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Amblyomma americanum (Acari: Ixodidae) Ticks Are Not Vectors of the Lyme Disease Agent, *Borrelia burgdorferi* (Spirocheatales: Spirochaetaceae): A Review of the Evidence

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Abstract

In the early 1980s, *Ixodes* spp. ticks were implicated as the key North American vectors of *Borrelia burgdorferi* (Johnson, Schmid, Hyde, Steigerwalt and Brenner) (Spirocheatales: Spirochaetaceae), the etiological agent of Lyme disease. Concurrently, other human-biting tick species were investigated as potential *B. burgdorferi* vectors. Rashes thought to be erythema migrans were observed in patients bitten by *Amblyomma americanum* (L.) (Acari: Ixodidae) ticks, and spirochetes were visualized in a small percentage of *A. americanum* using fluorescent antibody staining methods, sparking interest in this species as a candidate vector of *B. burgdorferi*. Using molecular methods, the spirochetes were subsequently described as *Borrelia lonestari* sp. nov. (Spirocheatales: Spirochaetaceae), a transovarially transmitted relapsing fever *Borrelia* of uncertain clinical significance. In total, 54 surveys from more than 35 research groups, involving more than 52,000 ticks, have revealed a low prevalence of *B. lonestari*, and scarce *B. burgdorferi*, in *A. americanum*. In Lyme disease-endemic areas, *A. americanum* commonly feeds on *B. burgdorferi*-infected hosts; the extremely low prevalence of *B. burgdorferi* in this tick results from a saliva barrier to acquiring infection from infected hosts. At least nine transmission experiments involving *B. burgdorferi* in *A. americanum* have failed to demonstrate vector competency. Advancements in molecular analysis strongly suggest that initial reports of *B. burgdorferi* in *A. americanum* across many states were misidentified *B. lonestari*, or DNA contamination, yet the

early reports continue to be cited without regard to the later clarifying studies. In this article, the surveillance and vector competency studies of *B. burgdorferi* in *A. americanum* are reviewed, and we conclude that *A. americanum* is not a vector of *B. burgdorferi*.

Keywords

Amblyomma americanum; *Borrelia burgdorferi*; prevalence; vector competency

Sylvatic transmission cycles of the spirochetal bacterium *Borrelia burgdorferi* (Johnson, Schmid, Hyde, Steigerwalt and Brenner) (Spiro cheatales: Spirochaetaceae), the etiological agent of Lyme disease, are widespread in the eastern United States. *Ixodes scapularis* Say (Acari: Ixodidae) ticks are recognized as the key vector in the eastern half of the United States; however, other human-biting ticks – particularly *Amblyomma americanum* (L.) (Acari: Ixodidae) feed on many of the same wildlife reservoirs, leading to concerns that this tick species also may contribute to the risk of acquiring Lyme disease. Here we review the literature describing 30 yr of surveillance of *A. americanum* for *Borrelia* spp. and summarize the results of multiple vector competency studies undertaken on this tick. This body of research overwhelmingly indicates that *A. americanum* is not a vector of *B. burgdorferi*.

Discovery of *B. burgdorferi* and Investigation of Possible Vector Ticks

Lyme disease was first recognized and described in the United States in 1975 (Steere et al. 1977). Willy Burgdorfer and colleagues discovered the spirochetal agent, later described as *B. burgdorferi*, in *I. scapularis (dammini)* ticks from Shelter Island, NY, and demonstrated the vector competency of this tick species (Burgdorfer et al. 1982). The agent was later found to also be transmitted by human-biting *Ixodes pacificus* Cooley and Kohls (Acari: Ixodidae) in the western United States and by other human-biting *Ixodes* genus ticks in Eurasia (Lane et al. 1991, Rudenko et al. 2011). Additionally, several *Ixodes* spp. that do not regularly feed on humans have been implicated as responsible in maintaining sylvatic transmission of *B. burgdorferi* (Clark et al. 2002). During the early years of Lyme disease research, other common species of anthropophilic ticks were also investigated as potential vectors of the agent of the disease. Some of the publications from the 1980s and 1990s (reviewed below and summarized in Table 1) described spirochetes in *A. americanum* and identified them as *B. burgdorferi.* However, these early investigations used microscopy and fluorescent antibody staining methods that depend on subjective interpretation and can detect related spirochete species (Barbour et al. 1996), or remnants of dead spirochetes (Bockenstedt et al. 2012). Many of the studies involved ticks removed while feeding on animal hosts, and spirochetes detected were likely in the bloodmeal in the ticks' midgut rather than representing infections within the ticks' bodies. Also, contamination is suspected in some investigations of *A. americanum*, as results were not repeatable and suspect-positives shared identity with the *Borrelia* strain used for positive control (Nelson 1995, Piesman and Happ 1997). Subsequent studies (vide infra) provided further evidence that the presence of other spirochetes, degraded spirochetes, or contamination most likely account for the reports of *B. burgdorferi* in these early studies.

Concurrently with these tick surveillance studies, experiments to evaluate the ability of *A. americanum* to acquire and transmit *B. burgdorferi* were conducted, and added no evidence to support the role of *A. americanum* in Lyme disease. Seven studies in the 1980s and 1990s failed to demonstrate vector competency of *A. americanum* (Piesman and Sinsky 1988, Mather and Mather 1990, Mukolwe et al. 1992, Ryder et al. 1992, Oliver et al. 1993, Sanders and Oliver 1995, Piesman and Happ 1997). Three subsequent studies suggested and demonstrated a salivary mechanism for the apparent inability of *A. americanum* to acquire or support colonization by *B. burgdorferi* (Ledin et al. 2005, Soares et al. 2006, Zeidner et al. 2009).

In 1996, phylogenetic analysis of *Borrelia* DNA sequences amplified from *A. americanum* led to the identification of *Borrelia lonestari* sp. nov. (Spirocheatales: Spirochaetaceae), a relapsing fever species distinct from *B. burgdorferi* (Armstrong et al. 1996, Barbour et al. 1996) and other Lyme disease group spirochetes. This new spirochete became a focus of research after it was detected in both an *A. americanum* removed from a patient suffering a skin rash and in the medium containing a biopsy of the rash (James et al. 2001), leading to speculation that it was the etiological agent of Southern Tick Associated Rash Illness (STARI), which occurs following bites of *A. americanum* (Masters et al. 2008). Many of the subsequent investigations of *A. lonestari* in *A. americanum* involved screening of ticks using genus-wide or broadly reactive polymerase chain reaction (PCR) primers capable of also detecting *B. burgdorferi*. Consequently, publications reporting these investigations provide separate data on the prevalences of both *B. lonestari* and *B. burgdorferi* infection in *A. americanum*, as long as sufficient measures – such as nucleotide sequencing, or confirmation with multiple PCR targets – were taken to discriminate among *Borrelia* species in *Borrelia*-positive samples (Table 2).

Since the mid-1980s, at least 35 different research groups have published 54 studies describing the investigation of more than 52,000 *A. americanum* for *B. burgdorferi.* Scarce (0.5%) *A. americanum* were reported as suspect-positive, and *B. burgdorferi* was never isolated in culture from these ticks. Ten studies evaluating the vector competency and the mechanisms of *B. burgdorferi* evasion by *A. americanum* concluded that this tick species has no relevance to the transmission of *B. burgdorferi.* These studies are discussed in detail below.

Initial Investigations of *B. burgdorferi* in *A. americanum*

Detection by investigators in the 1980s and 1990s of a low prevalence (~1%) of *Borrelia* in more than 18,000 *A. americanum* (Table 1) demonstrated that the ticks were being exposed to spirochetes in host bloodmeals and presaged the discovery of *B. lonestari,* a spirochete that can be maintained in *A. americanum*. The methods of analysis of ticks for *Borrelia* spp. were typically darkfield microscopy and direct fluorescent antibody testing (DFA) or indirect fluorescent antibody testing (IFA) using polyclonal antibody (PAb) or monoclonal antibody (MAb). In one study, PCR was also used as a final step on samples positive by IFA. Analysis by darkfield or IFA with PAb will detect related *Borrelia* species (Barbour et al. 1996), so the identity of the species in these studies was not established (Schulze et al. 1984, 1986; Magnarelli et al. 1986; Levine et al. 1989; Rawlings and Teltow 1994; Luckhart et al.

