The Lyme Disease Spirochete, *Borrelia burgdorferi*, in Tick Species Collected from Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*) Trapped in the Warren and Barren Counties of South Central Kentucky

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THE LYME DISEASE SPIROCHETE, Borrelia burgdorferi, IN TICK SPECIES COLLECTED FROM RACCOONS (Procyon lotor) AND OPOSSUMS (Didelphis virginiana) TRAPPED IN THE WARREN AND BARREN COUNTIES OF SOUTH CENTRAL KENTUCKY

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Master of Science

By
Kristina Tackett
December 2009
THE LYME DISEASE SPIROCHETE, *Borrelia burgdorferi*, IN TICK SPECIES COLLECTED FROM RACCOONS (*Procyon lotor*) AND OPOSSUMS (*Didelphis virginiana*) TRAPPED IN THE WARREN AND BARREN COUNTIES OF SOUTH CENTRAL KENTUCKY

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The incidence of tick-borne zoonoses such as Ehrlichiosis, Rocky Mountain Spotted Fever, and Lyme disease has steadily increased in the southeastern United States in recent years. According to the Centers for Disease Control and Prevention (CDC), the southeastern states accounted for 1,200 of the 27,000 total cases of Lyme disease reported in the U.S. in 2007. Although *Ixodes scapularis* is the most commonly recognized vector for the Lyme disease spirochete *Borrelia burgdorferi*, *Dermacentor variabilis* (a common vector for Rocky Mountain Spotted Fever) also has been shown to be a viable host for this pathogen. The purpose of the present study was to use PCR and DNA sequencing technologies to determine if *Borrelia burgdorferi sensu lato* is present in ticks and whole blood samples removed from raccoons and opossums trapped in south-central Kentucky.

Raccoons and opossums were trapped in Barren and Warren counties of Kentucky between June 2007 and June 2008. Ticks were removed and stored in 70% ethanol. Sterile blood samples were collected into three 10 ml tubes containing the anticoagulant K$_2$EDTA and stored at 4°C. Genomic DNA was extracted from ticks and blood samples using a QIAamp DNA mini kit and a QIAamp DNA blood mini kit (Qiagen).
respectively. DNA samples were analyzed by polymerase chain reaction (PCR) for the presence of *B. burgdorferi* using oligonucleotide primers specific for the OspA gene.

A total of 976 ticks were collected. Three different species were obtained from raccoons; *Dermacentor variabilis, Amblyomma americanum*, and *Ixodes sp.*

*Dermacentor variabilis* was the only tick species found on opossums. Twenty-five percent (163/642) of the tick DNA samples were positive for *Borrelia burgdorferi*. Prevalence of *B. burgdorferi* by tick species was 24.4% (141/577) in *D. variabilis*, 40.6% (13/32) in *A. americanum*, and 27.6% (8/29) in *I. scapularis*. In the present study, 15.7% (8/51) of the total raccoon blood samples examined by PCR were positive for *B. burgdorferi*, while no opossum blood samples were positive. The high prevalence of *B. burgdorferi* in ticks common to raccoons and opossums observed in this study, as well as in a tick species that aggressively bites humans in the southeast U. S. (*A. americanum*), creates concern that there are ample opportunities for people to come in contact with the infected ticks on these animals. Future studies are urgently needed to fully assess the presence and prevalence of *B. burgdorferi* in Kentucky and other southeastern states in the U. S.
INTRODUCTION

Ticks

Ticks are currently considered to be second only to mosquitoes as vectors of human infectious diseases in the world (Parola and Raoult, 2001; Hill and Wikel, 2005). They are obligate hematophagous arthropods that parasitize every class of vertebrates in almost every region of the world (Parola and Raoult, 2001). Ticks serve as vectors of nematodes, protozoa, rickettsiae, spirochetes, bacteria, and viruses (Oliver, 1996). They have been recognized as human parasites for thousands of years, being described by ancient Greek writers such as Homer and Aristotle (Parola and Raoult, 2001). By 1996, 869 species of ticks had been recorded though only 10% of these impact humans directly (Oliver, 1996; Parola and Raoult, 2001). Approximately 80 species occur in the U.S. (Oliver, 1996). Ticks are members of the subclass Acari, which is the largest subclass in the class Arachnida, separating mites and ticks from other arachnids in that their abdomen region is fused with only the head region being flexible. Ticks are in the order Ixodida, and there are two major tick families: the Ixodidae, or “hard ticks”, which are the most important family in numerical terms and medical importance, and the Argasidae, or “soft ticks”, so called because of their flexible cuticle (Oliver, 1989; Parola and Raoult, 2001). The ixodid ticks are characterized by a sclerotized dorsal plate known as the scutum and the remainder of the body is able to expand during feeding (Sonenshine, 1991; Parola and Raoult, 2001). A third recognized family of ticks is Nuttalliellidae which is represented by a single tick species, Nuttalliella, confined to southern Africa (Oliver, 1989; Parola and Raoult, 2001).

Ticks have 4 life stages: the egg, larval, nymphal, and adult. The life cycle is usually completed in 2-3 years depending on environmental conditions including
temperature, relative humidity, and photoperiod (see Fig. 1) (Oliver, 1989; Parola and Raoult, 2001). Ticks range in size from 2-30mm (Parola and Raoult, 2001). Adults and nymphs have 4 pairs of legs while larvae have only 3 pairs (Oliver, 1989; Parola and Raoult, 2001). All stages have no antennae, and their bodies are not divided into distinct head, thorax, and abdominal regions like insects (Parola and Raoult, 2001). The anterior part of the body, the capitulum, bears the mouthparts, including cutting organs and a median immobile organ (the hypostome) with numerous backward directed teeth that serve a “harpoon-like” function to anchor the tick to the host’s skin (see Fig. 2) (Parola and Raoult, 2001). Ticks also have peripheral sensory organs such as hair-like structures on the body, legs, and mouthparts, as well as a sensory complex located on the dorsal surface of the tarsus of the first leg which contains a cluster of olfactory and gustatory receptors known as the Haller’s organ (Parola and Raoult, 2001). The Haller’s organ is important in enabling ticks to locate their hosts and to communicate with other ticks, responding to stimuli such as CO₂, phenols, and aromatic chemicals (Oliver, 1989; Parola and Raoult, 2001). Ticks also possess a circulatory system in which all organs and tissues are bathed by a circulating fluid called hemolymph, a blood analogue that lacks red blood cells (Sonenshine, 1991; Parola and Raoult, 2001).
Figure 1. Life cycle of the hard ticks. (CDC, 2008)

Figure 2. Diagram of hard tick characteristics. (Keirans and Litwak, 1989)
At each stage the tick feeds only once, but this feeding may involve a great variety of hosts that occupy very diverse habitats (Parola and Raoult, 2001). In the U.S. they are seasonally active, seeking hosts when environmental conditions are most suitable since they are extremely sensitive to climate (Oliver, 1989; Parola and Raoult, 2001; Estrada-Pena, 2002). There are two typical host-seeking behavior patterns: ambush, in which the ticks climb up vegetation and wait for passing hosts with their front legs held out, or attack, in which the ticks run toward a nearby host (Oliver, 1989; Parola and Raoult, 2001). *Ixodes* ticks, however, typically wait in the burrows or nests of their hosts (Oliver, 1989; Parola and Raoult, 2001). Before feeding, a tick may wander around on its host for several hours before inserting only its hypostome into the skin and releasing salivary substances to create a feeding pool (Parola and Raoult, 2001). The salivary secretions include a cement to anchor the mouthparts to the skin of the host and an anesthetic to make the bite painless, as well as enzymes, vasodilators, anti-inflammatory substances, antihemostatics, and immunosuppressive substances (Oliver, 1989; Parola and Raoult, 2001; Ramamoorthi et al., 2005). Prolonged attachment (5-7 days) of certain species of ticks may result in paralysis of the host caused by neurotoxic substances produced by the salivary glands (Parola and Raoult, 2001). Clinical signs include weakness in the lower extremities, which ascends within hours or days to involve the trunk musculature, upper extremities, and head (Parola and Raoult, 2001). Removal of the tick leads to rapid recovery within 24hrs (Parola and Raoult, 2001).

