

## First Culture Isolation of *Borrelia lonestari*, Putative Agent of Southern Tick-Associated Rash Illness

Andrea S. Varela,<sup>1</sup> M. Page Luttrell,<sup>2</sup> Elizabeth W. Howerth,<sup>3</sup> Victor A. Moore,<sup>1</sup>  
William R. Davidson,<sup>2,4</sup> David E. Stallknecht,<sup>1,2</sup> and Susan E. Little<sup>1\*</sup>

Department of Medical Microbiology and Parasitology,<sup>1</sup> Southeastern Cooperative Wildlife Disease Study,<sup>2</sup> and Department of Pathology,<sup>3</sup> College of Veterinary Medicine, and Warnell School of Forest Resources,<sup>4</sup> University of Georgia, Athens, Georgia 30602

Received 25 August 2003/Returned for modification 5 October 2003/Accepted 5 November 2003

**Southern tick-associated rash illness (STARI) is a Lyme disease-like infection described in patients in the southeastern and south-central United States, where classic Lyme disease is relatively rare. STARI develops following the bite of a lone star tick (*Amblyomma americanum*) and is thought to be caused by infection with an “uncultivable” spirochete tentatively named *Borrelia lonestari*. In this study, wild lone star ticks collected from an area where *B. lonestari* is endemic were cocultured in an established embryonic tick cell line (ISE6). The cultures were examined by dark-field microscopy for evidence of infection, and spirochete identity and morphology were evaluated by flagellin B and 16S rRNA gene sequence, by reaction to *Borrelia*-wide and *B. burgdorferi*-specific monoclonal antibodies, and by electron microscopy. Live spirochetes were first visualized in primary culture of *A. americanum* ticks by dark-field microscopy 14 days after the cell culture was inoculated. The sequences of the flagellin B and 16S rRNA genes of cultured spirochetes were consistent with previously reported sequences of *B. lonestari*. The cultured spirochetes reacted with a *Borrelia*-wide flagellin antibody, but did not react with an OspA antibody specific to *B. burgdorferi*, by indirect fluorescent antibody testing. Electron microscopy demonstrated organisms that were free and associated with ISE6 cells, with characteristic *Borrelia* sp. morphology. This study describes the first successful isolation of *B. lonestari* in culture, providing a much needed source of organisms for the development of diagnostic assays and forming a basis for future studies investigating the role of the organism as a human disease agent.**

Lyme disease, caused by *Borrelia burgdorferi* sensu lato, is the most common tick-borne disease of humans worldwide and the most frequently reported vector-borne disease in the United States. The hallmark of acute Lyme disease is erythema migrans, which is present in 60 to 90% of patients (13, 45), although several other nonspecific multisystemic symptoms also occur (9). *B. burgdorferi* is maintained in nature through a cycle involving rodent reservoir hosts and *Ixodes* sp. tick vectors (1, 28, 46). The disease is endemic throughout much of the Northeast, mid-Atlantic states, Midwest, and West Coast; however, in much of the South, where *Ixodes* sp. ticks are seldom found on humans (17, 18), epidemiologic evidence and case reporting suggest that classic Lyme disease is relatively rare, despite the presence of *B. burgdorferi* in wild rodent populations and ticks (16, 36, 37, 38, 40).

Since the mid-1980s, physicians have described a Lyme disease-like illness in patients from the southeastern and south-central United States in which an erythema migrans rash and mild flu-like symptoms develop following the bite of a lone star tick, *Amblyomma americanum* (2, 3, 12, 19, 31, 43). This disease is alternatively referred to as southern tick-associated rash illness (STARI), Master's disease, or southern Lyme disease (23, 30). *A. americanum* has been shown to be an incompetent vector for *B. burgdorferi*, and serologic testing of STARI patients does not support a diagnosis of classic Lyme disease

despite microscopic evidence of spirochetes in biopsy samples of affected skin, leading researchers to speculate that another *Borrelia* sp. may be responsible (16, 24, 29, 31, 33, 35, 39, 42, 48, 49). However, all attempts to culture spirochetes from patients with STARI and from ticks have failed.

Molecular evidence of a novel *Borrelia* sp. has been reported from lone star ticks, from white-tailed deer, and from the skin of a patient with STARI, as well as from a lone star tick removed from that patient (2, 10, 11, 23, 32, 47). The organism, tentatively named *Borrelia lonestari*, has been described only by PCR amplification of the flagellin B gene and 16S ribosomal DNA (rDNA). Complete understanding of *B. lonestari* and its role in STARI has been hampered by the inability to culture the etiologic agent. Here, we report the first culture isolation of *B. lonestari* and provide a microscopic and ultrastructural description of the organism in cell culture.

### MATERIALS AND METHODS

**Tick specimens.** Adult *A. americanum* ticks were collected, using dry ice (CO<sub>2</sub>) traps (25), from Whitehall Experimental Forest, an 800-acre forest owned by the University of Georgia in Clarke County, Ga., during March and April 2003. This population of ticks had been previously confirmed to harbor *B. lonestari* (A. S. Varela, V. A. Moore, and S. E. Little, unpublished data). The ticks were maintained at 94% humidity in chambers containing saturated potassium nitrate for ~2 months before they were cultured.

**Culture isolation.** Procedures for the coculture of *B. burgdorferi* in a tick cell line modified from previous studies were used to isolate *B. lonestari* from wild-caught ticks (26, 27, 34). Ten adult *A. americanum* ticks (five female and five male) collected from Whitehall Experimental Forest on 12 March 2003 were washed under sterile conditions by vortexing them for 3 min in successive solutions of 3% hydrogen peroxide, 95% alcohol, 0.1% sodium hypochlorite, and 1× phosphate-buffered saline (PBS; pH 7.2). After being washed, the ticks were

\* Corresponding author. Mailing address: Department of Medical Microbiology and Parasitology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602. Phone: (706) 542-8447. Fax: (706) 542-0059. E-mail: slittle@vet.uga.edu.