1991, 1992; Kollars et al. 2000). Analysis using IFA and MAb is designed to be specific for *B. burgdorferi* (Barbour et al. 1983b), and IFA with MAb H5332 has been shown not to react with *B. lonestari* (Varela et al. 2004a); however, incorrect technique can produce false positives. False-positive results using a monoclonal antibody to the ospA protein (H5332) may arise due to insufficient blocking or washing, or concentrations of primary or secondary antibodies that are too high, all of which can result in nonspecific binding. Ideally, as a negative control studies should use an irrelevant mouse monoclonal antibody of the same immunoglobulin type as the monoclonal antibody against *B. burgdorferi*. Buffer or medium by itself may be inadequate as a negative control. Most of the studies reviewed here do not present IFA methods in enough detail to evaluate whether sufficient measures were taken to avoid false positives and none involved use of a negative control slide with *B. lonestari* antigen because the organism had not been characterized or cultured at the time the studies were conducted (Varela et al. 2004a). Furthermore, IFAs with MAbs rely on subjective interpretation and can potentially detect dead cells from host bloodmeals (Allan et al. 2010; Bockenstedt et al. 2012); it has been suggested by researchers investigating *B. burgdorferi* xenodiagnosis that ticks might acquire dead organisms during feeding (Marques et al. 2014). In the studies using the *B. burgdorferi*-specific MAb H5332, spirochetes were more often detected in *A. americanum* collected from animal hosts (Levine et al. 1991, Ouellette et al. 1997), but in some of the studies, spirochetes were detected by MAb H5332 in ticks collected from vegetation (Simpson & Hinck 1993, Ouellette et al. 1997, Feir et al. 1994). Results of MAb IFA in the study from Feir et al. (1994) were not consistently confirmed by subsequent PCR of the samples, and contamination was suspected (Nelson 1995). In one IFA study using MAb H5332, the origin of collection of the single positive tick was not reported, and this study included ticks from both vegetation and animal hosts (Sonenshine et al. 1995). Five studies reported attempts to culture *B. burgdorferi* from *A. americanum* ticks; none were successful (Schulze et al. 1986, Teltow et al. 1991, Rawlings and Teltow 1994; Rich et al. 2001; Clark et al. 2002).

Investigations of *A. americanum* from New Jersey

The first investigations of possible *B. burgdorferi* transmission by *A. americanum* were conducted in the 1980s in New Jersey by entomologists from the New Jersey State Department of Health (Schulze et al. 1984, Schulze et al. 1986). In the first study, *A. americanum* females were removed from two different patient's erythema migrans-like lesions, prompting a field survey in which spirochetes were detected in 9.1% (4/44) of *A. americanum* collected on the property where the second patient lived (Schulze et al. 1984). However, the authors observed no motile spirochetes in the infected *americanum* and remarked that 'It is not known if nonmotile spirochetes were the result of examining dead or moribund ticks, loss of pathogen viability during transtadial passage, or from indigenous substances within the tick hostile to spirochete vigor or survival'. The second study by the same lead author reported detection of *B. burgdorferi* in 4.6% (35/756) of adult and nymphal *A. americanum* and 15.6% (5/32 pools of 15 larvae) of larvae, when identifying spirochetes by darkfield microscopy and DFA testing using fluorescein isothiocyanate (FITC) labeled rabbit anti-*B. burgdorferi* antiserum. These methods are liable to detect antibodies to infections with other *Borrelia* spp. (Barbour et al. 1996), or antigens of non-viable, degraded *B. burgdorferi* spirochetes (Bockenstedt et al. 2012). The researchers were not able to

establish cultures in Barbour-Stoenner-Kelly (BSK) medium – the standard medium used for cultivating *burgdorferi* (Barbour et al. 1983a). Subsequently, spirochetes found in *A. americanum* collected by the New Jersey group were used to develop the PCR methods instrumental in the first investigation and description of *B. lonestari* (Barbour et al. 1996).

In later studies by the same New Jersey research group (Schulze et al. 2005, 2006), the findings from the 1980s were no longer described as *B. burgdorferi,* but rather as 'spirochetes', and the authors proposed that the spirochetes previously detected in *A. americanum* were *B. lonestari,* not *B. burgdorferi*. The finding of larval pools infected with spirochetes in Schulze et al. (1986) further suggests that the organism was the transovarially transmitted relapsing fever spirochete *B. lonestari*, rather than *B. burgdorferi*, which has not been found to be vertically transmitted by ticks (Stromdahl et al. 2003, Rollend et al. 2013). Investigation of *A. americanum* adults in Schulze et al. (2005), using PCR with primers designed to amplify all *Borrelia* spp. (Barbour et al. 1996), followed by sequencing of representative positive amplicons to confirm the species, reported 9.1% (11/121) infected with *B. lonestari* and none infected with *B. burgdorferi*. Another investigation of *A. americanum* adults by Schulze et al. (2006), using primers for *B. burgdorferi/B. lonestari* (Stegall-Faulk et al. 2003), followed by sequencing of amplicons, revealed 5.8% (6/103) infected with *B. lonestari* and none infected with *B. burgdorferi*.

Investigations of *A. americanum* from North Carolina and Virginia

Magnarelli et al. (1986), a group of researchers from Connecticut and North Carolina, examined *A. americanum* collected from whitetailed deer in North Carolina by DFA staining using FITC-labeled rabbit antibody against *B. burgdorferi* and detected *Borrelia* spirochetes in 1.4% (7/512) of the ticks. White-tailed deer are reservoir incompetent for *B. burgdorferi* (Telford et al. 1988) and are also zooprophylactic, likely because deer blood complement lyses *B. burgdorferi* in feeding ticks (Bouchard et al. 2013, Roome et al. 2017). Although *B. burgdorferi* is sometimes detected in *I. scapularis* collected from deer, these are likely fragments of dead or dying spirochetes, or another species more able to remain viable in whitetailed deer, such as *Borrelia miyamotoi* sp. nov. (Spirocheatales: Sp irochaetaceae) (Han et al. 2016) or *B. lonestari* (Moyer et al. 2006, Varela-Stokes 2007). DFA and polyclonal rabbit antiserum used in these analyses are liable to cross-react with other *Borrelia* spp. or identify dead and degraded *B. burgdorferi* spirochetes.

Levine et al. (1989), from North Carolina State University, used darkfield microscopy and DFA with polyclonal antisera to screen 1,836 North Carolina *A. americanum* adults and nymphs collected in 1984–1987 from vegetation. Spirochetes were found in nine *A. americanum* ticks using darkfield microscopy, but only one tick reacted to the polyclonal antisera. This same research group next removed ticks from hosts and vegetation in North Carolina and Virginia in 1987 and examined them for *Borrelia* with IFA using a *B. burgdorferi* species-specific MAb H5332 (Levine et al. 1991). *Borrelia* was not detected in 151 questing *A. americanum*, but was found in 6.0% (4/67) of *A. americanum* removed from animals (four larvae removed from two raccoons). The MAb H5332 was designed to be specific for *B. burgdorferi* (Barbour et al. 1983b) and has been found to be nonreactive with *B. lonestari* (Varela et al. 2004a), so, as is always the case for detection of pathogens in

engorged ticks collected from hosts, this might indicate spirochetes or their remnants in host blood, rather than viable infection of the tick itself. Sonenshine et al. (1995), at Old Dominion University, collected ticks from vegetation and animal hosts at several sites in eastern Virginia in 1991–1994 and used darkfield microscopy and IFA with MAb H5332 to detect *B. burgdorferi* in 0.2% (1/546) of *A. americanum*. Ticks examined in this study were either questing or removed from animal hosts; the origin of the positive tick was not specified in the article, so it is possible the spirochetes came from host blood.

The North Carolina State University group again collected *A. americanum* from raccoons and by flagging vegetation in North Carolina from 1990 to 1993 (Ouellette et al. 1997). Examination by IFA using *B. burgdorferi*-specific MAb H5332 revealed a very low prevalence of spirochetes, 0.2% (6/2,985 in the questing ticks), and a slightly higher prevalence of 1.7% (45/2,739) in ticks removed from raccoons. These investigators also sampled the raccoons and were able to culture spirochetes from the blood of 26% (23/87) of the animals; however, none of the IFA-positive ticks were removed from culture-positive raccoons. The spirochetes detected in the questing ticks may have been undigested fragments of spirochetes from previous infected bloodmeals (Allan et al. 2010, Bockenstedt et al. 2012).

