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Detection of Lyme *Borrelia* in Questing *Ixodes scapularis* (Acari: Ixodidae) and Small Mammals in Louisiana

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Abstract

Lyme borreliosis is caused by spirochetes from the *Borrelia burgdorferi* sensu lato species complex. In the United States, *B. burgdorferi* sensu stricto (s.s.; Johnson, Schmid, Hyde, Steigerwalt, and Brenner) is the most common cause of human Lyme borreliosis. With >25,000 cases reported annually, it is the most common vector-borne disease in the United States. Although approximately 90% of cases are contained to the northeastern and Great Lake states, areas in Canada and some southern states are reporting rises in the number of human disease cases. Louisiana records a few cases of Lyme each year. Although some are most certainly the result of travel to more endemic areas, there exists evidence of locally acquired cases. Louisiana has established populations of the vector tick, *Ixodes scapularis* (Say), and a wide variety of potential reservoir animals, yet Lyme *Borrelia* has never been described in the state. Using culture and polymerase chain reaction, we investigated the presence of Lyme *Borrelia* in both mammals and questing ticks at a study site in Louisiana. Although culture was mostly unsuccessful, we did detect the presence of *B. burgdorferi* s.s. DNA in 6.3% (11 of 174) of ticks and 22.7% (five of 22) of animal samples. To our knowledge, this is among the first evidence documenting *B. burgdorferi* s.s. in Louisiana. Further investigations are required to determine the significance these findings have on human and animal health.

Keywords

Lyme disease; *Borrelia*; tick; LA; small mammal

With >25,000 human cases reported annually, Lyme borreliosis is the most common vector-borne disease in the United States (Bacon et al. 2008). It is caused by spirochetes from the *Borrelia burgdorferi* sensu lato (s.l.) species complex (collectively referred to as Lyme *Borrelia*). In the United States, *B. burgdorferi* sensu stricto is the most common Lyme *Borrelia* spirochete implicated in human disease, its main vectors being *Ixodes scapularis* (Say) and *Ixodes pacificus* (Cooley and Khols) (Radolf et al. 2012). The nymphal life stage of these ticks is overwhelmingly responsible for the majority of reported human cases (Barbour and Fish 1993). Lyme borreliosis is a zoonotic disease, and humans are considered dead-end hosts; therefore, reservoir hosts like mice, chipmunks, and shrews are essential in perpetuating the spirochetes' enzootic transmission cycle (Fish 1995).

Although the majority of Lyme borreliosis cases occur in northeastern and Great Lakes states (Bacon et al. 2008), areas not historically considered Lyme endemic are starting to report more cases. For example, researchers in Canada have mapped the encroachment of *I.*

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scapularis and subsequently Lyme *Borrelia* spirochetes over the past two decades (Ogden et al. 2006, 2010). On the southern side of this expansion, states like Virginia are becoming Lyme "hotspots," with a reported 1,300% increase in cases from 1997 to 2007 (Anonymous 2011a, Levi et al. 2012). Louisiana reported 133 cases of Lyme borreliosis from 1998 to 2011; however, for many of these cases, it is impossible to determine whether the patient had actually acquired the infection in Louisiana or from travel to more Lyme-endemic areas (Anonymous 2011b). In 2012, a young child was diagnosed with Lyme borreliosis by physicians associated with Tulane Medical Center. This case met the Centers for Disease Control's surveillance definition and had no history of travel outside the state (Black 2012; B. F. Leydet, personal communication). Like most other states in the southern United States, the lower incidence of Lyme borreliosis in Louisiana is likely multifactorial. Although Lyme *Borrelia* spirochetes are endemic to many states in the South, differences from the traditional northeastern Lyme *Borrelia* ecology probably play a significant role in human exposure and disease diagnosis. For example, host diversity in the South is higher, leading to more refractory hosts and changes in the vector's ecology; multiple complex cryptic Lyme *Borrelia* cycles involving nonhuman biting ticks exist; and the population and overlap of *Amblyomma americanum* (L.), the vector of southern tick–associated rash illness, further complicate the situation (LoGiudice et al. 2003, Oliver et al. 2003, Goddard and Piesman 2006, Pepin et al. 2012). These reasons among others likely play an integral role in the lower infection prevalence and significantly reduced human encounters with nymphal *I. scapularis* across the southern United States (Felz et al. 1996, Oliver 1996).

The spread of Lyme borreliosis highlights the importance of surveillance in areas once considered low risk. Surprisingly, even with established state-wide populations of *I. scapularis* (Mackay and Foil 2005), a plethora of potential reservoir hosts (Lowery and Life 1974), and evidence that Louisiana populations of *I. scapularis* are competent vectors of *B. burgdorferi* (Jacobs et al. 2003), almost nothing exists in the literature regarding the presence of Lyme *Borrelia* in Louisiana. Therefore, this study investigated the presence of Lyme *Borrelia* in ticks and mammals at a site in Louisiana.