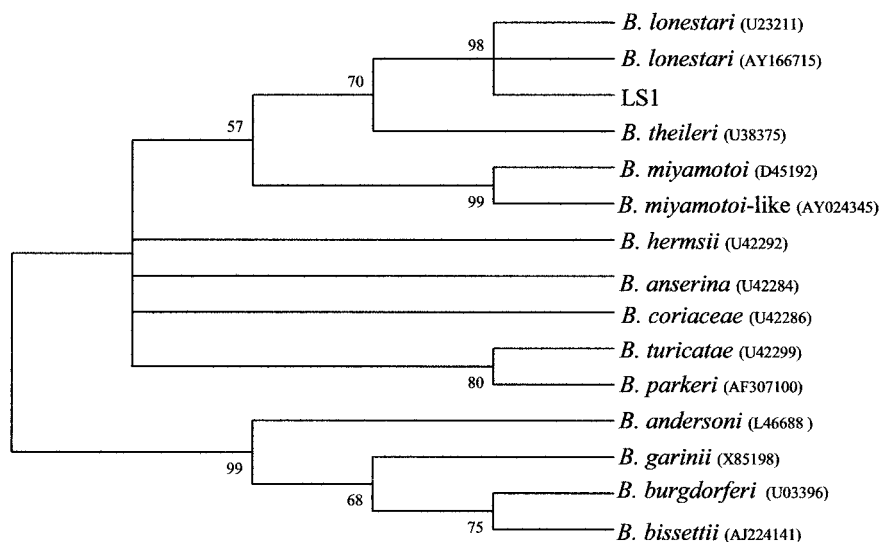


FIG. 1. Bootstrap consensus (1,000 times) of phylogenetic tree of *Borrelia* sp. 16S rDNA gene sequences generated using maximum-parsimony analysis with close-neighbor interchange. The number at each node indicates the percentage of times that node was supported by bootstrap analysis.

placed in a sterile petri dish and individually dissected using fine forceps and a no. 11 scalpel blade. The instruments were sterilized between individual tick dissections with a bead sterilizer, and the scalpel blades were changed between ticks. Tissues from each dissected tick were pooled in a sterile 1.5-ml tube with 1 ml of complete Barbour-Stoenner-Kelly II (BSKII) medium prepared by the College of Veterinary Medicine, University of Georgia, as previously described (6, 7) using bovine serum albumin fraction V (catalog number 81003; ICN Bio medicals, Costa Mesa, Calif.). The medium was supplemented with 6% rabbit serum (catalog number 16120099; Invitrogen, Carlsbad, Calif.), phosphomycin (0.02 mg/ml), rifampin (0.05 mg/ml), amphoterin (2.5 µg/ml), and 1.4% gelatin, hereafter referred to as BSKII/G. The contents of the tube were mixed well by vortexing, transferred to a sterile 4.5-ml snap cap tube with ~3 ml of BSKII/G medium, and maintained at 34°C overnight to test for contamination.

When no evidence of contamination was observed after 24 h, the snap cap tube was centrifuged at 75 × g for 5 min to pellet the contents. After 4 ml of supernatant was removed, the pellet was resuspended in the remaining 0.5 ml of BSKII/G and added to 4.5 ml of L-15B300 medium consisting of L-15B (catalog number 41300; Invitrogen) supplemented with 5% fetal bovine serum (catalog number F0643; Sigma, St. Louis, Mo.), 10% tryptose phosphate broth (catalog number T9157; Sigma), and 0.1% bovine lipoprotein concentrate (catalog number 191475; ICN Biomedicals) (34). This 5-ml suspension was transferred to a 12.5-cm<sup>2</sup> flask with a confluent culture of ISE6 cells, an embryonic *Ixodes scapularis* tick cell line (kindly provided by Uli Munderloh, University of Minnesota). The culture was fed every 3 to 4 days, and the spent medium was transferred to a separate sterile tube and centrifuged at 210 × g for 5 min to pellet spirochetes and cells. After all but 0.5 to 1 ml of medium was removed, the pellet was resuspended and saved at -20°C for PCR assay and/or used to prepare slides for dark-field microscopy, for indirect fluorescent antibody (IFA) testing, or for subsequent passages. Subsequent passages of the original isolate were maintained in L-15B300 with 10% BSK-H (catalog number B3528; Sigma); the BSK-H medium was supplemented with 6% rabbit serum and antibiotics as described for the BSKII medium above.

Upon detection of live spirochetes under dark-field microscopy and confirmation of their identity by PCR, samples of the primary culture of the isolate, designated strain LS-1, were passaged into additional flasks of ISE6 cells to maintain the culture and into cell-free culture tubes with BSK/G or L-15B300 with or without supplemental BSK/G; culture aliquots were also frozen in liquid nitrogen for future use. As a positive control for IFA tests, *B. burgdorferi* SH2-82 was cultured in cell-free BSK/G medium and passaged to ISE6 cells with L-15B300 and 10% BSK/G. All cocultures in ISE6 cells were fed twice weekly, and the spent media were assessed by dark-field microscopy for the presence of spirochetes. Samples of cultured tick spirochetes in cell-free tubes were monitored for color change as evidence of growth and harvested after 30 days in culture to check for live spirochetes.

**Molecular analysis.** DNA was extracted from 100 µl of tick cell culture 3, 14, and 17 days after inoculation into ISE6 cell monolayers, using the GFX Genomic Blood Purification kit (Amersham Pharmacia Biotech, Piscataway, N.J.). The sample collected on day 3, which contained remnants of the tick tissue inoculum, was tested to determine whether the original inoculum contained *Borrelia* spp. Two DNA targets were amplified and sequenced to identify the organisms: a 330-bp region of the flagellin B gene and a 1,336-bp region of the 16S rRNA gene. The external primers FLALL and FLARL and the internal primers FLALS and FLARS were used in a nested-PCR assay to amplify the *flaB* target as previously described (32). Due to failure to amplify the 16S rDNA target using a previously described protocol (10), we used a heminested-PCR assay to increase amplification of the template. The primary reaction mixture consisted of 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 0.5 µM primers 8F (5'-AGTTTGATCATGCTCAG-3') and 16RnA, 200 µM each deoxynucleoside triphosphate, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, and 10 mM Tris-HCl (pH 9.0), with 5 µl of DNA template in a final reaction volume of 100 µl (10). The same reagent concentrations and 2 µl of primary product were combined in a secondary reaction using primers 16RnA and 16RnR. Thermal cycling for amplification of the 16S rDNA followed the program used by Barbour et al. (10). Water controls were included in each step of each reaction, and DNA extraction, primary and secondary PCR, and gel visualization were performed in separate laboratories to reduce the risk of contamination. Amplicons were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide under UV transillumination.

Amplicons were purified and concentrated using a Microcon 100 microconcentrator (Amicon, Inc., Beverly, Mass.) and submitted to MWG Biotech (High Point, N.C.) for direct sequencing of both forward and reverse strands. The resultant sequences were aligned using the ClustalX multiple-alignment program and directly compared to published 16S rDNA and *flaB* sequences of *B. lonestari* (= *Borrelia barbouri*) and other *Borrelia* spp. available in GenBank. To further compare the 16S rDNA sequence from our isolate with those of other *Borrelia* spp., phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis program version 2.1. For phylogenetic analysis, sequence alignment was performed using the ClustalX multiple-alignment program on the 16S rDNA fragment sequenced from the LS-1 isolate, corresponding to positions 190 to 1076 of the *B. lonestari* 16S rDNA sequence U23211. The lengths of analyzed 16S rDNA sequences were limited by the amount of sequence data available for *Borrelia theileri* and thus did not include the entire available LS-1 sequence. Accession numbers for all *Borrelia* sp. sequences used in the construction of the phylogenetic tree are provided in Fig. 1.