Investigations of *A. americanum* from Texas

Investigations in 1988–1989 by the Texas Department of Health reported isolation of spirochetes identified by culture in 3/354 *A. americanum* pools collected from vegetation, animal hosts, and humans in Texas (Teltow et al. 1991). These cultured spirochetes were inoculated into mice in preparation for testing the vector competence of three human-biting tick species, including *A. americanum*. However, the isolates proved not to be infectious for mice, so vector competence trials were precluded. These isolates had pulsed field gel electrophoretic patterns and plasmid profiles that were indistinguishable from high-passage *B. burgdorferi* strain B31, which is suggestive of cross-contamination of cultures during primary isolation (Piesman and Happ 1997). In a second study of *A. americanum* collected from vegetation and animal hosts by the same research group (Rawlings and Teltow 1994), spirochetes were detected in 1.0% (54/5,195) of ticks using DFA testing that did not discriminate among *Borrelia* species. Attempts to culture spirochetes from additional ticks collected in this study failed. Spirochetes in *A. americanum* collected in this study were also used in the first investigation and description of *B. lonestari*, where authors (including G. J. Teltow, author of the reports mentioned in this paragraph) proposed that ‘the majority if not all of the spirochetes previously noted in *A. americanum* ticks were *B. lonestari* sp. nov. and not *B. burgdorferi*’ (Barbour et al. 1996). In more recent studies of human-biting ticks submitted to the Texas Department of State Health Services, all spirochetes found by PCR in *A. americanum* were determined by nucleotide sequencing to be *B. lonestari*, including 0.8% (4/367) in 2004–2008 (Williamson et al. 2010) and 1.4% (8/591) in 2008–2014 (Mitchell et al. 2016). In another investigation of *A. americanum* from Texas, Yuan (2010), at University of Texas at Houston, reported *B. lonestari*, and no *B. burgdorferi*, in 1.5% (3/186) of ticks tested by PCR and multilocus sequence typing (MLST).

Investigations of *A. americanum* from Alabama and Mississippi

Luckhart et al. (1991), of Auburn University, detected spirochetes in 4.0% (6/150) of *A. americanum* removed in 1988 and 1989 from white-tailed deer and vegetation in Alabama using DFA and IFA with anti-*B. burgdorferi* PAb, which are not *B. burgdorferi*-specific tests (as described above). The six positive ticks were recovered feeding on deer, so detection of other *Borrelia* species or fragments of dead spirochetes cannot be ruled out. In another Alabama study using DFA and IFA with anti-*B. burgdorferi* PAb, the same Auburn University group reported spirochetes from 6.4% (8/125) of *A. americanum* removed from hunter-killed deer (three of the positives were from the same deer), but again, the species identity of these spirochetes was not established (Luckhart et al. 1992). Almost a decade later, Auburn researchers used *Borrelia* genus-wide primers and sequencing (Barbour et al. 1996) to identify 1.0% (2/202) of questing *A. americanum* as being infected with *B. lonestari*, not *B. burgdorferi*; PCR of these ticks using *B. burgdorferi*-specific primers was negative (Burkot et al. 2001).

Collaborators from the CDC and the Mississippi Department of Health used DFA to examine 68 *A. americanum* collected in 1999 and 2000 from vegetation, deer, dogs, and humans in Mississippi; none were positive for *Borrelia* spp. spirochetes (Goddard et al. 2003).

Investigations of *A. americanum* from Arkansas

A U.S. Army entomology group examined ticks collected from vegetation, human, and animal hosts in Arkansas in 1990 using IFA with the *B. burgdoferi*-specific MAb H5332 (Kardatzke et al. 1992). No *B. burgdoferi* infections were detected in 471 *A. americanum*. Selected samples of IFA-negative ticks were also tested using DFA and FITC-labeled rabbit anti-*B. burgdorferi* PAb to determine whether other spirochetes were missed by the more specific test, and none were detected. Simpson and Hinck (1993), Arkansas State University, also investigated *A. americanum* from Arkansas for *B. burgdorferi*. Two hundred *A. americanum* were collected in 1989–1991 from vegetation and animal hosts and were first examined for spirochetes by darkfield microscopy. Those slides on which spirochetes were detected were then examined using IFA with MAb H5332. Spirochetes were observed in five females and two nymphs of 200 (3.5%) *A. americanum*, and authors reported that the majority of the IFA-positive *A. americanum* were collected from vegetation. Again, this might indicate spirochetes or their remnants in host blood, rather than viable infection of the tick itself (Allan et al. 2010, Bockenstedt et al. 2012). Simpson and Hinck did not provide details of IFA methods and reference an article that describes DFA (Anderson and Magnarelli 1984); therefore, it is not possible to evaluate their precautions to avoid false positives.

Investigations of *A. americanum* from Missouri

Using IFA with MAb H5332, Feir et al. (1994) visualized spirochetes in 1.9% (33/1752) of *A. americanum* collected from vegetation in 1989 in Missouri, and subsequent PCR of the tick smear material from the IFA slides amplified *B. burgdorferi* in a number of these ticks (both *Dermacentor variabilis* (Say) (Acari: Ixodidae) and *A. americanum* were investigated, but the number of PCR-positive of each species is not reported). However, the PCR was not

congruent with the IFA results; some IFA-negative samples were PCR-positive, and some IFA-positive samples were PCR negative. Perhaps the concentration of the secondary antibody, at a 1:20 dilution, was high enough to cross-react with other *Borrelia* species. Although two primer sets, one for a 371 bp chromosomal target, and one for 16S rRNA, were used on a sample of the *A. americanum* ticks, *burgdorferi* was only amplified using the chromosomal target PCR. Sequencing was performed on only one *A. americanum* tick smear PCR amplicon. A contemporaneous critique of this article questioned the author's methods and conclusions because *B. burgdorferi* was never isolated from the ticks and their PCR analysis used material removed from IFA slides, not ticks (Nelson 1995). Two subsequent articles, both co-authored by E. M. Masters, also an author of Feir et al. (1994), cite the Feir article as evidence of *B. lonestari*, not *B. burgdorferi* stating 'Approximately 2% of *A. americanum* ticks are infected with a spirochete different from both *B. burgdorferi* and the other *Borrelia* genospecies recognized as causes of Lyme disease in Eurasia. Barbour et al. proposed the name *B. lonestari* species novum. This borrelial species appears to be closely related to *B. theileri*, the cause of bovine borreliosis.' (Wormser et al. 2005b). The second article (Wormser et al. 2005a) contains a similar statement. In the face of the inadequate evidence from the Feir article itself, and the re-evaluation of the results by co-author Masters, it is remarkable that this article continues to be cited as support for the role of *A. americanum* in the transmission of *B. burgdorferi* (Rudenko et al. 2016).

Four subsequent investigations of Missouri *A. americanum* using PCR found only *B. lonestari* and no *B. burgdorferi*. Researchers from Georgia Southern University tested *A. americanum* collected from animals and vegetation in Missouri in 1995 and 1996 with IFA using both PAb and MAb (H5332). Five ticks (5/436 = 1.1%) were positive with the PAb, but negative with the MAb, and authors suggested that this might indicate infection with *B. lonestari* (Kollars et al. 2000). A study by Centers for Disease Control and Prevention (CDC) and U.S. Army investigators using PCR primers for both the 16S rDNA and the *fla*B gene of all *Borreliae* (Barbour et al. 1996), plus sequencing, identified a minimum infection rate of 5.6% (12/214) for *B. lonestari* and no *B. burgdorferi* in pools of *A. americanum* adults and nymphs (Bacon et al. 2003). Additional analysis of another population of Missouri *A. americanum* by the same research team revealed only *B. lonestari*; all 654 (114 pools) of *A. americanum* were tested using primers specific for *B. lonestari* and also primers specific for the *ospA* gene of *B. burgdorferi* (Demaerschalck et al. 1995). Twenty-two pools (yielding a maximum likelihood estimate of 3.8% [22/654]) were positive for *B. lonestari* with none positive for *B. burgdorferi* (Bacon et al. 2005). In 2010, a Missouri team from Washington University and St. Louis Children's Hospital examined 1,383 questing nymphal *A. americanum* using PCR that amplified the 16SrDNA gene of *B. lonestari* and *B. burgdorferi* and the 23S-5S intergenic spacer region of *B. burgdorferi*; 1.3% (18/1383) contained *B. lonestari*, and none were positive for *B. burgdorferi* (Allan et al. 2010). In yet another study of ticks from Missouri, Yuan (2010) investigated *A. americanum* adults collected from vegetation and detected neither *B. burgdorferi* nor *B. lonestari* in 42 ticks using primers that amplified the 16S-23S intergenic spacer region, the *recG* gene and the *uvrA* gene of *B. burgdorferi* and *B. lonestari.*

Two other studies of Missouri *A. americanum* report PCR detection of *Borrelia* spp. and *B. burgdorferi* (Cyr et al. 2005, Hudman and Sargentini 2016), but problems with methodology

undermine the credibility of these results. Cyr et al. (2005) present insufficient evidence to support their report of *B. burgdorferi* in a small sample of *A. americanum* collected from vegetation, humans, and dogs. Oddly, sequencing of the positive amplicons is described in the Materials and Methods section, but results of the sequencing are not reported. Detection of 4 of 16 *A. americanum* positive for *B. burgdorferi* was remarkable, and some explanation of these findings should have been put forward. Hudman and Sargentini (2016), of A.T. Still University, investigated *A. americanum* collected from vegetation in Missouri and described detection of *B. burgdorferi* in 0.3% (5/1,880) of adult and nymphal ticks using the primers from Barbour et al. (1996) and sequencing. However, the authors recognized that the evidence of one gene only was insufficient to confirm the identity of *B. burgdorferi* and therefore reported these samples as positive for *Borrelia* spp. only. Furthermore, the primers used were not specific for *B. burgdorferi*; both internal and external primer sets of the nested PCR used in this study would amplify *Borrelia* spp. other than *B. burgdorferi* (Barbour et al. 1996).