During the first 24-36 hours of attachment, there is little to no ingestion of blood (Parola and Raoult, 2001). This initial slow feeding period (usually 3-4 days) is followed by a period of rapid engorgement that normally lasts 1-3 days while the ticks, particularly
females, may increase their body weight by up to 120-fold (Oliver, 1989; Parola and Raoult, 2001). During this time the ticks rapidly concentrate the blood meal by removing water and electrolytes in their feces or during transpiration (Parola and Raoult, 2001). Adult males generally feed only briefly and sparingly. Once sated, the tick detaches, drops from the host, and finds a resting place to digest the blood meal and molt to the next feeding stage or enter diapause if needed (Oliver, 1989; Parola and Raoult, 2001). Mating occurs on the host, and females detach and drop off the host to digest the blood meal and to lay from 400 to more than 20,000 eggs, depending on the species (Oliver, 1989). The females die shortly after laying their eggs in a sheltered environment (Oliver, 1989; Parola and Raoult, 2001). It has been estimated that Ixodid ticks actually spend greater than 90% of their life unattached from a host (Parola and Raoult, 2001).

The major ticks of public health importance in the U.S. are *Ixodes scapularis, I. pacificus, Dermacentor variabilis, D. andersonii, Amblyomma americanum, Ornithodoros hermsi,* and *O. turicatae.* Other tick species that rarely feed on humans also play important roles in maintaining pathogens in zoonotic cycles (Walker, 1998). Ixodid ticks have a number of attributes that enhance their vector potential as compared to Argasids. They feed for relatively long periods (several days) during which they remain firmly attached to the host, their bite is usually painless, and they may go unnoticed for lengthy periods of time (Oliver, 1989; Parola and Raoult, 2001). They also transmit the most diverse array of infectious agents of medical and veterinary importance (Hill andWikkel, 2005). Tick control is best based on the concept of integrated pest management where different control methods are adapted to one area or against one tick species with consideration to their environmental effects (Parola and Raoult, 2001).
The three tick species that most commonly feed on humans are *Dermacentor variabilis* (the American dog tick), *Amblyomma americanum* (the Lone star tick), and *Ixodes scapularis* (the black-legged tick). Though spirochetes have been isolated from all three species, *D. variabilis* and *A. americanum* appear to be incompetent laboratory vectors (Clark et al., 2002). *Ixodes scapularis* is believed to be the primary vector of *B. burgdorferi*. Ticks often feed in clusters, and *I. scapularis* larvae appear to obtain *B. burgdorferi* from the skin itself rather than from the blood during feeding (Walker, 1998). In the unfed infected nymphal tick, *Borreliae* are only present in the midgut and during feeding they can multiply to 100,000 bacteria (Walker, 1998). Here they aggregate near the microvillar brush border and in the intercellular spaces of the gut epithelium (Burgdorfer et al., 1989). Populations of *I. scapularis* are well established in several areas of the northeast, southeast, and midwest (Estrada-Pena, 2002). These populations have been increasing as indicated by mammal ectoparasite surveys, human tick bite submissions, increasing numbers of Lyme disease cases, and tick sampling (Daniels et al., 2000). *Ixodes scapularis* can also carry and transmit the agents that cause ehrlichiosis and babesiosis (Hill and Wikel, 2005; Bratton and Corey, 2005).

*Dermacentor variabilis* is the primary vector for the agent of Rocky Mountain spotted fever, but it can also carry and transmit the agents of tularemia and ehrlichiosis (Burg, 2001). *Dermacentor variabilis* becomes active in late spring with peak activity in the summer in July (Burg, 2001). They are able to move a considerable distance independently but, like other ticks, travel the largest distances when on a host (Burg, 2001). *Amblyomma americanum* is the primary vector for the agent of ehrlichiosis, but may also transmit the agents of tularemia and the recently discovered southern tick-
associated rash illness (STARI). They have also been found to contain borrelia and rickettsiae in the southeast (Mixson et al., 2006). *Amblyomma americanum* ticks reportedly have saliva with borreliacidal activity against *B. burgdorferi*, but it is unclear whether *B. lonestari* exhibit the same sensitivity (Ledin et al., 2005). The bite of *A. americanum* often causes inflammation and irritation (Ledin et al., 2005). They are the species of tick that most aggressively attaches to humans in the southeast (Oliver, 1996). Comprehensive studies have not been carried out to determine whether *D. variabilis* or *A. americanum* are associated with the risk of Lyme disease (Armstrong et al., 2001).

A single tick may harbor more than one pathogen at a time and may transmit several different pathogens with one bite (Ginsberg, 2008). Co-infections are common, and in fact, in the U.S., 23% of Lyme disease patients also have babesiosis and 10-30% of patients also have ehrlichiosis (Steere, 2004; Bratton, 2005). Coinfection can also increase the severity of symptoms of disease (Ginsberg, 2008).

**Tick-borne Disease**

Tick-borne diseases have become an increasingly serious public health concern. Ticks are the number one vector of infectious agents in the United States (Hill and Wikel, 2005). Tick-borne diseases of the United States include Lyme disease, Rocky Mountain spotted fever, ehrlichiosis, tularemia, babesiosis, Colorado tick fever, and relapsing fever (Bratton and Corey, 2005). The etiologic agents for these diseases are quite diverse and include spirochetes, gram-negative bacilli, obligately intracellular rickettsiae and ehrlichiae, viruses, and intracellular protozoa (Walker, 1998). This varying array of pathogens is just one characteristic of tick-transmitted infectious diseases that pose a difficult set of problems for public health officials. An assortment of tick species, each having its own geographic distribution, vertebrate hosts, seasonal activity, and vector
biology are involved as well. Collectively, these attributes tend to disperse focal outbreaks of these zoonotic diseases across large geographic areas, in contrast to other diseases such as the *Legionella* outbreak of 1976, which was a point source outbreak, or the Kikwit Ebola outbreak, which was communicable by human-to-human contact (Walker, 1998). The dispersed distribution of tick-borne diseases leads to the impression among the public, many physicians, and government officials that these diseases are not a major problem (Walker, 1998).