**IFA testing.** Samples of ISE6 cell cultures infected with LS-1 and separate ISE6 cell cultures infected with *B. burgdorferi* SH2-82 were fixed on microscope slides with acetone and tested by IFA assay for reactivity with a *Borrelia*-wide monoclonal antibody (MAb) against flagellin (H9724) and a *B. burgdorferi*-

TABLE 1. Comparison of *B. lonestari* flagellin B gene sequences from GenBank with those amplified from our isolate

Source <sup>a</sup> (reference)	State	GenBank accession no.	Nucleotide difference at position <sup>b</sup>			
			330	331	332	348
Cell culture <sup>c</sup>	GA	AY442141	*	*	*	A
LST (4)	MO	AY166716	*	*	*	A
LST (10)	TX	U26074	*	*	*	G
LST (10)	NJ	U26075	*	*	*	A
LST (11)	AL	AF298653	*	*	*	A
LST (11)	AL	AF298654	*	*	*	A
Human (23)	NC, MD	AF273670	A	G	A	A
LST (23)	NC, MD	AF273671	A	G	A	A
WTD (32)	NC	AF538851	*	*	*	A
WTD (32)	GA	AF538852	A	G	A	A

<sup>a</sup> LST, lone star tick; WTD, white-tailed deer.  
<sup>b</sup> Numbers correspond to nucleotide position of *B. lonestari flaB* sequence U26074. An asterisk indicates no corresponding base at the position.  
<sup>c</sup> Sequence of the isolate reported in this study; originally isolated from *A. americanum* from Georgia.

specific MAb which recognizes OspA (H5332) (both MAbs were kindly provided by Tom G. Schwan, Rocky Mountain Laboratories, Hamilton, Mont.) (5, 8). Slides were incubated with undiluted primary antibody for 25 min and then washed twice in 1× PBS for 5 min each, rinsed in distilled water for 5 min, and allowed to dry. A 1:30 dilution of fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody was applied, and the slides were incubated for 25 min and subsequently washed in PBS as described above and then counterstained with eriochrome black T. After drying in the dark, the slides were examined with a compound microscope under UV illumination.

**Light and electron microscopy.** For measuring the lengths of spirochetes, samples prepared by cyto centrifugation from culture passages were fixed in methanol, stained with Giemsa stain, and examined by light microscopy. For scanning and transmission electron microscopy (SEM and TEM), a sample of spirochetes was prepared from the primary isolate, LS-1, cocultured in ISE6 cells. Approximately 5 ml of medium from the original flask, containing nonadherent ISE6 cells and spirochetes, was initially centrifuged at 83 × g for 1 min. Two milliliters of supernatant was removed for subsequent passage of cell-free spirochetes. The remaining 3 ml was resuspended; 0.5 ml was retained for dark-field examination, PCR, and further passage, and 2.5 ml was centrifuged at 210 × g for 5 min to pellet spirochetes and ISE6 cells. The supernatant was removed, and the pellet was fixed in 2% glutaraldehyde–2% (para)formaldehyde–0.2% picric acid in 0.1 M cacodylate-HCl buffer (pH 7.0 to 7.3).

The fixed cell suspension was divided for TEM and SEM preparation. For TEM, the portion was centrifuged at 9,550 × g, the fixative was removed, and the pellet was resuspended in buffer. This was repeated once, and then the sample was centrifuged a third time, the buffer was removed, and the pellet was resuspended in 50°C molten agar. The agar suspension was centrifuged at 9,550 × g for 5 min and allowed to solidify at 4°C. When solid, the pellet was cut into sections no greater than 1 mm thick, placed in buffer solution, and subsequently

TABLE 2. Comparison of *B. lonestari* (= *B. barbouri*) 16S rRNA gene sequences from GenBank with those amplified from our isolate

Source <sup>a</sup> (reference)	State	GenBank accession no.	Nucleotide difference at position <sup>b</sup>			
			522	698	1065	1201
Cell culture <sup>c</sup>	GA	AY442142	C	T	G	C
LST (4)	MO	AY166715	C	T	A	C
LST (10)	TX	BLU23211	C	C	G	T
LST (41)	MD	U38374	T	NR <sup>d</sup>	NR <sup>d</sup>	NR <sup>d</sup>

<sup>a</sup> LST, lone star tick.  
<sup>b</sup> Numbers correspond to nucleotide position of *B. lonestari* 16S rDNA sequence U23211.  
<sup>c</sup> Sequence of the isolate reported in this study; originally isolated from *A. americanum* from Georgia.  
<sup>d</sup> NR, not reported.

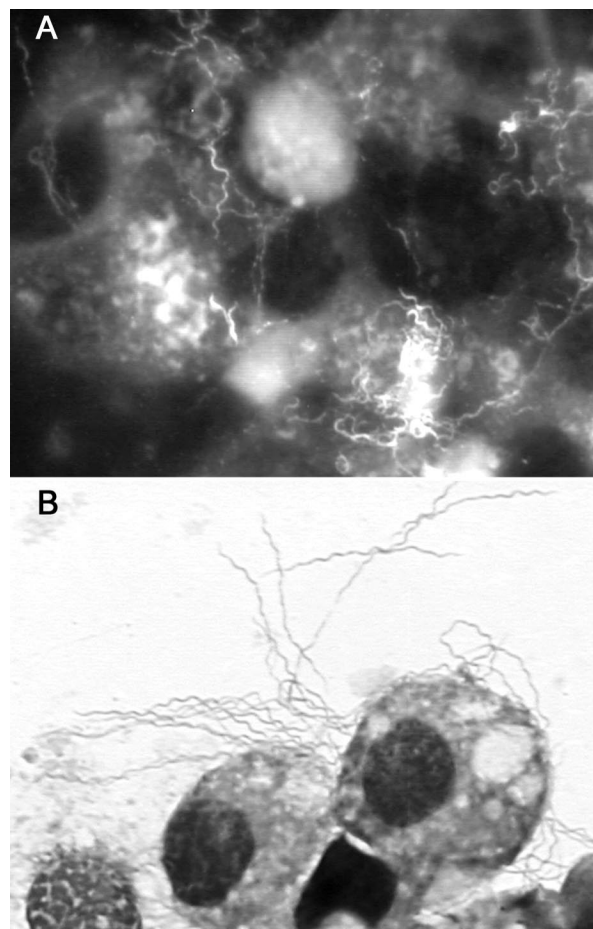


FIG. 2. Immunofluorescence antibody and Giemsa staining of cultured spirochetes. (A) Immunofluorescence antibody staining of spirochetes isolated from lone star ticks using *Borrelia*-wide anti-flagellin antibody (H9724). (B) ISE6 tick cell cultures of spirochetes were Giemsa stained; spirochetes are present free and attached to ISE6 cells. Magnification, ×1,000.

postfixed in 1% OsO<sub>4</sub>–0.1 M cacodylate-HCl buffer. The sections were rinsed four times in deionized water for 10 min each time, stained en bloc with 0.5% uranyl acetate (aqueous) for 1 h, and rinsed with deionized water three times for 2 min each time. After being dehydrated with increasing concentrations of alcohol, the sections were infiltrated with Epon-Araldite (EMS, Hatfield, Penn.) and then polymerized at 75°C prior to examination with a JEOL JSM-1210 transmission electron microscope.

