the several relapsing fever spirochetes.

DISCUSSION

Our analysis supports Filippova's hypothesis that the vectors and agents of Lyme disease comprise a monophyletic clade indicating evolution by descent within this group (11). The one exception is *B. miyamotoi*, originally isolated from *Ixodes persulcatus* (12, 13), the main Eurasian vector of *B. burgdorferi* sensu lato. Although this association may reflect a secondary host shift from the metastriate-associated borreliae, it may be that *I. persulcatus* is not the main vector but that sympatric Japanese ticks such as the metastriate *Haemaphysalis longicornis* serve in this capacity. Further studies on the epizootiology of *B. miyamotoi* may resolve this interesting exception to the otherwise strongly supported congruence between *Ixodes* ticks and Lyme disease-like borreliae.

The metastriate tick-transmitted *B. theileri* and the *Amblyomma* spirochete clearly group outside the other ixodid-borne borreliae, and certain features of their biology corroborate this finding. Bovine borreliosis due to *B. theileri* is characterized by prominent spirochetemias in the peripheral blood (7, 25), as is relapsing fever. Both *B. theileri* and many of the argasid-transmitted spirochetes may be efficiently maintained by transovarial transmission. In the vector, these spirochetes may easily be found in the hemolymph (25), in sharp contrast to the *Ixodes*-transmitted borreliae, which less frequently produce disseminated infections in the tick hosts (6). The *Amblyomma* agent is detectable within ticks as frequently by the hemolymph test as it is by indirect immunofluorescence of gut contents (Fisher exact test, $P = 0.376$) (our unpublished results), suggesting that other features of *B. theileri*'s biology may be shared, such as the presence of spirochetemia in the as-yet-undescribed reservoir and its possible maintenance in ticks by transovarial transmission.

The degree of sequence similarity between the *Amblyomma* agent and *B. theileri* is similar to that between different isolates of *B. hermsii* and may reflect a very recent divergence of the two metastriate-transmitted spirochetes. Because *A. americanum* feeds mainly upon deer in all three instars, we previously reasoned that any spirochete maintained by this tick might be closely related to that maintained by the cattle tick *Boophilus* (26). Indeed, deer served as important enzootic hosts for the tick *B. microplus*, and the eradication program for this economically important ectoparasite of cattle (because of its role as a vector of Texas cattle fever or bovine babesiosis) targeted cervids throughout the southern United States (19). Because cattle and deer are sympatric in many sites, *Boophilus* and the agents that it maintained could have established parallel transmission cycles in deer.

Although specific names have been applied to the *Amblyomma* agent (*B. lonestari, B. barbouri,* and a *Borrelia* sp. similar to *B. nr. miyamotoi*), given its genetic similarity to *B. theileri*, any formal description would require justifying the application of a new name. More importantly, because the *Amblyomma* agent is as yet uncultivatable, determining its role in the etiology of southern tick-associated rash infection (Masters' disease) may be facilitated by the use of *B. theileri* as a surrogate. These spirochetes attain a great density within the blood of cattle and may be purified for use as an antigen. In addition, the knowledge that *B. theileri* is maintained transovarially and may be found as disseminated infections within the vector, as well as producing transient spirochetemia in the ungulate host, may help advance studies that support or refute the role of borreliae in the epidemiology of Lone Star tick-associated, Lyme disease-like infections of humans.

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