Investigations of *A. americanum* from South Carolina

A group from Georgia Southern University attempted culture of *Borrelia* from 210 *A. americanum* adults collected from vegetation in South Carolina in 1994 and 1995, but no isolations were obtained (Clark et al. 2002).

Transmission/Vector Competency Trials

Arthropod species may be incompetent as vectors for a pathogen for three possible reasons: 1) failure to acquire the pathogen while feeding on an infected host; 2) inability of the pathogen to persist in the vector; or 3) inability of the tick to subsequently transmit the pathogen to another vertebrate host even if the arthropod can acquire and maintain infection (Ledin et al. 2005). Since 1988, there have been nine studies to our knowledge using animal experiments to assess vector competence of *A. americanum* for *B. burgdorferi*, and in none was vector competence demonstrated. The strains of *B. burgdorferi* used in these trials have been diverse and from a wide range of geographic areas. Five of these animal transmission experiments used strains of *B. burgdorferi* from northeastern Lyme disease-endemic areas, JDI and SH2-82 (Piesman and Sinsky 1988, Mather and Mather 1990, Mukolwe et al. 1992, Ryder et al. 1992, Soares et al. 2006). One experiment used the SI-1 strain of *B. burgdorferi* from a cotton mouse and *I. scapularis* ticks from Georgia (Oliver et al. 1993). Another experiment used the MI-6 strain of *B. burgdorferi* from Florida (later identified as *Borrelia bissettii* sp. nov. (Spirocheatales: Spirochaetaceae) by Lin et al. 2002), the northeastern strain SH2-82 as a positive control, and ticks collected in Georgia (Sanders and Oliver 1995). Another study involved 34 strains isolated from the northeastern, southeastern, midwestern, Rocky Mountain, Pacific, and southwestern regions (Piesman and Happ 1997). In two of these experiments, *A. americanum* larvae acquired spirochetes during feeding upon infectious hosts, but all of these larvae became spirochete-negative before molting to the nymphal stage (Piesman and Sinsky 1988, Mather and Mather 1990). In another study involving *A. americanum* larvae fed on hamsters infected with *B. burgdorferi*, a single nymph (1 per 60 nymphs) retained infection through the molt and was positive by IFA for *B. burgdorferi*. However, nymphal *A. americanum* that had fed on infected hosts did not

transmit infection when fed on uninfected hamsters (Ryder et al. 1992). In one study, researchers not only fed *A. americanum* larvae by themselves on infected mice but also fed *A. americanum* and *I. scapularis* together on the mice. Co-feeding with *I. scapularis* significantly increased bacterial uptake by *A. americanum* during feeding, but no spirochetes were detectable in the *A. americanum* by 14 d after feeding (Soares et al. 2006). There has been no successful transmission of *B. burgdorferi* between infected and naïve hosts by *A. americanum* (Mukolwe et al. 1992, Ryder et al. 1992, Oliver et al. 1993, Sanders and Oliver 1995, Piesman and Happ 1997). In all nine studies, successful experimental transmission of *B. burgdorferi* by *I. scapularis* acted as a positive control on the experimental conditions. Subsequently, discovery of a protein in the saliva of *A. americanum* that destroyed *B. burgdorferi* has provided a biological explanation for the observed lack of vector competency suggesting that the incompetency of *A. americanum* is mostly due to their inability to acquire viable *B. burgdorferi* spirochetes (Ledin et al. 2005, Zeidner et al. 2009).

Discovery of *B. lonestari* and Surveillance for *Borrelia* spp. in *A. americanum* Using PCR

Repeated detection of *Borrelia* in *A. americanum* with microscopy and immunofluorescent methods (described above) – juxtaposed with repeated failure of *A. americanum* to maintain and transmit *B. burgdorferi* in laboratory studies – strongly suggested infection of *A. americanum* by a distinct *Borrelia* species. Evidence of this hypothesized novel *Borrelia* species was first published in 1996 for *A. americanum* from Maryland (Armstrong et al. 1996) and from Missouri, New Jersey, New York, North Carolina, and Texas (Barbour et al. 1996). Researchers from Harvard School of Public Health conducted a detailed epidemiological/entomological study of ticks and tick-bite victims in Maryland and investigated *A. americanum* using IFA with polyclonal rabbit antiserum to *B. burgdorferi* sensu lato, which identified spirochetes in 1.0% (7/685) of ticks (Armstrong et al. 2001). These IFA-positive samples were analyzed using *fla* gene PCR, and amplicon sequencing plus phylogenetic analysis revealed a spirochete close to the relapsing fever spirochete *Borrelia theileri* (Laveran) (Spirocheatales: Spirochaetaceae), which later was identified as *B. lonestari*. None of the ticks contained *B. burgdorferi*, and attempts to cultivate the spirochete in BSK II and Kelly's medium failed (Armstrong et al. 1996, Rich et al. 2001). Another research group also conducted molecular analyses of the *Borrelia* detected in *A. americanum* from a variety of locations and described the spirochete as *B. lonestari* (Barbour et al. 1996); *Borrelia* sequences detected in *A. americanum* by both groups were identical (Rich et al. 2001).

The description of *B. lonestari* (Armstrong et al. 1996, Barbour et al. 1996, Rich et al. 2001) and its detection in both an *A. americanum* tick removed from a patient suffering a skin rash and the supernatant of the patient's skin biopsy sample (James et al. 2001) raised the possibility of *B. lonestari* being the etiologic agent of a condition that became known as STARI (Masters et al. 2008). This prompted numerous PCR surveys of *Borrelia* in *A. americanum* throughout its range. Many of the investigators screened ticks using broadly reactive or generic *Borrelia* primers, often *flaB* gene nested primers from the article first describing *B. lonestari* (Barbour et al. 1996). These primers amplify *Borrelia* strains

potentially associated with human illness, including *Borrelia americana* sp. nov. (Spirocheatales: Spiro chaetaceae), *Borrelia andersonii* sp. nov. (Spirocheatales: Spirochae taceae), and *B. bissettii* (Stromdahl et al. 2015), as well as *B. burgdorferi*, *B. lonestari* and *B. miyamotoi*. Other research teams used different primer sets specific for *B. lonestari* and for *B. burgdorferi.*

Ultimately, no further human case studies linked *B. lonestari* with STARI patients, and it is no longer thought to be a human pathogen (Philipp et al. 2006; Wormser et al. 2005a,b). Nevertheless, the surveillance performed in these numerous studies, summarized in Table 2, provides extensive evidence of the absence of *B. burgdorferi* in *A. americanum.* Over 33,000 *A. americanum,* from locations throughout the range of the tick, have been tested for *Borrelia* spp. in this manner in 37 studies from more than 25 research groups, yielding a prevalence of ~1.7% of *B. lonestari*. In six of these surveys, from four research groups, PCR detection of 35 samples (~0.1%) positive for *B. burgdorferi* was reported. Only three of these PCR-positives from one study were characterized in detail (Rudenko et al. 2016), and laboratory contamination was suspected in another (Stromdahl et al. 2001, 2015).