Further complications arise because the precise incidence of these tick-borne diseases, even Lyme disease, is not known due to difficulties in establishing diagnoses, insufficient physician knowledge of the diseases, and deficiencies in or absence of reporting systems (Walker, 1998). The vector capacity of ticks is also being affected by anthropogenic pressures, of which the most significant factors are a gradual increase in winter temperatures and environmental pollution by heavy metal ions (Alekseev and Dubinina, 2008). Heavy metal ions, such as cadmium, can substitute the calcium in the chitinous exoskeleton which increases tolerance against desiccation, allowing infected ticks to quest longer and have an increased locomotory activity (Alekseev and Dubinina, 2008). Also, while expending energy to remove the ion from its system, the tick becomes more susceptible to infection with disease agents, which allows for more intense pathogen replication and a higher prevalence of multi-infection (Alekseev and Dubinina, 2008). All of these factors play a role in the maintenance of tick-borne diseases, which are dependent on the relative abundance of reservoir hosts and vector-competent ticks as well as the intensity of host-vector interactions (Rudenko et al., 2009).
Lyme Disease

Lyme borreliosis is the most common vector-borne infectious disease reported in the United States. It accounts for more than 90% of all reported vector-borne diseases, including those vectored by mosquitoes, and has been reported in 46 of the 48 contiguous states of the U.S. (Oliver, 1996; Oliver et al., 2000). The annual incidence of Lyme disease cases reported to the Centers for Disease Control and Prevention (CDC) has increased steadily from 9,000 cases in 1992 to 19,000 cases in 2006 (Bacon et al., 2007). In 2007, 27,000 cases were reported making the national average 9.1 cases per 100,000 people (CDC/DVBID, 2008). A majority of the cases (93%) occurred in 10 states in the northeastern and north-central states, including Connecticut, Delaware, Massachusetts, Maryland, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin, where the average is 34.7 cases per 100,000 people (Bacon et al., 2007).

The case definition for Lyme disease according to the CDC is either physician-diagnosed erythema migrans (EM) of greater than or equal to 5 cm in diameter or at least one objective late manifestation (i.e. musculoskeletal, cardiovascular, neurologic) with laboratory confirmation of infection with the causative agent, *Borrelia burgdorferi* (Bacon et al., 2007). Laboratory confirmation in 1996 required either isolation of *B. burgdorferi* from clinical specimens or demonstration of diagnostic levels of IgM and IgG antibodies to *B. burgdorferi* in serum or CSF with a significant change in the IgM or IgG antibody response in paired serum samples. However, in 1997 the modified requirements were isolation of *B. burgdorferi*, demonstration of diagnostic levels of IgM or IgG antibodies using a two-tier approach, or detection of IgG antibodies using a single-tier immunoblot approach (Bacon et al., 2007). A case is considered confirmed
when EM is present with known exposure to a tick or when there is laboratory evidence of infection accompanied by a late manifestation. A case is considered probable when any case of physician-diagnosed Lyme disease has laboratory evidence, or suspected when EM is present without known exposure or laboratory evidence (CDC/DISSS, 2008).

History of Lyme disease

Lyme disease is named for the location of a cluster of juvenile cases of rheumatoid arthritis that were investigated in Lyme, Connecticut in 1975. In these cases it became clear that the arthritis was a late manifestation of a multisystem disease caused by an agent associated with a tick bite (Walker, 1998; Parola and Raoult, 2001; Steere, 2004). In 1982, Burgdorfer and colleagues discovered and isolated the causative agent and, in 1984, named it *Borrelia burgdorferi* (Walker, 1998; Parola and Raoult, 2001; Steere, 2004). However, the cases in Connecticut were not the first appearance of Lyme disease. The earliest cases in America were first described in Cape Cod in the 1960’s, and *B. burgdorferi* DNA has been successfully isolated from museum tick specimens from the late 19th century (Persing et al., 1990; Steere, 2004). A disease with similar symptoms and a rash that was named “erythema chronicum migrans” (currently erythema migrans) was described in Sweden in 1909 (Oliver et al., 1993; Walker, 1998; Parola and Raoult, 2001). Lyme disease agents have probably been present in Europe and North America for thousands of years and are widely established in the remaining forested areas (Steere, 2004).

During the European colonization of North America, forests were cleared to less than 20% of the current forest area for farming (Walker, 1998; Steere, 2004). Lyme
disease appeared in the U.S. in the late 20th century because it was around this time that the agricultural land reverted back to woodland (Walker, 1998; Steere, 2004). It was also during this time that white-tailed deer proliferated after being hunted almost to extinction, improving conditions for the ecology of Lyme disease (Walker, 1998; Steere, 2004). Rural wooded areas then became populated with humans and deer and soon developed into “wooded suburbs” where deer were without predators and hunting was prohibited (Steere, 2004).

*Borrelia burgdorferi*

*Borrelia burgdorferi* is the main species of *Borrelia* that causes Lyme disease in the United States. This species is referred to as *B. burgdorferi sensu stricto* and is the primary genospecies that has been cultured from humans with Lyme disease (Lin et al., 2001; Lin et al., 2004; Oliver et al, 2008). However, there are a total of thirteen genospecies of *Borrelia* that cause Lyme disease, collectively known as the *B. burgdorferi sensu lato* complex (Salyers and Whitt, 2002; Rudenko et al. 2009). The thirteen genospecies include *Borrelia burgdorferi sensu stricto, B. afzelii, B. garinii, B. bissettii, B. valaisiana, B. lusitaniae, B. andersonii, B. turdi, B. japonica, B. spielmanii, B. sinica,* and *B. californiensis* (Anderson et al., 1996; Rudenko et al., 2009). A genospecies is defined as a line of homozygous self-fertilizing organisms which perpetuates by inbreeding or cloning (Anderson et al., 1996; Lin et al., 2001; Rudenko et al., 2009). However, each species has a different distribution throughout the world, is differently associated with vectors and hosts, and has different pathogenicity patterns (Rudenko et al., 2009). In addition to *B. burgdorferi s. s., B. andersonii* and *B. bissettii* have been reported in the U.S. and during the last decade have been identified and
described in the southeastern states of Georgia, Florida, and South Carolina (Salyers and Whitt, 2002; Lin et al., 2001; Lin et al., 2004; Oliver et al., 2008; Rudenko et al., 2009). *Borrelia andersonii* and *B. bissettii* were mainly isolated from ticks and small mammals from New York, California, Colorado, and the southeastern U.S. (Oliver et al., 2008). It has been speculated that there is greater genetic and phenotypic heterogeneity among the southern isolates as compared to the northern isolates based on restriction fragment length polymorphism patterns and phylogenetic analysis of the rrf-rrl intergenic spacer sequences (Lin et al., 2001; Oliver et al., 2008). Clinical studies in the south have not been extensive, and some human cases may not have been accurately diagnosed (Oliver et al., 2008). Recently, Rudenko et al. (2009) identified a fourteenth genospecies, *B. carolinensis*, which adds further support to the idea that spirochete diversity may be greater in the southeastern U.S. than in the northern states.