The fixed portion retained for SEM was rinsed three times in 0.1 M buffer for 15 min each time and allowed to adhere to a coverslip as follows. Briefly, a drop of poly-L-lysine (in PBS) was placed onto a 12-mm-diameter round coverslip and allowed to set at room temperature for 1 h and was then rinsed in running water. A drop of washed cell suspension was placed on the coated coverslip, and the coverslip was stored in a humidity chamber to allow the cells to settle. The coverslip was critical-point dried, mounted on an aluminum stub with Electrodag 502 (Ted Pella, Inc., Redding, Calif.) to dry, and overlaid with sputter coat prior to examination with a JEOL JSM-5800 scanning electron microscope.

A separate sample for negative staining was taken from a second passage of LS-1 spirochetes in ISE6 cell culture. Medium with nonadherent ISE6 cells and spirochetes was centrifuged at 302 × g for 10 min, and the pellet was resuspended in sterile filtered PBS and centrifuged at 302 × g for 10 min. The washed pellet was resuspended in Karnovsky's type fixative, fixed at 4°C for 1 h, and centrifuged at 302 × g for 10 min. The pellet was washed in sterile filtered PBS, centrifuged at 302 × g for 10 min, and resuspended once more with a small (~50-μl) volume of PBS. A 40-μl volume of suspension in a drop of deionized water was airfused (pounds per square inch gauge 10) on a copper grid at 43,110



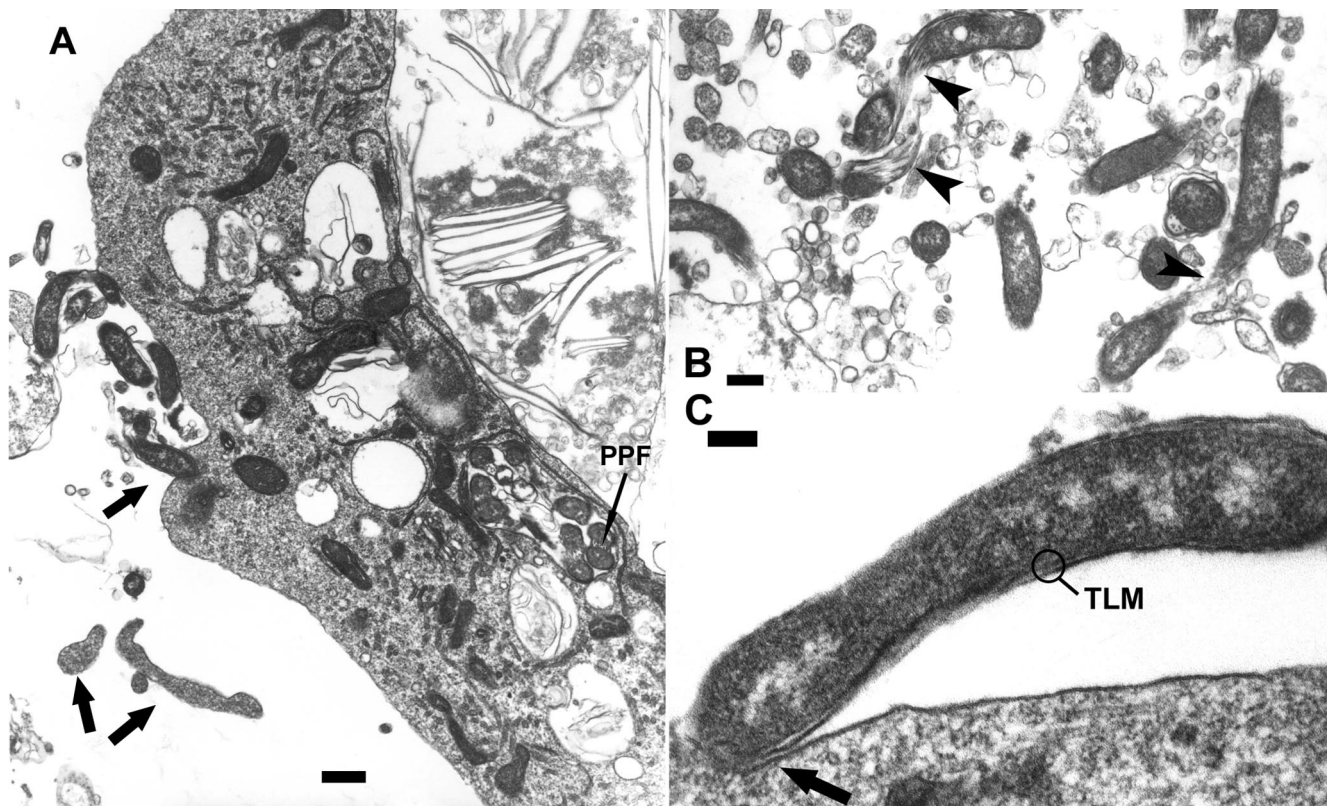


FIG. 3. Transmission electron micrographs of cultured spirochetes. (A) Spirochetes are present free and associated with ISE6 cells (arrows). Transverse sections of intracellular spirochetes showing periplasmic flagella (PPF) are evident within membrane-bound vacuoles. (B) Bundles of flagella can be seen running along oblique and transverse sections of spirochetes (arrowheads). (C) Spirochete and ISE6 cell in close association, with loss of resolution at the site of contact (arrow). The trilaminar membrane (TLM), consisting of an outer sheath, cell wall, and inner cytoplasmic membrane, is also seen more directly. Bars, 500 (A), 200 (B), and 100 (C) nm.

× g for 5 min and stained with 2% phosphotungstic acid (pH 7.0) for 30 s for TEM examination.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the *B. lonestari* flagellin gene and the 16S rRNA gene reported in this paper are AY442141, and AY442142, respectively.