Investigations Identifying Only *B. lonestari* and Not *B. burgdorferi* Using Primers From Barbour et al. (1996) and Sequencing of All Amplicons

Nine of the surveys, from seven different research groups from seven states (Alabama, Florida, Georgia, Missouri, Nebraska, New Jersey, and Texas), using flagellin gene primers from Barbour et al. (1996) and sequencing of all amplicons, identified *B. lonestari* and no other *Borrelia* spp. in 1.7% (79/5,771) of *A. americanum* removed from vegetation and humans (Burkot et al. 2001, Bacon et al. 2003, Varela et al. 2004b, Williamson et al. 2010, Gleim 2013, Maegli et al. 2016, Gleim et al. 2016, Mitchell et al. 2016, Sayler et al. 2016). Bacon et al. (2003) took extra steps to verify their results by using an additional PCR for a *Borrelia* genus-specific 16S rRNA gene and a PCR for *B. burgdorferi ospA* as a control for false positives. Similarly, Mitchell et al. (2016) tested all *A. americanum* in their study with primers for the 16S rDNA of genus *Borrelia*, and, as with the PCR with the primers from Barbour et al. (1996), none were found positive for *B. burgdorferi*. Sayler and the University of Florida research group cited above conducted a second study using the primers from Barbour et al. (1996) to investigate 777 additional Florida *A. americanum*, and none of the ticks tested were positive (Sayler et al. 2017). To investigate the possibility of false negatives in this study, genomic DNA from all tested tick specimens was visualized on agarose gels and quantified using a Qubit Fluorometer; intact, high molecular weight DNA was verified in all samples.

Investigations Identifying Only *B. lonestari* and Not *B. burgdorferi* Using Primers From Barbour et al. (1996) and Multilocus PCR Electrospray Ionization Mass Spectrometry

In another study, from U.S. Army entomology (Stromdahl and Hickling 2012), 1,621 *A. americanum* removed from humans were tested using the primers from Barbour et al. (1996). This effort yielded 24 *Borrelia*-positive samples that were then tested further using specific PCRs for *B. lonestari* (Bacon et al. 2004) and *B. burgdorferi* (Straubinger 2000). Nine of 1,621 (0.6%) were positive in the *B. lonestari* PCR, but none were positive for *B. burgdorferi*. Ten of the 15 tick samples that were positive in the generic *Borrelia* PCR, but

negative in both the *B. lonestari* and *B. burgdorferi*-specific PCRs, were sent to Ibis Biosciences for further analysis using a multilocus PCR electrospray ionization mass spectrometry (PCR/ESI-MS) *Borrelia* identification and genotyping assay (Crowder et al. 2010). PCR/ESI-MS analysis determined that two samples were *Borrelia*-negative and four were positive for *B. lonestari*. The remaining three samples, and one of the *B. lonestari*-positive samples, were positive for the *B. burgdorferi* flagellin primer but negative for seven other *Borrelia* primers. Attempts to clone and sequence the flagellin amplicon from these samples were unsuccessful. The PCR/ESI-MS assay targets the same region of the flagellin gene used in the initial screening (Barbour et al. 1996), so amplicon contamination from the positive control could have been responsible for these flagellin primer detections. In total, 0.8% (13/1,621) of *A. americanum* adults and nymphs were confirmed positive for *B. lonestari* by multiple PCRs, three suspects were amplicon contamination or perhaps amplification of remnants of *Borrelia*, five suspects were not identified to species, and no *B. burgdorferi* was found.

Investigations Identifying Only *B. lonestari* and No *B. burgdorferi* Using Primers From Barbour et al. (1996) and Sequencing of a Selection of Amplicons

Seven of the studies in Table 2 also involved the use of flagellin gene primers from Barbour et al. (1996) and reported detection of *B. lonestari* only, and no *B. burgdorferi*, in 2.1% (297/13,858) of *A. americanum* from human, animals, and vegetation, but in these cases, only representative amplicons were sequenced, so detection of other *Borrelia* cannot be ruled out. Most of these researchers (Stromdahl et al. 2003; Schulze et al. 2005, 2011; Mixson et al. 2006; Castellaw et al. 2010; Killmaster et al. 2014) reported all PCR-positives as *B. lonestari*. Fryxell et al. (2012) sequenced 66% (107/161) of the amplicons from PCR-positive ticks and reported those not sequenced as '*Borrelia* spp.'.

U.S. Army entomologists (Stromdahl et al. 2003) investigated ticks from the entire geographic range of *A. americanum* in the United States and detected 78 *Borrelia* positives. Of these, 68 were sequenced, and all were *B. lonestari*. Furthermore, all 78 of these positive samples tested negative in a PCR specific for *B. burgdorferi ospA* (Rosa et al. 1991). Mixson et al. (2006), from CDC and other academic and public health laboratories, used the Barbour flagellin primers to investigate *A. americanum* from nine states: Florida, Georgia, Iowa, North Carolina, New Jersey, New York, Oklahoma, Rhode Island, and South Carolina. Ticks from all states except Florida, Iowa, Oklahoma, and Rhode Island were PCR-positive. Only selected samples were sequenced, and all were identified as *B. lonestari*. Similarly, New Jersey public heath entomologists detected *Borrelia* in *americanum* from New Jersey using the Barbour flagellin primers, selected samples were sequenced, and all were identified as *B. lonestari* (Schulze et al. 2005, 2011). Castellaw et al. (2010) used the same primers to test *A. americanum* from Mississippi and detected *Borrelia* in 2.6% (5/191) of the ticks. Three of these were sequenced and were identified as *B. lonestari*. Killmaster et al. (2014) also used the Barbour primers to test 4,236 *A. americanum* from Georgia and reported 59 ticks positive for *B. lonestari* and none for *B. burgdorferi*, but only 10% were confirmed by sequencing.

Fryxell et al. (2012) used the primers from Barbour et al. (1996) to test 657 *A. americanum* removed from deer and dogs in Arkansas. Of these, 161 produced amplicons and sequencing of 107 identified all as *B. lonestari*, and none as *B. burgdorferi*. A single unidentified nymphal *Amblyomma* spp. removed from a deer was identified by sequencing as *B. burgdorferi*; however, over 100 ticks of another *Amblyomma* species, *Amblyomma maculatum* Koch (Acari: Ixodidae), were collected from deer and dogs in this survey, and *B. burgdorferi* was identified and confirmed by sequencing in two of these, so it cannot be assumed that the tick was *A. americanum*. This was the first reported detection of *B. burgdorferi* in *A. maculatum*, and a subsequent PCR study examining unfed ticks was undertaken to investigate the potential of this tick to transmit *B. burgdorferi* (Lee et al. 2014). In this study, no *B. burgdorferi* was detected in PCR of 306 adult *A. maculatum* using primer sets for both the *flaB* and 16S rRNA genes, and 97 adult *A. maculatum* using only the 16S rRNA PCR. However, two ticks contained a novel reptile-associated *Borrelia*. This suggests that the *B. burgdorferi* found in the Fryxell study had been acquired during the bloodmeal and was not being maintained by the tick.

Investigations Identifying No *B. burgdorferi* Using Primers Other than Barbour et al. (1996)

Nine reports of *A. americanum* describing the detection of only *B. lonestari* (and no other *Borrelia* spp.), or no *Borrelia*, used primer sets other than those of Barbour et al. (1996), and together they revealed a *B. lonestari* prevalence of 1.3% (96/7372; Stegall-Faulk et al. 2003, Bacon et al. 2005, Schulze et al. 2006, Jordan et al. 2009, Allan et al. 2010, Yuan 2010, Fritzen et al. 2011, Stromdahl et al. 2015, https://www.tickreport.com/stats (Accessed 26 September 2017).

Primers designed by researchers at Middle Tennessee State University that amplify the flagellin gene of *B. lonestari* and *B. burgdorferi* were used to test Tennessee *A. americanum* (Stegall-Faulk et al. 2003). Two of 453 (0.4%) were positive at the gel band size indicating *B. lonestari*, and identity of both as *B. lonestari* was confirmed with dot blot hybridization of PCR products. One of the samples was sequenced to further confirm identity. Bacon et al. (2005) tested 114 pools (654 total ticks) of *A. americanum* using primers specific for *B. lonestari* and also primers specific for the *ospA* gene of *B. burgdorferi* (Demaerschalck et al. 1995). Twenty-two pools were positive for *B. lonestari* with none positive for *B. burgdorferi*. Schulze et al. (2006) also used the primers from Stegall-Faulk et al. (2003) to test *A. americanum* from New Jersey. Six of 103 (5.8%) *americanum* samples produced gel bands at the size indicating *B. lonestari*, and none were positive for *B. burgdorferi*. All six samples were sequenced to confirm identity. Different flagellin primers designed by the Middle Tennessee State University research group to amplify both *B. lonestari* and *B. burgdorferi* were used to test 399 *A. americanum* collected in Tennessee, but no tick samples were positive for either target (Jordan et al. 2009). As described earlier in this review, the Missouri team from Washington University and St. Louis Children's Hospital examined 1,383 *A. americanum* using PCR for *B. lonestari* and *B. burgdorferi*; 1.3% (18/1383) contained *B. lonestari*, and none were positive for *B. burgdorferi* (Allan et al. 2010). Universal 16S rDNA primers (Pichon et al. 2003) and primers for the 23S-5S intergenic spacer of *Borrelia* (Rijpkema et al. 1995) were used for the PCR screen, and a reverse line blot assay for *B. burgdorferi* and *B. lonestari* reconfirmed the positives.