The borreliae belong to the eubacterial phylum of spirochetes, which are long, thin, corkscrew-shaped bacteria, approximately 0.25 μm in diameter and 25 μm in length (Walker, 1998; Salyers and Whitt, 2002; Steere, 2004). The cell wall consists of a cytoplasmic membrane surrounded by peptidoglycan and a loosely associated outer membrane (Salyers and Whitt, 2002; Steere, 2004). There are 7 to 11 flagella that are imbedded between the cytoplasmic and outer membranes and are also known as axial filaments (Salyers and Whitt, 2002; Walker, 1998). *Borrelia burgdorferi* has a small linear chromosome that is just under one megabase as well as nine circular and twelve linear plasmids that constitute 40% of its DNA (Fraser et al., 1997; Steere, 2004). Some of these plasmids are indispensable for life and could be thought of as mini chromosomes because they encode several membrane lipoproteins, including outer surface protein
(Osp) A, OspB, and OspC that are vital to *Borrelia* pathogenesis (Salyers and Whitt, 2002; Walker, 1998; Steere, 2004; Neelakanta et al., 2007). A very unique feature of *B. burgdorferi* is that it does not require iron, allowing it to circumvent some of the usual host defenses (Steere, 2004). In nature, the spirochetes live in an enzootic cycle involving ticks of the *Ixodes ricinus* complex and a wide range of animal hosts, including mammals, birds, and reptiles (Steere, 2004; Hanincova et al., 2006; Jordan et al., 2009).

*Borrelia* is an extracellular pathogen and causes infection by migrating through tissues, adhering to host cells, and evading immune clearance (Steere, 2004). It has been found that *Borrelia* in North America are never transmitted before 24 hours of tick attachment and rarely transmitted during the second day of feeding (Hojgaard et al., 2008). The delay in transmission is due to the change in expression of outer surface proteins from OspA to OspC. Prior to the blood meal, the borreliae express OspA and are not infectious, but after a blood meal OspA is decreased and OspC is increased (Walker, 1998; Salyers and Whitt, 2002). OspA seems to mediate adherence and colonization of *B. burgdorferi* to the tick midgut during the first 15 hours of feeding (Walker, 1998; Yang et al., 2004). The increase in production of OspC is in response to blood components and an increase in temperature when tick feeding begins (Walker, 1998; Hojgaard et al., 2008). The switch to OspC allows the spirochetes to be released from a tick midgut protein (called TROSPA) and invade through the gut wall into the hemocoel (Walker, 1998). By 48 hours, the spirochetes migrate to the salivary glands, bind to the tick salivary gland protein Salp15, and transfer to the host (Walker, 1998; Hojgaard et al., 2008).
Borreliae have a slow rate of growth, about 20-33 hours, as compared to other organisms (Oliver, 1996). The incubation period in humans is normally 7 to 14 days but can vary from 3 to 36 days, and it can survive in blood processed for transfusion and held at 4°C for up to 48 days, though there have been no known cases of Lyme resulting from transfusions (Center for Food Security, 2005).

Clinical Characteristics and Disease Pathogenesis

Lyme disease presents in stages with remissions, exacerbations, and different manifestations at each stage. Stage one is designated as an early localized infection of the skin presenting as erythema migrans, also known as a “bull’s eye rash”, followed within days or weeks by stage two early systemic infection, and months to years later by stage three persistent infection (Steere, 2004). However, infection is variable with some patients having only localized infection while others show symptoms only later in the course of infection.

The erythema migrans rash is an annularly expanding rash or lesion that is the hallmark of the disease and forms at the site of the tick bite after a 3-32 day incubation period (Steere, 2004). The “bull’s eye” appearance of the rash is due to central clearing, which is only observed in 37% of patients, and the diameter of the rash varies from 5 cm to 73 cm (Walker, 1998). The rash only occurs in 70-80% of cases, while the remaining 20-40% of patients either do not develop erythema migrans or present it to a physician who does not recognize it (Walker, 1998; Steere, 2004). In the U.S., this rash is often accompanied by flu-like symptoms, such as fever (39% of cases), fatigue and malaise (54%), headache and stiff neck (35%), arthralgias (44%), and myalgias (44%) (Walker, 1998; Steere, 2004). Differential diagnoses for erythema migrans include cellulitis, tinea,
granuloma, annulare, allergic reaction, erythema multiforme, contact dermatitis, urticaria, and fixed drug reaction (Walker, 1998). The first line of defense against *Borrelia* during localized infection may be complement-mediated lysis of the organism, and histologic examination of erythema migrans skin lesions reveal mild to marked perivascular infiltrates of lymphocytes, DCs, macrophages, and some plasma cells (Steere, 2004).

*B. burgdorferi* widely disseminates in the early systemic stage days to weeks after disease onset and has been found in blood, cerebrospinal fluid, and, in small numbers, myocardium, retina, muscle, bone, spleen, liver, meninges, and brain (Steere, 2004). Disseminated infection is marked by arthritis in 60% of patients, secondary hematogenous skin lesions in 18% of patients, transient carditis with heart block in 5% of patients, and neuroborreliosis in 15% of untreated patients (Walker, 1998; Steere, 2004). The neuroborreliosis presents as palsy of the facial nerve in 50-75% of patients (Parola and Raoult, 2001; Steere, 2004). Other possible clinical manifestations include acute lymphocytic meningitis, cranial neuropathy, radiculoneuritis, migratory musculoskeletal pain in joints, bursae, tendon, muscle, or bone, and rarely eye manifestations (Walker, 1998; Parola and Raoult, 2001; Steere, 2004).

*B. burgdorferi* may survive in the body within localized niches for several years after systemic symptoms have become minimal to non-existent. Months to years after disease onset, about 60% of untreated patients experience intermittent attacks of arthritis, primarily in the knee, and persistent joint inflammation occurs in about 10% of patients months to years after they receive a standard course of antibiotic treatment (Steere, 2004). A small percentage of patients develop disabling musculoskeletal pain,
neurocognitive symptoms, or fatigue, similar to chronic fatigue or fibromyalgia, a condition which has become known as chronic Lyme disease (Steere, 2004).

Differences in clinical manifestations of the disease in patients from the U.S. and those from Eurasia are reportedly due to the differences in organotropism (affinity for particular tissues, organs, or organ systems) of the *Borrelia* species that cause Lyme disease (Parola and Raoult, 2001). *B. afzelii*, a species found in Europe, is associated more with EM and milder disease than *B. burgdorferi* sensu stricto, and infection with *B. garinii*, also found in Europe, is more frequently associated with neurological abnormalities while *B. burgdorferi* is more often associated with rheumatological disorders (Parola and Raoult, 2001).

**Diagnosis**

Diagnosis is carried out on the basis of clinical presentations, epidemiological findings, and serological testing. For the classic presentation, the characteristic EM rash with a confirmed history of tick bite, diagnosis is usually based on the clinical findings alone, but a history of recognized tick bite is only obtained in 14-32% of cases (Walker, 1998). In many cases, laboratory tests are performed to confirm the presence of *B. burgdorferi*, though they are not required. Cultivation of *B. burgdorferi* in BSK II medium is the gold standard for confirmation of a Lyme disease diagnosis. BSK II components include *N*-acetylglucosamine, yeast extract, bovine serum albumin, and rabbit serum as well as sources of amino acids, nucleotides, and vitamins. Samples from the outer margin of the EM rash yield cultures from 57-86% of patients, and blood samples have a recovery yield of about 50% in early disseminated cases (Walker, 1998;
Parola and Raoult, 2001; Aguero-Rosenfeld, 2003). \textit{B. burgdorferi} can also be visualized directly with fluorescent antibody or silver staining methods (Aguero-Rosenfeld, 2003).