## RESULTS

**Culture isolation.** Live spirochetes from the primary culture were first visualized both free in the medium and attached to ISE6 cells under dark-field microscopy 14 days after the flask was inoculated and then twice weekly thereafter from spent cell medium that was removed when the flask was fed. A thawed aliquot that had been frozen for 3 days in liquid nitrogen was proven viable after passage into a confluent monolayer of ISE6 cells. Spirochetes passed into culture tubes with cell-free medium were not viable after 30 days in culture.

**Molecular characterization.** *flaB* sequences of cultured spirochetes collected 3, 14, and 17 days postinoculation were identical to each other and to previously published *flaB* sequences of *B. lonestari* (Table 1), including the original *flaB* sequence of *B. lonestari* described from *A. americanum* collected in New Jersey (10) and *flaB* sequences subsequently described from ticks from Alabama and Missouri and deer from Georgia and North Carolina (4, 11, 32). The 3-bp insert at nucleotides 330 to 332 that has been described from several other *flaB* sequences of *B. lonestari* (23, 32, 47) was not present

in our isolate. The 16S rDNA sequence from our isolate also was consistent with sequences previously reported from *B. lonestari* (= *B. barbouri*) (Table 2) and differed by 1 base from the 16S rDNA sequence of *B. lonestari* reported from *A. americanum* from Missouri and by 2 bases from the 16S rDNA sequence of *B. lonestari* reported from *A. americanum* from Texas (4, 10).

Phylogenetic trees constructed using 16S rDNA sequences from our isolate and other *Borrelia* spp. resulted in *B. lonestari* forming a group with *Borrelia miyamotoi* and *B. theileri* that is more closely related to the relapsing fever *Borrelia* spp. than either group is to *B. burgdorferi* and its allies (Fig. 1), as previously described (10, 44).

**IFA testing.** The anti-flagellin MAb H9724, which recognizes all *Borrelia* spp., bound to whole-cell antigen from the LS-1 strain spirochetes (Fig. 2A) as well as to that from *B. burgdorferi* SH-1 spirochetes in ISE6 cell culture. The anti-OspA MAb specific to *B. burgdorferi* recognized *B. burgdorferi* but not the LS-1 isolate (data not shown).

**Light and electron microscopy.** The morphology of *B. lonestari* was typical for *Borrelia* spp. (7, 15, 20, 21, 22). Spirochetes were seen free and attached to or closely associated with ISE6 cells and other spirochetes and within cells on TEM (Fig. 2B, 3A, and 4B). Under light microscopy, spirochetes were 11 to 25  $\mu\text{m}$  long and 0.23 to 0.26  $\mu\text{m}$  wide. The average length of

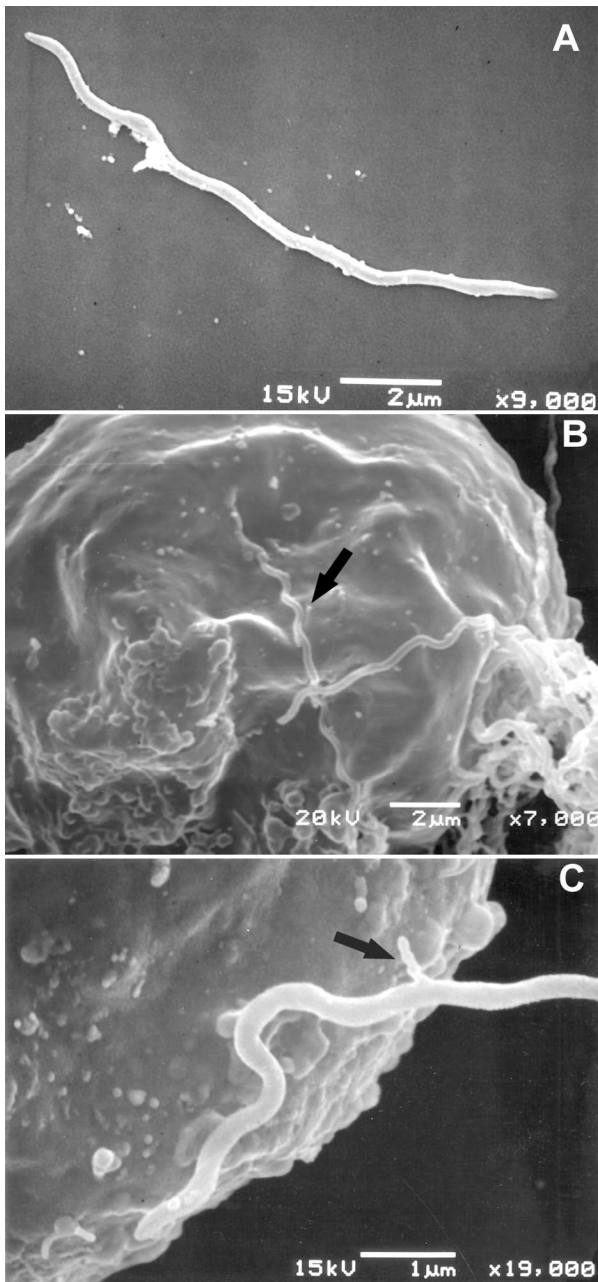


FIG. 4. Scanning electron micrographs of cultured spirochetes. (A) Single spirochete with characteristic tapered ends, relatively flat wavelength, and a cytoplasmic bleb. (B) The arrow points to a spirochete with a counterclockwise helix (left handed; moving away from the observer); also evident are spirochetes entangled with each other and closely associated with the ISE6 cell. (C) Greater magnification of a cytoplasmic bleb (arrow).

most spirochetes was 15.48  $\mu\text{m}$ . Several apparently longer spirochetes (37 to 40  $\mu\text{m}$ ) were suspected to be in the process of dividing or attached to another spirochete in tandem.