Yuan (2010) at University of Texas at Houston used primers designed to amplify the 16S-23S intergenic spacer of both *B. burgdorferi* and *B. lonestari* (Bunikis et al. 2004), plus MLST modified to identify the *recG* and *uvrA* genes from *B. burgdorferi* and *B. lonestari* (Margos et al. 2008) and reported *B. lonestari* in 1.5% (3/186) of *A. americanum* from Texas, no *Borrelia* in 42 *A. americanum* from Missouri, and no *B. burgdorferi* in any of these 228 ticks.

A group from the Tennessee Department of Health and the Kentucky Department for Public Health also used primers for the *Borrelia* 16S-23S intergenic spacer from Bunikis et al. (2004) to detect *B. lonestari* in 0.9% (1/108), and no *B. burgdorferi* in *A. americanum* collected from animals in Kentucky. The identity of the *Borrelia* spp. in this sample of ticks was confirmed by sequencing. These ticks were also assessed with *B. burgdorferi*-specific *ospA* primers from Demaerschalck et al. (1995), and none of the ticks were positive (Fritzen et al. 2011).

Researchers from U.S. Army entomology, CDC, two academic laboratories, and Ibis Biosciences used *Borrelia* flagellin gene primers from Clark et al. (2013) to screen 1,097 *A. americanum* removed from humans throughout the range of the tick (Stromdahl et al. 2015). Nine suspect-positives (five pools and four individuals) were all confirmed as *B. lonestari* by subsequent PCRs targeting the 16S rRNA qPCR for *Borrelia* (Tsao et al. 2004), the 16S-23S intergenic spacer region of *Borrelia* (Bunikis et al. 2004), and in the eight *Borrelia* PCRs of the Ibis Biosciences PCR/ESI-MS system (Crowder et al. 2010).

The Laboratory of Medical Zoology (LMZ) at the University of Massachusetts offers a tick identification and pathogen testing service. The crowd-sourced program provides a public surveillance database of human-biting ticks, their feeding status, and submitted and tested by LMZ (https://www.tickreport.com/stats). From 2006 until 2017, the LMZ tested 2,483 human-biting *A. americanum* for *B. burgdorferi* using a TaqMan qPCR assay (Xu et al. 2016). None of these *A. americanum* were positive for *B. burgdorferi*, while 31 (1.11%) were positive for *B. lonestari*.

Investigations Identifying *B. lonestari*, *B. burgdorferi*, Or Both in *A. americanum*

Six of the molecular surveys listed in Table 2 report identification of *B. burgdorferi* in *A. americanum* (Stromdahl et al. 2001, Clark 2004, Cyr et al. 2005, Taft et al. 2005, Clark et al. 2013, Rudenko et al. 2016). In the earliest article, the *B. burgdorferi*-positive PCR of *A. americanum* was likely due to contamination, as authors explained in subsequent publications (Stromdahl et al. 2001, 2015). Four of the surveys that reported *B. burgdorferi* presented insufficient evidence (PCR of one gene only, sequencing in one direction only, products not sequenced) to definitively confirm the identity of the PCR products (Clark 2004, Cyr et al. 2005, Taft et al. 2005, Clark et al. 2013). In the sixth study, Rudenko et al. (2016) confirmed species identity by MLST/MLSA of up to 10 genes, but only a very small number of ticks (3) ticks were so characterized.

Stromdahl et al. (2001) used PCR to investigate *A. americanum* collected in 1997 for *B. burgdorferi* and reported a minimum infection rate of 11.7% (26/222), though only 3.2% (7/222) produced amplicons in PCRs for two different gene targets. In a later publication,

these PCR results were re-evaluated and contamination was suspected because all occurred in the first year of the study when PCR was initially implemented, and no positive results were obtained across large numbers of samples in all subsequent years (Stromdahl et al. 2015). Amplicons from the 1997 tick PCRs were not sequenced.

In a study that involved ticks collected from vegetation in Florida, Clark (2004) reported detection of *B. burgdorferi* in 1.3% (5/396) of *A. americanum*. Identity of positive amplicons was confirmed by sequencing in one direction only, and the various PCR methods used on each positive sample are not precisely described. Of 252 *A. americanum* ticks tested using *flaB* primers from Johnson et al. (1992), five tested positive for *B. burgdorferi*. It appears that these were also tested using primers from Barbour et al. (1996, Tables 3 and 5; Clark 2004), and only one tick was positive for *B. burgdorferi*. Some of the samples positive with the primers from Johnson et al. (1992) were tested using nested *ospA* primers (Guttman et al. 1996, Guy et al. 1991) and 5S-23S primers (Rijpkema et al. 1995), but no *A. americanum* ticks were positive for *B. burgdorferi*. Most of the samples positive with the primers from Johnson et al. (1992) were tested using nested p66 primers (Rosa et al. 1991), and two *A. americanum* were positive for *B. burgdorferi*.

Cyr et al. (2005), as described earlier in this review, presented insufficient evidence to support their report of a improbably high incidence of *B. burgdorferi* in a small sample of *A. americanum* (4/16 = 25%); the 95% confidence interval for finding 4 of 16 ticks positive is 0.08–52%. The investigators stated that this occurred because the tick collections for their study were purposely made in areas of Missouri suspected of being ‘hot spots’ for Lyme disease, and PCR using the same primers produced positive results testing skin biopsies from Missouri patients suspected of having Lyme disease. This result is inconsistent with the much larger surveys reported here. In the absence of confirmation by another test, this report should be viewed cautiously. Novel findings of *B. burgdorferi* in tick species should be supported by characterization of multiple gene targets and evaluation of cross-reactivity with other *Borrelia*, but in this instance, only one gene target, 16S rDNA, was used for the tick samples and for the human samples that corroborated the findings in the tick samples. The sequencing data from the positive ticks and skin samples were not presented therefore could not be analyzed. A search for articles citing Cyr et al. (2005) identified only one other study using these primers and again, authors report ‘anomalous’ infection rates detected in *I. scapularis* ticks collected in Maryland (Carroll and Cyr 2005). These novel primers should have been assessed in conjunction with other, more frequently used, assays for *Borrelia* spp.

Taft et al. (2005) tested ticks using primers of their own design targeting the flagellin gene of both *B. burgdorferi* and *B. lonestari* and reported 1.5% (4/269) of *A. americanum* positive for *B. burgdorferi* and 2.2% (6/269) positive for *B. lonestari*. The *B. burgdorferi*-positive *A. americanum* were two adults and two nymphs collected from vegetation. The positive samples were reconfirmed via dot blot hybridization with probes internal to the amplicon that differentiated *B. burgdorferi* and *B. lonestari*; however, the *B. burgdorferi*-positive samples were not sequenced, so these tick are best considered ‘suspect-positive’.

Clark et al. (2013) described *A. americanum* as a vector of the agent of Lyme disease and presented as evidence the detection of *B. burgdorferi* by PCR in two *A. americanum* ticks

removed from two patients. Confirmation of the identity of *B. burgdorferi* in these two PCR-positive *A. americanum* relied on the detection and sequencing of one gene only (*flaB*). The 429 nucleotide *flaB* sequence amplified from one of the ticks (collected from Patient 4) was 100% identical to the *B. burgdorferi* B31 PCR control (NC_001318.1 sequence, GenBank), but was 99.8% (429/430-bp) similar to the *flaB* sequence from the blood of Patient 4 from whom the tick was collected. The 456 nucleotide *flaB* sequence amplified from the other tick (collected from Patient 7) was 99.6% (453/455-bp) identical to the B31 PCR control and 99.6% (451/453) identical to *B. burgdorferi* in an EM biopsy from Patient 7. The number of sequencing reads used to determine their sequences is not stated for this study, so it is unclear whether the sequence differences between the patient samples and the B31 control were real or due to sequencing errors. The *B. burgdorferi* sequences amplified from the Patient 4 blood sample and the Patient 7 skin sample did not match those obtained from the same patient's attached *A. americanum* tick, so there is no evidence that the *B. burgdorferi* DNA found in the patient samples came from *B. burgdorferi* in the attached tick.