When no EM is present, however, diagnosis is based on recognition of the symptoms and a two-tier serological approach that requires a positive or equivocal result by enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay (IFA) followed by Western blot (Steere, 2004). The initial ELISA or IFA tests for either IgM or IgG antibodies, but if the initial tests are negative and there is strong evidence of Lyme, tests should be repeated with a second serum sample taken 2-4 weeks after initial testing (Walker, 1998). An immunoblot is recommended as a confirmation only after a positive or equivocal result has been obtained by ELISA or IFA. The criteria for confirmation are as follows: detection of IgM antibodies to at least two of three \textit{B. burgdorferi} proteins, 24kDa (OspC), 39kDa (BmpA), and 41kDa (flagellin), or detection of IgG antibodies to at least five of ten \textit{B. burgdorferi} proteins; 18, 24, 28, 30, 39, 41, 45, 58 (not GroEL), 66, and 93 kDa (Walker, 1998). The most common marker for Lyme by IgM or IgG immunoblotting is the 39kDa protein (Walker, 1998).

Serologic testing presents a new set of problems for the diagnosis of Lyme disease due to occurrence of both false positive and false negative results. The serological tests are highly sensitive for the detection of \textit{Borrelia} antibodies, as well as for similar antibodies produced against other diseases with similar characteristics. For example, false positives are often observed in patients with borrelial relapsing fever, syphilis, human granulocytotropic ehrlichiosis, infectious mononucleosis, and autoimmune diseases (Walker, 1998). In addition, in some areas where Lyme disease is endemic, 10% of the population has antibodies to \textit{B. burgdorferi} from previous exposure.
which can also be detected by serological testing (Walker, 1998). For these reasons, it is recommended that serological tests have a sensitivity of 95% or greater to be used in the two-tier approach for diagnosis (Walker, 1998). However, the tests should be used in support of a diagnosis that was based on clinical observations, history of exposure to ticks, and symptoms to prevent false positive diagnosis (Walker, 1998). An inter-laboratory comparison was performed between 516 labs to evaluate the reliability of ELISA and IFA in routine clinical use and found a progressive decline in specificity from 97% in 1992 to 30% in 1994 (Walker, 1998). A similar comparison at the CDC and five academic centers showed serology that, under optimal conditions, identified antibodies in 59-66% of early EM cases, 63-75% of early neuroborreliosis cases, 89-95% of late Lyme arthritis cases, and 91-100% of late neuroborreliosis cases (Walker, 1998). Confirmation by Western blot improved the results by 20-30% (Walker, 1998).

A third test that promises the greatest sensitivity, specificity, and timeliness is the polymerase chain reaction (PCR). The PCR reaction is still primarily a research tool and is not readily available for clinical use. The major drawback of PCR-based techniques is DNA contamination (Walker, 1998). Targets for PCR diagnosis are the 49kb plasmid encoding OspA and OspB, the flagellin gene, a chromosomal gene designated clone 2H1, a fragment of the RNA polymerase C gene, and the 23S rRNA gene (Walker, 1998; Chang et al., 1998). The best yield for PCR, as with culture, is from skin specimens from the EM lesions. This approach allows for the detection of \textit{B. burgdorferi} in 60-90% of cases with sensitivities exceeding 80% (Walker, 1998; Aguero-Rosenfeld, 2003). PCR also appears to be the best method for testing synovial fluid of patients with Lyme arthritis, detecting \textit{B. burgdorferi} in 96% of cases (Walker, 1998). PCR results are more
frequently positive for patients with untreated or partially treated arthritis and more frequently negative for patients who have had prolonged antibiotic treatment (Walker, 1998; Aguero-Rosenfeld, 2003). This method has also detected the agent in CSF in 78% of cases (Walker, 1998). PCR also holds great promise for specimens preserved in alcohol or ones that have been dried (Persing et al., 1990).

Overdiagnosis is also an important issue. In one instance, 788 patients were referred to a medical center with a positive diagnosis of Lyme disease. Of these, only 23% had active infection, mostly arthritis or neuroborreliosis, and 20% had a different illness but also had evidence of a prior *B. burgdorferi* infection (Walker, 1998). In a separate instance, a pediatric study reported that 38% of cases were overdiagnosed, 8% were underdiagnosed, and 54% were correctly diagnosed (Walker, 1998).

*Treatment*

The typical treatment for the early stages of Lyme disease is a 200 milligram dose of doxycycline every day for 20-30 days (Parola and Raoult, 2001). Doxycycline is in the tetracycline class of antibiotics that target the ribosome and prevent protein synthesis (Salyers and Whitt, 2002). It can be given orally for longer periods of time than most antibiotics without adverse effects, which makes it the choice for treatment of Lyme disease. However, physicians are advised to base their decision on whether or not to begin prophylaxis for Lyme disease on positive identification of the tick as a nymphal *Ixodes scapularis*, the degree of endemicity for Lyme disease in the geographic region, and the duration of tick attachment (Parola and Raoult, 2001; Hojgaard et al., 2008). When an engorged nymphal tick is found, a single dose of doxycycline is sufficient to prevent infection, but removal of any tick within 24 hours after attachment is usually an
adequate preventative measure (Steere, 2004). For presumed EM with no serologic
testing, and because serologic tests are insensitive during the first two weeks of infection,
a 10 to 20 day treatment with doxycycline, or amoxicillin for children, is recommended
(Steere, 2004). A single course of antibiotic is normally sufficient to cure early stages of
Lyme disease. For patients diagnosed in the later chronic stages of Lyme disease,
treatment is normally by oral or intravenous antibiotic therapy, usually with ceftriaxone
or penicillin, for several months to a year to treat persistent infection in which \textit{B.}
burgdorferi is present in localized niches that may have a protective function, such as the
cytosol of synovial cells (Steere, 2004).

\textbf{Reservoirs}

The principal reservoir for \textit{B. burgdorferi} in nature is the white-footed mouse,
\textit{Peromyscus leucopus}. However, there are other wild mammals with evidence of
infection including white-tailed deer (\textit{Odocoileus virginianus}), eastern chipmunks
(\textit{Tamias striatus}), gray squirrels (\textit{Sciurus carolinensis}), opossums (\textit{Didelphis virginiana}),
and raccoons (\textit{Procyon lotor}), although the reservoir competence of these hosts is
unknown (Center for Food Security, 2005). White-tailed deer do not contribute directly
to the dispersal of \textit{B. burgdorferi} but do serve in maintaining populations of adult \textit{I.}
scapularis which heavily parasitize the deer (Norris et al., 1996; Hanincova et al., 2006).
Rudenko et al. (2009) showed that the cotton mouse, \textit{P. gossypinus}, and the eastern wood
rat, \textit{Neotoma floridana}, were the primary reservoir hosts for \textit{B. carolinensis}. There may
be several parallel enzootic cycles that may or may not overlap, and the cotton mouse (\textit{P.}
gossypinus), hispid cotton rat (\textit{Sigmodon hispidus}), and eastern woodrat (\textit{Neotoma}
\textit{floridana}) are natural reservoir hosts to \textit{B. burgdorferi sensu stricto} and \textit{B. bissettii} in the
southeast U.S. (Oliver et al., 2006; Oliver et al., 2008). As many as 69% of *Peromyscus gossypinus* trapped in coastal localities in the southeast (including North Carolina, South Carolina, Alabama, and Mississippi) were shown to be naturally infected (Oliver et al., 1993; Rudenko et al., 2009).