Several spirochetes were intimately associated with ISE6 cells and appeared to be indenting the cell surface; in these areas, the spirochete-ISE6 cell interface seemed less distinct, suggesting that the spirochetes were in the process of entering the cells (Fig. 3A and C). Within ISE6 cells, multiple spiro-

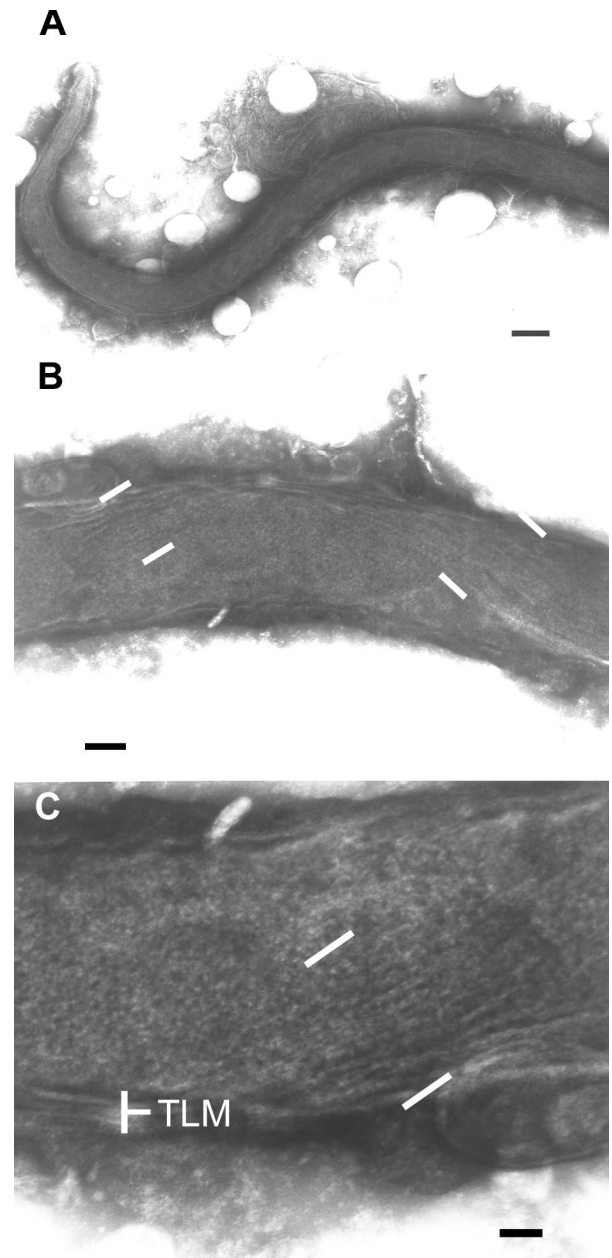


FIG. 5. Transmission electron micrographs of negatively stained spirochetes. (A) Spirochete with a prominent outer membrane extending over one end. (A and B) Flagella can be seen running along the axis of the spirochete. (B) Flagellar bundles are evident on either side of the curvature of the spirochete (bars). (C) Greater magnification of the spirochete from panel B reveals the trilaminar membrane (TLM) with evidence of multiple flagella running periplasmically and more clearly demonstrates a single flagellar bundle (bars). Bars, 200 (A), 100 (B), and 50 (C) nm.

chetes were seen inside membrane-bound vacuoles (Fig. 3A). Transverse sections of spirochetes revealed a trilaminar membrane consisting of an outer membrane (sheath), a cell wall, and an inner cytoplasmic membrane, analogous to what has been described for other *Borrelia* spp. (7, 49) (Fig. 3C and 5C). Periplasmic flagella were seen as bundles on one side of the spirochete, between the outer membrane and the cell wall of



the spirochete in transverse sections, and emerging along the submembranous surfaces of organisms in oblique sections.

The majority of spirochetes appeared to have a flat, wavelike shape, with widely variable wavelengths (1.5 to 2.36  $\mu\text{m}$ ) and amplitudes (0.45 to 0.53  $\mu\text{m}$ ). In spirochetes that appeared helical, the spirals were directed counterclockwise (Fig. 4B). Flagella were not apparent on SEM, but protrusions resembling cytoplasmic blebs, as well as less distinct membrane-associated debris, were observed on the surfaces of spirochetes (Fig. 4A and C). A trilaminar membrane was confirmed on TEM of negatively stained spirochetes, with a prominent outer membrane extending over the end of an organism (Fig. 5A). Bundles of 6 to 11 flagella were present in the submembranous space directed parallel to the long axis (Fig. 5B). The widths of flagellar bundles ranged from 0.105 to 0.156  $\mu\text{m}$ .

## DISCUSSION

Our cell culture and molecular, immunological, and electron microscopy data all indicate that we have made the first isolation of *B. lonestari* in culture; we have designated this strain LS-1. We were able to cultivate this fastidious organism using a tick cell line and a source of organisms from naturally infected lone star ticks. The spirochetes were found to possess molecular characteristics consistent with the organism tentatively named *B. lonestari* and morphological and IFA staining properties distinct from those of *B. burgdorferi*.

The flagellin B gene sequence of our *B. lonestari* isolate (LS-1) is identical to *B. lonestari flaB* sequences previously reported from lone star ticks and white-tailed deer in that it lacks a 3-base insert at positions 330 to 332 (4, 10, 11, 32). Although the LS-1 isolate differs in this respect from the single *flaB* sequence of *B. lonestari* reported from a human clinical case (23), the significance of this 3-base insert is not clear. Nucleotide differences have been observed at four base positions in the 16S rRNA gene among the reported sequences of *B. lonestari* (= *B. barbouri*). Our LS-1 isolate 16S rRNA gene sequence matched one of the two published sequences for *B. lonestari* at three of these base positions and both *B. lonestari* sequences at the fourth position (base 522) (10). The published sequence for *B. barbouri* contained a single base difference at position 522, but because this was a partial sequence (355 bp) that did not include bases 698, 1025, and 1201, nucleotide similarity could not be evaluated at those positions (41). While it is important to note them, these differences are minor, and both the 16S rRNA and flagellin B gene sequences of the LS1 spirochetes support the identification of the organisms we have cultured as *B. lonestari*.

We also report here the first ultrastructural description of *B. lonestari*. The LS-1 organisms had typical spirochete morphology and characteristics consistent with *Borrelia* spp. (7, 22). Their lengths, although variable, also fell within the range of *Borrelia* spp. The ends of LS-1 organisms, like those of *Borrelia anserina* and *Borrelia recurrentis*, were tapered, but the tips were relatively blunt in comparison to those of *B. burgdorferi*; unlike *B. anserina*, strain LS-1 was not regularly waved (15, 22). Cytoplasmic protrusions or blebs on the surfaces of LS-1 organisms were consistent with protrusions that have been described for other spirochetes (7, 14).

TEM showed multiple sections of spirochetes inside vacu-

ole-like, membrane-bound organelles within the ISE6 cells. Transverse organism sections that shared the same periplasmic flagellar bundle were evidently the same organism; other apparently unassociated sections may have been the same organism or another organism within the same organelle. The presence of multiple membrane-bound organelles within the same ISE6 cell, as well as single spirochetes that appeared to be free within the cytoplasm, suggests that *B. lonestari* may replicate within membrane-bound organelles of cells and then emerge from the organelles and cells. Spirochetes seen in close association with ISE6 cells on SEM, often indenting the host cell membrane, may represent the attachment and subsequent invasion of ISE6 cells, suggesting a possible dependence on the host tick cell. The interactions between these spirochetes and their growth in culture are among many significant life history characteristics that await investigation.