Materials and Methods

The study area chosen was Tunica Hills Wildlife Management Area in West Feliciana Parish, LA. Forest cover was categorized as upland hardwood rolling bluff land, which supports a large population of animals, including species of small rodents, birds, wild turkey, and white-tailed deer. Questing ticks were collected by flagging with 1-m^2 white felt cloth flags along defined hiking paths and animal trails. Flagging was conducted once or twice a week for 2–3 h throughout the year (2009–2010), but only *I. scapularis* populations were used in this study. Animal trapping was conducted when *I. scapularis* nymphs were expected to be feeding on hosts (from spring to early fall in 2010). Animals were trapped using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI) placed along animal tracks and trails. In total, 33 traps were set at night at least once a week during the aforementioned trapping period. This resulted in 1,056 trap nights. Captured animals were anesthetized with a combination of ketamine and dexmedetomidine, measured, weighed, and sexed. Ectoparasites were removed and placed in tubes alive for later processing and identification by using a standard key (Diamant and Strickland 1965). Two 2-mm ear-punch biopsies (EPB) were taken, and blood was drawn for culture and polymerase chain reaction (PCR) analysis. Animals were released at capture site after recovery from anesthesia. *Borrelia* culture isolation attempts were performed on blood, EPB, and questing ticks in BSK-H complete media (Sigma, St. Louis, MO) supplemented with rifampin (50 μg/ml), phosphomycin (50 μg/ml), and amphotericin B (2.5 μg/ml) as described previously (Barbour 1984, Sinsky and Piesman 1989). An indirect immunofluorescence assay was performed on cultures with evidence of spirochete growth by using monoclonal antibodies specific for *B.*

burgdorferi (H5332) as described previously (Barbour et al. 1983). DNA extractions were performed on 2-mm EPBs from small mammals and half of each adult blacklegged tick (*I. scapularis*) by using the GeneElute Genomic DNA Miniprep kit (Sigma). EPBs and tick halves were minced using single-use sterile scalpels, and DNA was extracted as directed by the manufacturer. PCR and thermocycling were performed with a nested primer set targeting a 389-bp portion of the chromosomally located *flaB* gene as described previously (Clark et al. 2005). *Borrelia flaB* PCR has been successful in the sensitive detection and delineation of *B. burgdorferi* s.l. species by sequence analysis of PCR products, from both field and culture samples (Wodecka et al. 2010, Wodecka 2011, Yang et al. 2012). Environmental controls used for detection of DNA contamination in all experiments included simultaneous extraction tubes with no tissue added for all DNA extractions, and molecular biology– grade water used as a negative template in all downstream PCRs. PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) following manufacturer's instructions. PCR products were bidirectionally sequenced by the GeneLab division of the BioMMED core facility at Louisiana State University's School of Veterinary Medicine. Sequences were aligned using CustalX and compared with existing sequences in the National Center for Biotechnology Information Genebank using the Basic Local Alignment Tool (Altschul et al. 1990, Thompson et al. 1997). In all aforementioned testing, appropriate positive controls included a culture of clonal *B. burgdorferi* B31 (5A11) and extracted genomic DNA from the culture. Negative controls as described previously in the text repeatedly showed no evidence of contamination throughout this study.

Results

In total, 174 adult blacklegged ticks (*I. scapularis*), 7 mice (*Peromyscus* spp.), 6 eastern woodrats (*Neotoma floridana* Ord), 4 raccoons [*Procyon lotor* (L.)], 4 eastern gray squirrels (*Sciurus carolinensis* Gmelin), and 1 North American opossum (*Didelphis virginiana* Kerr) were screened by PCR and culture for evidence of Lyme *Borrelia.* PCR testing of ticks produced 11 (6.3%) *Borrelia* sequences. Five (22.7%) of the animals had PCR-positive EPBs; this included two mice (28.6%), a woodrat (16.7%), a raccoon (25%), and a squirrel (25%). No *I. scapularis* nymphs were collected from trapped animals. The 11 positive questing *I.scapularis* had identical sequences (Table 1). Animal sequences shared some similarity with the ticks in one polymorphic site; however, other single nucleotide polymorphisms differed among samples. All sequences generated in this study had nucleotide differences from *B. burgdorferi* strain B31, yet were 99% homologous when aligned (Table 1). One tick and one squirrel sample produced spirochetes in primary culture isolates; however, on multiple passages, the samples were lost to bacterial contamination. Aliquots of these cultures were subjected to immunofluorescence assay, in which they reacted to *B. burgdorferi*–specific OspA H5332 antibodies. These two cultures were also positive by PCR and had identical sequences to their respective tick half and EPB, which were subjected only to PCR as described previously.