While rodents appear to be the preferred reservoirs for *B. burgdorferi*, medium-sized mammals are hosts to more species of ticks than deer or mice (Kollars, 1993). Tick species that have been removed from raccoons and opossums include *Dermacentor variabilis* (90%), *A. americanum* (0.07%), *I. scapularis* (0.1%), *I. texanus* (5%), and *I. cookei* (2%) (Kollars, 1993; Pung et al., 1994). Studies have shown raccoons to be the principal host for adult *D. variabilis* in Tennessee, Kentucky, and Virginia (Kollars, 1993; Kollars and Kenglueciia, 2001). Raccoons and opossums are abundant and widely distributed in North America, and they occupy almost any available ecologic niche, with the greatest densities being found in urban and suburban environments (Yabsley et al., 2008). Raccoons are often heavily infested by ticks and in endemic areas seem to contribute to production of infected ticks (Norris et al., 1996; Ouellette et al., 1997). However, there is disagreement on the reservoir competence of raccoons. *Borrelia* has been isolated from naturally infected raccoons, but several experimental studies have indicated that raccoons are relatively incompetent reservoirs (Magnarelli et al., 1991; Norris et al., 1996; Ouellette et al., 1997; Yabsley et al., 2008). For example, Yabsley et al did not detect *Borrelia* in wild raccoon blood samples using an assay designed to detect all *Borrelia* species. Yabsley et al explained that it was possible that the raccoons were infected but that *Borrelia* were not circulating in the blood. Other studies, however, have confirmed that raccoons are competent reservoirs via tick transmission (LoGiudice
et al., 2003). Differences in experimental approach may account for the discrepancies in conclusions about reservoir competence. Yabsley et al inoculated raccoons via intravenous injection while LoGiudice et al infected raccoons by allowing infected ticks to feed on the raccoons, which mimics the course of disease transmission in nature. Several investigators have concluded that raccoons are competent reservoirs but their ability to infect vectors is apparently much less efficient than the white-footed mouse (Fish and Daniels, 1990; LoGiudice et al., 2003). *Borrelia* are passed to 40-90% of the larval ticks that feed on infected *Peromyscus leucopus* (Fish and Daniels, 1990; LoGiudice et al., 2003). Two other tick-borne pathogens that have been isolated from raccoons include *Babesia* and *Ehrlichia* species (Dugan et al., 2005; Birkenheuer et al., 2006). Opossums have also tested positive for *B. burgdorferi* by blood culture and serology (Fish and Daniels, 1990). However, opossums, as well as squirrels, seem to clear infection quickly (Weinhold, 2004). Like raccoons, opossums are widely distributed and highly adaptive to different environments including suburban areas. Their increasing frequency in suburban areas brings the raccoons and opossums, along with their associated ticks, into contact with humans, increasing the likelihood of Lyme disease transmission.

**Lyme disease in the Southeast**

There is debate about the transmission and presence of *B. burgdorferi* in the southeastern U.S. While it is present, it does not seem to be as readily transmitted in the southeast and the *I. scapularis* tick does not actively attach to humans. Since all of the components for the transmission of *B. burgdorferi* are available (such as *I. scapularis* ticks, reservoir hosts, and white-tailed deer), the low incidence of Lyme disease in the
southeast is intriguing (Jacobs et al., 2003). Most of what is known about the ecology of
*B. burgdorferi sensu lato* in the eastern U.S. was derived from studies conducted in the
northeast where the majority of human cases have occurred, but there are data that
confirm the presence of *B. burgdorferi sensu lato* in many areas where Lyme disease was
not previously thought to occur (Ryan et al., 2000; Clark, 2004; Rudenko et al., 2009).

Since Lyme disease has become reportable, hundreds of cases have been reported
from southeastern states and while some cases may have resulted from exposures
elsewhere in the country, many were locally transmitted (Clark, 2004). *Borrelia
burgdorferi* has been isolated from birds, rodents, and ticks in Florida, Georgia, South
Carolina and other southern states, but it is still unclear whether endemic infection
commonly occurs (Clark, 2004). Clinical cases have been reported in South Carolina but
there is disagreement about whether they are true cases due to the absence of *B.
burgdorferi* culture isolates (Oliver et al., 2000). In addition, *B. burgdorferi* and *I.
scapularis* seem to be more genetically varied in the south, which may cause
investigators to overlook them when using criteria based on northern isolates (Oliver,
1996; Jacobs et al., 2003). Infection in questing southern *Ixodes* ticks is rare or absent as
compared to the greater than 25% infection rate of northern populations (Jacobs et al.,
2003). Southern *I. scapularis* ticks are competent experimental vectors and could
theoretically serve as a bridge vector to humans (Walker, 1998; Jacobs et al., 2003).
However, *I. scapularis* ticks in the south do not actively quest during the summer season
when host activity is high and the host affinity is diverse, resulting in a prevalence of *B.
burgdorferi* in southern adult *I. scapularis* ticks of only 0-3% (Walker, 1998; Jacobs et
al., 2003). There are also other tick species that may have a more important role in
maintaining the enzootic cycle of *B. burgdorferi* in the south, such as *Ixodes minor* (Rudenko et al., 2009). Although data exist, the primary tick vectors and reservoir hosts for *B. burgdorferi sensu lato* in the southeast U.S. have not been determined unequivocally (Clark et al., 2002). Tick transmission studies and reservoir competence studies with southern isolates of *B. burgdorferi* under conditions that mimic the natural transmission of the spirochete will be needed.

Further complicating our understanding of Lyme disease in the south is the emergence of a southern tick-associated rash illness (STARI), also known as Master’s disease in honor of Dr. Edwin Masters, a family physician who first reported cases to the Missouri state Department of Health. The clinical presentation of STARI resembles classic Lyme disease, but STARI is associated with bites from *A. americanum* (Clark, 2004). These ticks were shown to contain a spirochete that was noncultivable in BSK medium and more closely related to relapsing fever *Borrelia*. The organism was tentatively named *Borrelia lonestari* (Clark, 2004). Since the mid 1980’s physicians have diagnosed this disease in patients from the southeast that have EM and mild flu-like symptoms following the bite of an *A. americanum* tick (Varela et al., 2004). Serologic testing, however, does not support a classic Lyme disease diagnosis despite microscopic evidence of spirochetes in biopsy samples (Varela et al., 2004). Since its discovery, *Borrelia lonestari* has been isolated from *A. americanum* from Alabama, Arkansas, Tennessee, Virginia, Delaware, Kansas, Kentucky, Maryland, New Jersey, and North Carolina (Burkot et al., 2001; Stromdahl et al., 2003). However, these isolations and characterizations have relied on PCR technologies since the agent is uncultivable (Varela et al., 2004).
Several dominant tenets in the past concerning Lyme disease in the southeastern U.S. suggest that Lyme disease does not occur due to absence of *B. burgdorferi* and a tick vector that readily transmits it to humans. However, in spite of these tenets, several studies indicate that *B. burgdorferi sensu lato* is present and widely distributed in the southern states (Rudenko et al., 2009). Some possible reasons for the low incidence of Lyme disease cases in the southeast are 1) underreporting of cases, 2) decreased infectivity and pathogenicity of some *B. burgdorferi* isolates, 3) differences in density of the human population with respect to that in the northeast, 4) diminished synchrony of the tick life cycle that prevents overwintering nymphs to feed on and infect a new cohort of reservoir hosts, 5) availability of lizards as hosts for infected ticks, and 6) differences in feeding behavior of nymphal *I. scapularis* suggesting that southern nymphae ticks rarely bite humans (Oliver 1996; Jacobs et al., 2003).