The findings in this study establish the critical foundation necessary for further investigations of *B. lonestari* that were previously hampered by the lack of a live isolate. Although culture isolation of *B. lonestari* from human cases of STARI is necessary to demonstrate a causative link to the illness, our results are an important step toward a greater understanding of STARI and the variable presentation of this Lyme disease-like illness. Cultured organisms are critical for the development of accurate human diagnostic assays; this will allow investigation of the clinical manifestations of infection in humans, as well as further our understanding of the epidemiology and natural history of *B. lonestari*, including host and vector competence, natural maintenance cycles, and geographical distribution. In addition, an in vitro source of organisms is now available for controlled experiments using animal hosts as models for human infection, as well as tick transmission studies. The knowledge gained from studies using cultured *B. lonestari* will be fundamental in differentiating between *B. lonestari* and *B. burgdorferi* infections and ultimately in revealing the true occurrence of Lyme disease and STARI in the southeastern and south-central United States.

## ACKNOWLEDGMENTS

We are grateful to Wayne Roberts and Mary Ard at the University of Georgia, Athens, for assistance with dark-field microscopy and electron microscopy, respectively; Tom G. Schwan, Rocky Mountain Laboratories, Hamilton, Mont., for supplying MAb; and Uli Munderloh, University of Minnesota, for providing the ISE6 tick cell line. We also thank Grace Chan, Amy Sexauer, and numerous personnel at the Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, for their assistance in field collections and laboratory studies.

## REFERENCES

- Anderson, J. F., and L. A. Magnarelli. 1993. Natural history of *Borrelia burgdorferi* in vectors and vertebrate hosts, p. 11–24. In H. S. Ginsberg (ed.), Ecology and environmental management of Lyme disease. Rutgers University Press, New Brunswick, N.J.
- Armstrong, P. M., S. M. Rich, R. D. Smith, D. L. Hartl, A. Spielman, and S. R. Telford III. 1996. A new *Borrelia* infecting Lone Star ticks. *Lancet* **347**:67–68.
- Armstrong, P. M., L. R. Brunet, A. Spielman, and S. R. Telford III. 2001. Risk of Lyme disease: perceptions of residents of a Lone Star tick-infested community. *Bull. W. H. O.* **79**:916–925.
- Bacon, R. M., R. D. Gilmore, Jr., M. Quintana, J. Piesman, and B. J. B. Johnson. 2003. DNA evidence of *Borrelia lonestari* in *Amblyomma americanum* (Acari:Ixodidae) in Southeast Missouri. *J. Med. Entomol.* **40**:590–592.
- Barbour A. G., S. L. Tessier, and W. J. Todd. 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. *Infect. Immun.* **41**:795–804.