Rudenko et al. (2016) reported 2.2% (13/590) of *A. americanum* to be PCR-positive using *B. burgdorferi* flagellin gene primers, but MLST of 10 gene targets could only confirm 3 of these positives, and not all 10 loci were amplified in each of those 3 samples. *B. burgdorferi* is readily amplified from *Ixodes* spp. ticks; weak PCR signals for *B. burgdorferi* in *A. americanum* tick samples may indicate amplification of remnants of a bloodmeal from a previous life stage (Allen et al. 2010, Bockenstedt et al. 2012, Marques et al. 2014), rather than active infection with viable spirochetes. It should also be emphasized that infrequent detection by molecular methods of *B. burgdorferi* DNA in *A. americanum* does not indicate that these ticks are capable of transmission.

Discussion and Conclusions

Early studies suggested the presence of *B. burgdorferi* in *A. americanum* ticks in the United States. However, in almost all cases, *Borreliae* were detected using methods that were not *Borrelia* species-specific; spirochetes that were detected were likely other species, or transient infections detected in engorged ticks collected from hosts infected with *B. burgdorferi* transmitted by other sympatric vector-competent tick species. More recently, extensive surveillance, using methods that discriminate among *Borrelia* species, has only rarely detected *B. burgdorferi* in *A. americanum* ticks, and most of these observations could have resulted from the presence of *B. burgdorferi* DNA that was most likely from an infectious host bloodmeal. Definitive experimental infections using a diverse array of *B. burgdorferi* strains have repeatedly failed to demonstrate vector competency of *A. americanum* for *B. burgdorferi*.

A hypothesis of 'selective compatibility'—i.e., that certain strains of *B. burgdorferi* in the southeastern United States may be better adapted to development in ticks other than *Ixodes* spp.—was proposed by Luckhart et al. (1991). Rudenko et al. (2016) revisited the possible role of *A. americanum* as a Lyme vector, citing as evidence findings of '*B. burgdorferi*' in *A. americanum* from New Jersey (Schulze et al. 1984), Missouri (Feir et al. 1994), and Texas (Teltow et al. 1991). However, authors of these three studies subsequently revised their findings to '*B. lonestari*' (Barbour et al. 1996; Schulze et al. 2005, 2006; Wormser et al.

2005a,b). Rudenko et al. (2016) reported detecting three questing *A. americanum* positive for *B. burgdorferi* by PCR and MLST and suggested that when compatible spirochete strains meet an appropriate tick population, maintenance and transmission could occur. If so, then these strains are likely very rare, as transmission did not occur in two laboratory experiments involving *A. americanum* and *B. burgdorferi* strains from the region of origin of these positive ticks (Oliver et al. 1993, Sanders and Oliver 1995). *Borrelia burgdorferi* has never been successfully cultured from *A. americanum*; motile spirochetes have never been described from *A. americanum,* and the studies reviewed in Tables 1 and 2 report no conclusive evidence of *B. burgdorferi* infection in extensive surveillance of more than 52,000 *A. americanum*. Throughout the entire range of *A. americanum* (Springer et al. 2014), the tick is constantly being exposed to *Borreliae*, although sympatric tick populations and host species compositions change. By some mechanism, most likely borreliacidal salivary components, the tick resists colonization by *B. burgdorferi*. While the existence of some compatible spirochete strains and *A. americanum* tick populations cannot be entirely ruled out, how transmission cycles could be maintained in nature at the vanishingly low prevalence levels detected in surveillance of *A. americanum* ticks remains an important question that needs to be addressed.

PCR and other detection methods have limitations when trying to answer the question of whether or not *A. americanum* has the ability to transmit/vector *B. burgdorferi* to humans. Most important to note is that the detection of *Borrelia* DNA does not mean living *Borrelia* are present. It has been shown that pieces of dead *B. burgdorferi* can elicit antigenic responses that are identified by immunofluorescent staining, and contain DNA that can be detected by PCR (Bockenstedt et al. 2012), and DNA from the blood cells of a previous host blood-meal often persists through the tick's molt and has been detected in between 45 and 63% of questing nymphal and adult *A. americanum* ticks (Allan et al. 2010, Harmon et al. 2015). Therefore, the rare detection of *B. burgdorferi* DNA in questing *A. americanum* should not be over-interpreted, and these rare detections need to be put in context with the comparatively high prevalence of infection in questing ticks of species known to be vector-competent. PCR data alone may reveal pathogen DNA, but the more useful information remains unknown, including whether the DNA came from live or dead bacteria within the tick's body, or dead bacteria residing in the tick's midgut that are remnants of the tick's last meal.

The possibility of false-positive test results is increased when using PCR (particularly nested PCR) to detect bacterial DNA because the products of DNA amplification that give the test result can be due to contamination or false priming if the PCR protocol is not sufficiently stringent (Lo and Chan 2006). There is greater confidence in PCR test results when multiple PCR targets are used and when amplicons are sequenced. Careful review of sequences of PCR amplicons is needed to rule out the possibility of contamination of PCR reactions by DNA from positive controls and strains grown in the laboratory, and to ensure that there is no carry-over contamination from other tested samples. PCR, sequencing, and analysis of highly variable DNA genes or multiple genes can assist in assuring that DNA contamination did not occur with the positive DNA control or other lab strains if they do not match the sequences of these strains. DNA sequencing should be done on both strands of a PCR

product, and if Sanger sequencing is employed, a sufficiently large number of reads should be used to determine the consensus sequence to assure sequencing errors did not occur.

Confirmation of a suspect-positive result by reproducing the result with multiple real-time PCR assays can have benefits. The ability to consistently reproduce positive results with other assays is suggestive that PCR amplicon contamination has not been occurring, but it cannot rule out genomic DNA contamination of a sample. Proper DNA extraction controls, PCR controls, and sequencing are needed to do this. Performing real-time PCR assays instead of standard or nested PCR in general reduces the likelihood that amplicon contamination will occur since the PCR products do not need to be pipetted from an open tube in order to be used in downstream PCRs or run on an agarose gel, both of which increase the chance of laboratory contamination with PCR products. In addition, real-time PCR assays that use a standard curve can provide information on the approximate DNA copy number present in a tick so as to provide information on whether the detected pathogen is present in biologically meaningful numbers.

Successful culture isolation of *B. burgdorferi* from a tick removed from an animal host does not distinguish between exposure to *B. burgdorferi* in the bloodmeal versus active infection of the tick, nor does it determine whether the spirochetes would survive transtadially until the tick feeds again at the next life stage, or be successfully transmitted to a new host during the next bloodmeal.

Culturing of host-seeking ticks, or of ticks removed from hosts and allowed to molt to the next life stage before culturing, could answer the question of whether some strains of *B. burgdorferi* can live through a molt. Transmission studies would then be required to verify whether these ticks could transmit the spirochete to their next bloodmeal host.

The role of *A. americanum* in the transmission of Lyme disease remains controversial in the minds of a very few scientists, yet public sentiment has kept the possibility of ‘Lyme disease from lone star ticks’ in the spotlight. Although advancements in molecular analysis strongly suggest that initial reports of *B. burgdorferi* in *A. americanum* across many states were in fact misidentified *B. lonestari* or DNA contamination, early reports continue to be cited without mention of the later clarifying studies; consequently, the search for a population of *A. americanum* that can transmit the Lyme disease spirochete has been ongoing. We suggest that the few studies implicating *A. americanum* as a vector of *B. burgdorferi* have not yet met the burden of proof for their assumption. This review of extensive surveillance and vector competency studies of *B. burgdorferi* in *A. americanum* studies supports the conclusion that *A. americanum* is not a consequential factor in Lyme disease ecology and epidemiology. Information pertaining to the geographical distribution of infected ticks is quite important so that practicing physicians adopt the appropriate level of concern in a given patient population. There is no epidemiological need whatsoever to invoke another vector to explain Lyme disease prevalence and distribution in the United States.

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Table 1.