The purpose of the present study was to use PCR and DNA sequencing technologies to determine if *Borrelia burgdorferi sensu lato* is present in ticks and whole blood samples removed from raccoons and opossums trapped in south-central Kentucky. Studies have suggested the presence of *B. burgdorferi* in the southeastern U.S. and *B. burgdorferi* has been isolated from birds, rodents, and ticks in Florida, Georgia, South Carolina and other southern states, but few studies have been performed in Kentucky (Clark, 2004; Rudenko et al., 2009). Most of what is known about the ecology and epidemiology of *B. burgdorferi sensu lato* in the eastern U.S. has been derived from studies conducted in the northeast where the majority of human cases have occurred. Therefore, there is an urgent need for additional studies of potential tick vectors and reservoir hosts of *B. burgdorferi* in the southeast U.S.
MATERIALS AND METHODS

Tick Collection and Identification

Raccoons and opossums were trapped from June 2007 to June 2008 with Havahart® 32”x10”x12” live animal traps from sites in the Warren and Barren counties of south central Kentucky (Fig. 3). Animals were euthanized using an overdose of the inhalant anesthesia, isoflurane. Sterile blood samples were collected into three 10 ml tubes containing the anticoagulant K$_2$EDTA using a Vacutainer® system and stored at 4°C. Animals were examined for all life stages of ticks. All ticks were removed with forceps and placed into vials of 70% ethanol for transport. Individual ticks were identified to species when possible following Keiran and Litwak’s “Pictorial key to the adults of hard ticks” (1989) and Ruedisueli and Manship’s “Tick Identification Key”. Identifications were confirmed by Dr. Byron Blagburn (Auburn University College of Veterinary Medicine). Individual ticks were placed into microfuge tubes containing fresh 70% ethanol and stored at 4°C prior to DNA extraction. Pictures of representative ticks were taken with a Syncroscopy unit microscope (provided by Dr. John Andersland, Western Kentucky University).
Figure 3. Map of trapping locations. Location of Warren and Barren counties in Kentucky (A). Magnification of box in A showing location of trapping sites within Warren and Barren counties (B).

DNA Isolation

Genomic DNA was extracted from individual adult ticks or pools of 2-20 nymphs using a QIAamp DNA mini kit (Qiagen) according to the manufacturer’s suggested supplemental protocol for the purification of total DNA from ticks. DNA yield was
determined using a Thermo Nanodrop ND-100 Spectrophotometer. DNA samples were stored at -20°C.

Genomic DNA was extracted from 200 µl of raccoon and opossum whole blood samples using a QIAamp DNA blood mini kit (Qiagen) according to the manufacturer’s instructions. DNA yield was determined using a Thermo Nanodrop ND-100 Spectrophotometer, and the DNA samples were stored at -20°C.

**Polymerase Chain Reaction (PCR)**

DNA samples were analyzed by polymerase chain reaction for the presence of *B. burgdorferi* using the oligonucleotide primers SL1 (5’- AATAGGTCTAATAATAGCC-TTAATAGC- 3’) and SL2 (5’-CTAGTGTTTTGCCATCTTCTTTGAAAA-3’) specific for the OspA gene (Demaerschalck et al., 1995; Sparagano et al., 1999). A 25 µl reaction was set up containing 10 µl of 2.5x MasterMix (5 Prime), 0.5 µl (2 µM) of each primer, 50ng of template DNA, and sterile nanopure water to bring the volume to 25 µl. A negative control was included with each reaction set containing sterile nanopure water in place of template DNA. A second negative control was included to confirm that the primer set did not amplify tick DNA. It contained DNA isolated from sterile lab-bred *I. scapularis* ticks (provided by Dr. Craig Banks, Institute of Arthropodology and Parasitology). A positive control with *B. burgdorferi* strain B31 genomic DNA (ATCC) as a template was also included in each set. The reaction mixture was loaded into an automated DNA thermal cycler to undergo 40 cycles of amplification. Amplification conditions were as follows: denaturation at 95°C for one minute, primer annealing at 57°C for 30 seconds, and extension at 72°C for one minute. Reactions were stored at -20°C until they were analyzed by agarose gel electrophoresis. To prevent
contamination, all procedures were carried out with aerosol barrier pipette tips in a PCR designated area. All PCR products were visualized on 2% agarose gels using ethidium bromide staining.

**Sequencing**

Two positive PCR samples randomly selected from each gel were purified with an Ultraclean PCR purification kit (MoBio). A Big Dye Terminator v3.1 (Applied Biosystems) reaction was set up as follows: 30ng of purified PCR product, 5 pmol of primer, 2 µl sequencing juice, and 2 µl sequencing buffer. Forward and reverse reactions were set up for each sample in the first run, after which it was determined that the reverse reaction was efficient to obtain sequences. The sequencing reaction conditions on the thermal cycler were as follows: 25 cycles of 96°C for 30 seconds, 55°C for 15 seconds, and 64°C for 4 minutes. Each sample was purified with a DyeEx 2.0 Spin kit (Qiagen), dried for 15 minutes with no heat in a speedvac, and rehydrated with 20 µl of HiDi formamide buffer before being loaded into the ABI 3130 Genetic Analyzer for sequencing by capillary electrophoresis. The sequences were edited before being analyzed by BLAST analysis and compared to sequences in the National Center for Biotechnology Information (NCBI) GenBank database. The sample ID and maximum identity were recorded for each sample.
RESULTS

Tick Collection and Identification

A total of 976 ticks were collected from wild-trapped raccoons and opossums between June 2007 and June 2008. There were five species of ticks recovered from raccoons including *Dermacentor variabilis*, *Amblyomma americanum*, *Ixodes scapularis*, *I. cookei*, and *I. texanus*. *Dermacentor variabilis* was the only species of tick recovered from opossums. Figures 4-6 show pictures taken of each species of tick except *I. cookei* and *I. texanus*. *Ixodes cookei* and *I. texanus* were used in the DNA isolation procedure before pictures could be taken. *Dermacentor variabilis* constituted 59% (577/976) of the ticks identified and *A. americanum* constituted 35% (341/976). Only 5.4% (53/976) of the identified ticks were *I. scapularis*, 0.4% (4/976) were *I. texanus*, and 0.1% (1/976) were *I. cookei*. Of the total ticks recovered, 38.9% were nymphs. The majority of ticks identified in this study, 802 (82.2%), were removed from raccoons. Table 1 lists information about the numbers of ticks collected, including life stages and the animals from which they were collected.

The average number of ticks removed from raccoons was highest during the late spring (May) to early summer months (June and July) with the highest average *D. variabilis* occurring in June 2007 (15.2) and the highest average *A. americanum* occurring in May 2008 (34.3). The majority of the *A. americanum* ticks recovered were nymphs (see Fig. 7). The average number of *D. variabilis* removed from opossums was highest during June (6.67) and July (5.8) of 2007 and May (11.4) of 2008 (see Fig. 8).
Figure 4. *Dermacentor variabilis*. Adult female dorsal view (A) and ventral view (B). Adult male dorsal view (C) and ventral view (D). Bar = 1mm.