6. Barbour, A. G. 1984. Isolation and cultivation of Lyme Disease spirochetes. *Yale J. Biol. Med.* **57**:521–525.
7. Barbour, A. G., and S. F. Hayes. 1986. Biology of *Borrelia* species. *Microbiol. Rev.* **30**:381–400.
8. Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schruppf, and S. L. Tessier. 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. *Infect. Immun.* **52**:549–554.
9. Barbour, A. G. 1988. Laboratory aspects of Lyme borreliosis. *Clin. Microbiol. Rev.* **1**:399–414.
10. Barbour, A. G., G. O. Maupin, G. J. Teltow, C. J. Carter, and J. Piesman. 1996. Identification of an uncultivable *Borrelia* species in the hard tick *Amblyomma americanum*: possible agent of a Lyme disease-like illness. *J. Infect. Dis.* **173**:403–409.
11. Burkot, T. R., G. R. Mullen, R. Anderson, B. S. Schneider, C. M. Happ, and N. S. Zeidner. 2001. *Borrelia lonestari* DNA in adult *Amblyomma americanum* ticks, Alabama. *Emerg. Infect. Dis.* **7**:471–473.
12. Campbell, G. L., W. S. Paul, M. E. Schriefer, R. B. Craven, K. E. Robbins, and D. T. Dennis. 1995. Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990–1993. *J. Infect. Dis.* **172**:470–480.
13. Centers for Disease Control and Prevention. 1997. Case definitions for infectious conditions under public health surveillance. *Morb. Mortal. Wkly. Rep.* **46**:20–21.
14. Charon, N. W., S. F. Goldstein, S. M. Block, K. Curci, J. D. Ruby, J. A. Kreiling, and R. J. Limberger. 1992. Morphology and dynamics of protruding spirochete periplasmic flagella. *J. Bacteriol.* **174**:832–840.
15. Cutler, S. J., J. Moss, M. Fukunaga, D. J. M. Wright, D. Fekade, and D. Warrell. 1997. *Borrelia recurrentis* characterization and comparison with relapsing-fever, Lyme-associated, and other *Borrelia* spp. *Int. J. Syst. Bacteriol.* **47**:958–968.
16. Feir, D., C. Reppell Santanello, B. Li, C. Xie, E. Masters, R. Marconi, and G. Weil. 1994. Evidence supporting the presence of *Borrelia burgdorferi* in Missouri. *Am. J. Trop. Med. Hyg.* **51**:475–482.
17. Felz, M. W., and L. A. Durden. 1996. Ticks parasitizing humans in Georgia and South Carolina. *J. Parasitol.* **82**:505–508.
18. Felz, M. W., and L. A. Durden. 1999. Attachment sites of four tick species (Acari: Ixodidae) parasitizing humans in Georgia and South Carolina. *J. Med. Entomol.* **36**:361–364.
19. Felz, M. W., F. W. Chandler, Jr., J. H. Oliver, Jr., D. W. Rahn, and M. E. Schriefer. 1999. Solitary erythema migrans in Georgia and South Carolina. *Arch. Dermatol.* **135**:1317–1326.
20. Goldstein, S. F., K. F. Buttler, and N. W. Charon. 1996. Structural analysis of the *Leptospiraceae* and *Borrelia burgdorferi* by high-voltage electron microscopy. *J. Bacteriol.* **178**:6539–6545.
21. Hovind-Hougen, K. 1984. Ultrastructure of spirochetes isolated from *Ixodes ricinus* and *Ixodes dammini*. *Yale J. Biol. Med.* **57**:543–548.
22. Hovind-Hougen, K. 1995. A morphological characterization of *Borrelia anserina*. *Microbiology* **141**:79–83.
23. James, A. M., D. Liveris, G. P. Wormser, I. Schwartz, M. A. Montecalvo, and B. J. B. Johnson. 2001. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. *J. Infect. Dis.* **183**:1810–1814.
24. Kirkland, K. B., T. B. Klimko, R. A. Meriwether, M. S. Schriefer, M. Levin, J. Levine, W. R. Mackenzie, and D. T. Dennis. 1997. Erythema migrans-like rash at a camp in North Carolina. *Arch. Intern. Med.* **157**:2635–2641.
25. Koch, H. G., and R. W. McNew. 1982. Sampling of Lone Star ticks (Acari: Ixodidae): dry ice quantity and capture. *Ann. Entomol. Soc. Am.* **75**:579–582.
26. Kurtti, T. J., U. G. Munderloh, G. G. Ahlstrand, and R. C. Johnson. 1988. *Borrelia burgdorferi* in tick cell culture: growth and cellular adherence. *J. Med. Entomol.* **25**:256–261.
27. Kurtti, T. J., U. G. Munderloh, D. E. Krueger, R. C. Johnson, and T. G. Schwan. 1993. Adhesion to and invasion of cultured tick (Acarina: Ixodidae) cells by *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) and maintenance of infectivity. *J. Med. Entomol.* **30**:586–596.
28. Levine, J. F., M. L. Wilson, and A. Spielman. 1985. Mice as reservoirs of the Lyme disease spirochete. *Am. J. Trop. Med. Hyg.* **34**:355–360.
29. Luekhart, S., G. R. Mullen, and J. C. Wright. 1991. Etiologic agent of Lyme disease, *Borrelia burgdorferi*, detected in ticks (Acari: Ixodidae) collected at a focus in Alabama. *J. Med. Entomol.* **28**:652–657.
30. Masters, E., S. Granter, P. Duray, and P. Cordes. 1998. Physician-diagnosed erythema migrans-like rashes following lone star tick bites. *Arch. Dermatol.* **134**:955–960.
31. Masters, E. J., and D. Donnell. 1995. Lyme and/or Lyme-like disease in Missouri. *Mo. Med.* **92**:346–353.
32. Moore, V. A., A. S. Varela, M. J. Yabsley, W. R. Davidson, and S. E. Little. 2003. Detection of *Borrelia lonestari*, putative agent of southern tick-associated rash illness, in white-tailed deer (*Odocoileus virginianus*) from the southeastern United States. *J. Clin. Microbiol.* **41**:424–427.
33. Mukolwe, S. W., A. A. Kocan, R. W. Barker, K. M. Kocan, and G. L. Murphy. 1992. Attempted transmission of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) (JDI strain) by *Ixodes scapularis* (Acari: Ixodidae), *Dermacentor variabilis*, and *Amblyomma americanum*. *J. Med. Entomol.* **29**:673–677.
34. Munderloh, U. G., S. D. Jauron, V. Fingerle, L. Leitritz, S. F. Hayes, J. M. Hautman, C. M. Nelson, B. W. Huberty, T. J. Kurtti, G. G. Ahlstrand, B. Greig, M. A. Mellencamp, and J. L. Goodman. 1999. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. *J. Clin. Microbiol.* **37**:2518–2524.
35. Oliver, J. H., F. W. Chandler, M. P. Luttrell, A. M. James, D. E. Stallknecht, B. S. McGuire, H. J. Hutvheson, G. A. Cummins, and R. S. Lane. 1993. Isolation and transmission of the Lyme disease spirochete from the southeastern United States. *Proc. Natl. Acad. Sci. USA* **90**:7371–7375.
36. Oliver, J. H. 1996. Lyme borreliosis in the southern United States: a review. *J. Parasitol.* **82**:926–935.
37. Oliver, J. H., T. M. Kollars, F. W. Chandler, A. M. James, E. J. Masters, R. S. Lane, and L. O. Huey. 1998. First isolation and cultivation of *Borrelia burgdorferi* sensu lato from Missouri. *J. Clin. Microbiol.* **36**:1–5.
38. Oliver, J. H., K. L. Clark, F. W. Chandler, Jr., L. Tao, A. M. James, C. W. Banks, L. O. Huey, A. R. Banks, D. C. Williams, and L. A. Durden. 2000. Isolation, cultivation, and characterization of *Borrelia burgdorferi* from rodents and ticks in the Charleston area of South Carolina. *J. Clin. Microbiol.* **38**:120–124.
39. Piesman, J., and R. J. Sinsky. 1988. Ability of *Ixodes scapularis*, *Dermacentor variabilis*, and *Amblyomma americanum* (Acari: Ixodidae) to acquire, maintain, and transmit Lyme disease spirochetes (*Borrelia burgdorferi*). *J. Med. Entomol.* **25**:336–339.
40. Rawlings, J. A., and G. J. Teltow. 1994. Prevalence of *Borrelia* (Spirochaetales) spirochetes in Texas ticks. *J. Med. Entomol.* **31**:297–301.
41. Rich, S. M., P. M. Armstrong, R. D. Smith, and S. R. Telford III. 2001. Lone star tick-infecting borreliae are most closely related to the agent of bovine borreliosis. *J. Clin. Microbiol.* **39**:494–497.
42. Sanders, F. H., Jr., and J. H. Oliver, Jr. 1995. Evaluation of *Ixodes scapularis*, *Amblyomma americanum*, and *Dermacentor variabilis* (Acari: Ixodidae) from Georgia as vectors of a Florida strain of the Lyme disease spirochete, *Borrelia burgdorferi*. *J. Med. Entomol.* **32**:402–406.
43. Schulze, T. L., G. S. Bowen, E. M. Bosler, M. F. Lakat, W. E. Parkin, R. Altman, B. G. Ormiston, and J. K. Shisler. 1984. *Amblyomma americanum*: a potential vector of Lyme disease in New Jersey. *Science* **224**:601–603.
44. Scoles, G. A., M. Papero, L. Beati, and D. Fish. 2001. A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. *Vector Borne Zoonotic Dis.* **1**:21–34.
45. Shapiro, E. D., and M. A. Gerber. 2000. Lyme disease. *Clin. Infect. Dis.* **31**:533–542.
46. Spielman, A. J., J. F. Levine, and M. L. Wilson. 1984. Vector capacity of North American Ixodes ticks. *Yale J. Biol. Med.* **57**:507–513.
47. Stegall-Faulk, T., D. C. Clark, and S. M. Wright. 2003. Detection of *Borrelia lonestari* in *Amblyomma americanum* (Acari: Ixodidae) from Tennessee. *J. Med. Entomol.* **40**:100–102.
48. Stromdahl, E. Y., S. R. Evans, J. J. O'Brien, and G. Gutierrez. 2001. Prevalence of infection in ticks submitted to the human tick test kit program of the U. S. Army Center for Health Promotion and Preventative Medicine. *J. Med. Entomol.* **38**:67–74.
49. Teltow, G. J., P. V. Fournier, and J. A. Rawlings. 1991. Isolation of *Borrelia burgdorferi* from arthropods collected in Texas. *Am. J. Trop. Med. Hyg.* **44**:469–474.