Initial investigations of *Borrelia* spp. in *A. americanum* using microscopy, immunofluorescent methods, culturing, and PCR

Date of collection	Location	Source of ticks[a]	Method of analysis	Total ticks tested	Ticks with spirochetes	References
1983	NJ	Vegetation	Darkfield, DFA (PAb)	44	4	Schulze et al. (1984)
1983–1984	NC	Animals	DFA (PAb)	512	7	Magnarelli et al. (1986)
1984	NJ	Vegetation	Darkfield, DFA (PAb), culture	756, 32 larval pools[b]	35, 5 larval pools	Schulze et al. (1986)
1984–1987	NC	Vegetation	Darkfield, DFA, (PAb)	1836	1	Levine et al. (1989)
1987	NC, VA	Vegetation, animals	Darkfield, IFA (MAb)	218	4	Levine et al. (1991)
1988–1989	AL	Mixed	DFA, IFA (PAb)	150	6	Luckhart et al. (1991)
1988–1989	TX	Mixed	Culture, darkfield, IFA (MAb)	354	3	Teltow et al. (1991)
1990	AR	Mixed	IFA (MAb), DFA (PAb)	471	0	Kardatzke et al. (1992)
1988–1990	AL	Animals	DFA, IFA (PAb)	125	8	Luckhart et al. (1992)
1989–1991	AR	Vegetation, animals	Darkfield, IFA (MAb)	200	7	Simpson and Hinck (1993)
1989	MO	Vegetation	IFA (MAb), PCR	1,752	33	Feir et al. (1994)
1990–1992	TX	Vegetation, animals	DFA (PAb), culture	5,195[c]	54	Rawlings and Teltow (1994)
1991–1994	VA	Vegetation, animals	Darkfield, IFA (MAb)	546	1	Sonenshine et al.(1995)
1990–1993	NC	Vegetation, animals	IFA (MAb)	5,724	51	Ouellette et al. (1997)
1995–1996	MO	Vegetation, animals	IFA (PAb, MAb)	436	5	Kollars et al. (2000)
1994–1995	SC	Vegetation, animals	Culture	210	0	Clark et al. (2002)
1999–2000	MS	Mixed	DFA (PAb)	68	0	Goddard et al. (2003)
Totals				18,597, 32 larvalpools	224	

Darkfield, darkfield microscopy; DFA, direct fluorescent antibody test; PAb, polyclonal antibody; IFA, indirect immunoflourescence assay; MAb, monoclonal antibody; PCR, polymerase chain reaction.

[a] Mixed = vegetation, human, and wildlife/domestic animals.

[b] Culture of spirochetes from ticks in this study was attempted, but none were isolated.

[c] Culture of spirochetes from >20,000 additional ticks in this study was attempted, but none were isolated.

Table 2.

Discovery of *B. lonestari* and surveillance for *Borrelia* spp. in *A. americanum* using PCR and other methods

Date of collection	Location	Source of ticks[a]	Method of analysis	Screening primer reference	Total tested	Reported PCR-positive		References
						B. burgdorferi	*B. lonestari*	
1995	MD	Vegetation	Darkfield, IFA, PCR	Armstrong et al. (1996)	297	0	1	Armstrong et al. (1996)
1989–1992	MO, NJ, NY, NC, TX	Humans, vegetation	DFA (PAb), PCR	Barbour et al. (1996)	875	0	Unspecified	Barbour et al. (1996)
1999	AL	Vegetation	PCR	Barbour et al. (1996)	202	0	2	Burkot et al. (2001)
1997	US	Humans	PCR	Rosa et al. (1991)	222	7[b]	–	Stromdahl et al. (2001)
1994	MD	Vegetation	Darkfield, culture, PCR	Rich et al. (2001)	388	0	2	Rich et al. (2001)
2001	MO	Vegetation	PCR	Barbour et al. (1996)	214	0	12 pools	Bacon et al. (2003)
1999–2000	TN	Vegetation	PCR	Stegall-Faulk et al. (2003)	453	0	2	Stegall-Faulk et al. (2003)
2000–2002	US	Humans, vegetation	PCR	Barbour et al. (1996)	6,334	0	78	Stromdahl et al. (2003)
1999–2000	FL	Vegetation	PCR	Barbouret al. (1996), Johnson et al. (1992)	396	5[c]	8	Clark (2004)
2001–2003	GA	Vegetation	PCR	Barbour et al. (1996)	398	0	4	Varela et al. (2004b)
2002	MO	Vegetation	PCR	Bacon et al. (2005),Demaerschalck et al. (1995)	654	0	22 pools	Bacon et al. (2005)
Unspecified	MO	Mixed	PCR	Cyr et al. (2005)	16	4[d]	–	Cyr et al. (2005)
2003–2004	NJ	Vegetation	PCR	Barbour et al. (1996)	121	0	11	Schulze et al. (2005)
1997–2000	US	Mixed	PCR, DBH	Taft et al. (2005)	269	4[e]	6	Taft et al. (2005)
1998–2005	US	Vegetation	PCR	Barbour et al. (1996)	2,038	0	54	Mixson et al. (2006)
2004	NJ		PCR	Stegall-Faulk et al. (2003)	103	0	6	Schulze et al. (2006)
2004–2005	TN	Vegetation	PCR	Haynes et al. (2005)	339	0	0	Jordan et al. (2009)
2005, 2007–2008	MO	Vegetation	PCR, RLB	Rijpkema et al. (1995),Pichon et al. (2003)	1,383	0	18	Allan et al. (2010)
2008	MS	Vegetation	PCR	Barbour et al. (1996)	191	0	3	Castellaw et al. (2010)
2004–2008	TX	Humans	PCR	Barbour et al. (1996)	367	0	4	Williamson et al. (2010)
2008–2010	MO, TX	Vegetation	PCR	Bunikis et al. (2004),Margos et al. (2008)	228	0	3	Yuan (2010)
2008	KY	Mixed	PCR	Bunikis et al. (2004)	108	0	1	Fritzen et al. (2011)
2008	NJ	Vegetation	PCR	Barbour et al. (1996)	281	0	19	Schulze et al. (2011)

Date of collection	Location	Source of ticks[a]	Method of analysis	Screening primer reference	Total tested	Reported PCR-positive		References
						B. burgdorferi	*B. lonestari*	
2006–2008	AR	Animals	PCR	Barbour et al. (1996)	657	0	107	Fryxell et al. (2012)
2010	US	Humans	PCR	Barbour et al. (1996)	1621	0	13	Stromdahl and Hickling (2012)
2009–2010	FL, GA	Humans	PCR	Clark et al. (2013)	3	2[f]	0	Clark et al. (2013)
2010–2011	GA	Vegetation	PCR	Barbour et al. (1996)	3061	0	38	Gleim (2013)
2005–2009	GA	Vegetation	PCR	Barbour et al. (1996)	4,236	0	59	Killmaster et al. (2014)
2012	NE	Vegetation	PCR	Barbour et al. (1996)	251	0	4	Maegli et al. (2016)
2013	US	Humans	PCR	Clark et al. (2013)	1097	0	6	Stromdahl et al. (2015)
2005–2006	GA	Humans	PCR	Barbour et al. (1996)	426	0	2	Gleim et al. (2016)
2014	MO	Vegetation	PCR	Barbour et al. (1996)	1,880	0	20	Hudman and Sargentini (2016)
2008–2014	TX	Humans	PCR	Barbour et al. (1996),Williamson (2010)	591	0	8	Mitchell et al. (2016)
2013	AL, FL, GA, TN, SC	Vegetation	PCR	Clark et al. (2005)	590	13[g]	–	Rudenko et al. (2016)
2010–2012	FL	Vegetation	PCR	Barbour et al. (1996)	260	0	5	Sayler et al. (2016)
2014	FL	Vegetation	PCR	Barbour et al. (1996)	777	0	0	Sayleretal. (2017)
2006–2017	US	Humans, mixed	PCR	TickReport real-time PCR assay	2,483	0	31	TickReport (https://www.tickreport.com/stats), accessed 26 September 2017
Totals					33,810	35	578	

Darkfield, darkfield microscopy; IFA, indirect fluorescent antibody testing; PCR, polymerase chain reaction; DFA, direct fluorescent antibody testing; PAb, polyclonal antibody; DBH, dot blot hybridization; RLB, reverse line blot.

[a] Mixed = vegetation, human, and wildlife/domestic animals.

[b] Contamination was suspected, and these results were re-evaluated in a later publication (Stromdahl et al. 2015).

[c] The identity of positive amplicons was confirmed by sequencing in one direction only.

[d] Only one gene target was used for PCR, and sequencing data were not presented.

[e] The positive samples were not sequenced.

[f] The identity of the PCR-positives relied on the detection and sequencing of one gene only.

[g] Multilocus sequence typing of 10 gene targets could only confirm 3 of these positives, and not all 10 loci were amplified in each of those three samples.