Discussion

Since 1998, Louisiana has recorded 133 cases of human Lyme borreliosis (Anonymous 2011b). Although a proportion of these certainly are acquired from travel to more Lyme-endemic parts of the country, there is evidence for locally acquired cases. The enzootic cycle of *B. burgdorferi* has been well studied in areas of the Northeast, yet many states in the South continue to be understudied. Large-scale U.S. surveys have concluded that the prevalence of infected *I. scapularis* nymphs in the South is minuscule. However, these conclusions were based on very low numbers of ticks (Pepin et al. 2012), and in Louisiana, almost no work has been conducted investigating the presence of Lyme *Borrelia.*

Conversely, tick surveys across other southern states have identified *B. burg-dorferi* infection in many populations of *I. scapularis* (Levine et al. 1991, Luckhart et al. 1991, Teltow et al. 1991, Rawlings and Teltow 1994, Sonenshine et al. 1995, Oliver et al. 2000, Clark et al. 2002, Clark 2004, Fryxell et al. 2012). In addition to natural cycles of *B. burgdorferi* involving *I. scapularis,* cryptic cycles of Lyme *Borrelia* have been documented in southern states, involving *Ixodes affinis* (Neumann), *Ixodes minor* (Neumann), and *Ixodes dentatus* (Neumann) transmitting multiple *B. burgdorferi* s.l. species (Oliver et al. 2003, Maggi et al. 2010). Competent reservoir animals dispersed across the southern United States have been shown to be infected with multiple species of Lyme *Borrelia* (Sonenshine et al. 1995, Oliver et al. 2003). Several ecological surveys conducted in southern states have led to the discovery of new Lyme *Borrelia* species, vectors, and reservoirs that have expanded our understanding of Lyme *Borrelia* ecology (Clark et al. 2002, Clark 2004, Lin et al. 2004).

Along with a recent survey of ticks collected off black bears in Louisiana (Leydet and Liang 2013), these are the first records of *B. burgdorferi* in Louisiana ticks and wildlife. This discovery, although novel, was not unexpected. Like other southern states, the prevalence of *B. burgdorferi* in Louisiana ticks at our study site is lower (6.3%) than the more endemic areas in the Northeast, where prevalence rates can be as high as 30 –50% (Adelson et al. 2004, Schulze et al. 2006, Pepin et al. 2012). The lack of nymphal *I. scapularis* collected from animals and by flagging during this study is also consistent with reports from states across the southern United States (Diuk-Wasser et al. 2006). The decreased success in cultivating *Borrelia* from ticks and mammals in this study is a phenomenon seen in other studies outside areas of higher endemicity. Could this be due to a lower detection sensitivity of culture versus molecular methods (Xu et al. 2013)? Or are Lyme *Borrelia* spirochetes in the South less likely to grow in traditional media (Clark 2004)? Although this remains unknown, further studies are warranted.

Because Louisiana consistently reports cases of human Lyme borreliosis each year, the detection of *B. burgdorferi* DNA in both vector ticks and competent reservoir hosts is significant. *I. scapularis* nymphs are the main vector for *B. burgdorferi* in the eastern United States, and although not detected in this study, can be found questing and attached to humans in Louisiana (B.F.L., unpublished data). It is also important to note that DNA evidence of bacteria does not confirm viability of the organism, and because cultures in this study were mostly unsuccessful, caution must be taken in the interpretation of these results. Still, molecular surveys demonstrating presence of a pathogen in known vectors and reservoirs are the critical first steps in our understanding of vector-borne disease agents in different geographical areas. More importantly, studies like this ultimately help relay new information to researchers and healthcare providers, who, in turn, help educate the public. Evidence from this study should prompt further state-wide surveys investigating the prevalence of Lyme *Borrelia* in Louisiana ticks and wildlife.

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Table 1

B. burgdorferi flaB sequences amplified from mammalian and tick gDNA samples in this study

Organism sampled	Common name	Homology to *B. burgdorferi* B31 (NC_001318.1)	Nucleotide differences from B31 (base pair site of polymorphism)
N. floridana	Woodrat	99%	T→C (738)
P. lotor	Raccoon	99%	T→C (738)
Peromyscus spp.	Field mouse	99%	T→C (51); T→A (687) and T→C (738)
Peromyscus spp.	Field mouse	99%	T→C (738)
S. carolinesis	Gray squirrel	99%	T→C (510); T→A (687) and T→C (738)
I. scapularis (questing)	Blacklegged tick	99%	T→C (738)

Sequence results aligned to the full (1,011 base pairs) *flaB* portion of the B31 chromosome (positions 147649–148659).