Figure 5. *Amblyomma americanum*. Adult female dorsal view (A) and ventral view (B). Adult male dorsal view (C) and ventral view (D). Bar = 1mm (A & B). Bar = 0.5mm (C & D).
Figure 6. *Ixodes scapularis*. Adult female dorsal view (A) and ventral view (B). Bar = 1mm.

Table 1. Number of ticks of each species collected out of 976 total ticks collected. Percent values are percentage of total ticks collected.

<table>
<thead>
<tr>
<th>Tick Species</th>
<th>Total n (%)</th>
<th>Nymphs n (%)</th>
<th>Adult Male n (%)</th>
<th>Adult Female n (%)</th>
<th>Raccoon n (%)</th>
<th>Opossum n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. v.</em></td>
<td>577 (59%)</td>
<td>0 (0%)</td>
<td>378 (38.7%)</td>
<td>199 (20.3%)</td>
<td>403 (41.2%)</td>
<td>174 (17.8%)</td>
</tr>
<tr>
<td><em>A. a.</em></td>
<td>341 (35%)</td>
<td>339 (34.8%)</td>
<td>1 (0.1%)</td>
<td>1 (0.1%)</td>
<td>341 (35%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><em>I. s.</em></td>
<td>53 (5.4%)</td>
<td>38 (3.9%)</td>
<td>0 (0%)</td>
<td>15 (1.5%)</td>
<td>53 (5.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><em>I. t.</em></td>
<td>4 (0.4%)</td>
<td>2 (0.2%)</td>
<td>0 (0%)</td>
<td>2 (0.2%)</td>
<td>4 (0.4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><em>I. c.</em></td>
<td>1 (0.1%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (0.1%)</td>
<td>1 (0.1%)</td>
<td>0 (0%)</td>
</tr>
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</table>

**Figure 7.** Average number of ticks removed from raccoons per month from June 2007 – June 2008.

**Figure 8.** Average number of ticks removed from opossums per month from June 2007 – June 2008.
**DNA Isolation**

Genomic DNA was successfully isolated from individual ticks or pools of 2-20 nymphal ticks using the Qiagen QIAamp DNA mini kit with yields ranging from 3.6 - 1919.6 ng/µl and an average yield of 180 ng/µl. All samples recorded an A260/A280 ratio of ≥ 1.8, confirming purity of isolated DNA. Genomic DNA was also successfully isolated from raccoon and opossum whole blood samples with yields ranging from 10 - 200 ng/µl.

**PCR**

Optimization of the PCR reaction with the SL primer set and pure genomic *B. burgdorferi* strain B31 DNA resulted in a PCR product of 307 bp. All samples producing an amplicon of 307 bp were considered positive. Out of 976 total ticks, 642 PCR reactions were performed (609 reactions with individual adult ticks and 33 reactions with pools of 2-20 nymphal ticks). A total of 163 out of 642 (25.4%) PCR samples were positive. Of these positive samples, 130 (80%) were from ticks removed from raccoons while the other 33 (20%) were from ticks removed from opossums. Positive DNA samples from ticks that were isolated from raccoons included 83% (108/130) *D. variabilis*, 10% (13/130) *A. americanum*, 6.2% (8/130) *I. scapularis*, and 0.8% (1/130) *I. cookei*. Positive DNA samples from ticks that were removed from opossums were 100% (33/33) *D. variabilis*. All of the positive samples from nymphs (13/642 or 2%) resulted from a single species, *A. americanum*. Table 2 gives detailed information about the samples that were positive by PCR. Figure 9 depicts one of the electrophoretic analyses of the PCR samples.
A total of 8 out of 92 (8.7%) blood samples were positive. All of these positives were from raccoon blood samples constituting 15.7% (8/51) of the total raccoon blood samples tested. Figure 10 depicts the results of the electrophoretic analysis of the raccoon and opossum blood PCR samples.

**Table 2.** PCR positive tick samples.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tick Life Stage</th>
<th>No. of PCR positive reactions/No. of PCR reactions for each species (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raccoon</td>
<td>Adult Male</td>
<td>68/577 (11.8%)</td>
</tr>
<tr>
<td></td>
<td>Adult Female</td>
<td>40/577 (6.9%)</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>0</td>
</tr>
<tr>
<td>Opossum</td>
<td>Adult Male</td>
<td>21/577 (3.6%)</td>
</tr>
<tr>
<td></td>
<td>Adult Female</td>
<td>12/577 (2.1%)</td>
</tr>
<tr>
<td></td>
<td>Nymph</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>141/577 (24.4%)</td>
<td>13/32 (40.6%)</td>
</tr>
</tbody>
</table>

Figure 9. Agarose gel electrophoresis (2% gel) of *B. burgdorferi* amplified from tick DNA. Lanes 1, 50, 51, and 100, 100bp DNA ladder. Lane 2, positive control. Lane 99, negative control. Lanes 3-49 and 52-98 represent individual tick samples.

Figure 10. Agarose gel electrophoresis (2% gel) of *B. burgdorferi* amplified from raccoon and opossum blood samples. Lanes 1, 50, 51, and 100, 100bp DNA ladder. Lane 2, positive control. Lane 99, negative control. Lanes 3-49 and 52-56 represent individual raccoon blood samples. Lanes 57-98 represent individual opossum blood samples.
Sequencing

A total of 19 positive samples were chosen for sequencing. Of these samples, 15 were positive tick samples and 4 were positive raccoon blood samples. Sequencing resulted in products ranging in size from 219 - 249 nucleotides. A majority, 18 samples, were identified as *B. burgdorferi sensu stricto* with max identities ranging from 97-99% similarity. One sample resulted in unusable sequence data due to unclean template.

Figure 11 shows a representative sequence alignment resulting from analysis of sequences of positive PCR samples from each of the genera of ticks examined. Figure 12 shows a representative sequence alignment following BLAST analysis for all of the positive raccoon blood samples that were sequenced.

**Figure 11.** Representative sequence alignment for PCR positive *Borrelia* samples amplified from *D. variabilis, A. americanum, and I. scapularis*. Lower case “t” within the query sequence represents a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.
**gb|ABJU01000032.1|**  Borrelia burgdorferi 80a gcontig_1107725642079, whole genome shotgun sequence. Length=9740

Score = 412 bits (223),  Expect = 5e-115
Identities = 242/244 (99%), Gaps = 1/244 (0%)
Strand=Plus/Minus

Query 1  AATTTTACTTTACTATTTGTCAGCTTTTACGCCTTCAAGTACTCCAGATCCATTGTTTTT  60
         |___________________________________________________________|
Sbjct 4482  AATTTTACTTTACTTTGTCAGCTTTTACGCCTTCAAGTACTCCAGATCCATTGTTTTT  4424

Query 61  ATCAGAAGTTCCCTTAAGCTCAAGCTTGCTACTGTTGCAATTAGATCGTACTTGCCGTC  120
         |___________________________________________________________|
Sbjct 4423  ATCAGAAGTTCCCTTAAGCTCAAGCTTGCTACTGTTGCAATTAGATCGTACTTGCCGTC  4364

Query 121  TTTGttttttCTTTGCTTACAAGAAACTTTCTTTCAACACCAGGCAAATCTACTGAAACGCT  180
         |___________________________________________________________|
Sbjct 4363  TTTGCTTTTTCTTTTCTTTACAAGAAACTTTCTTTCAACACCAGGCAAATCTACTGAAACGCT  4304

Query 181  GTTTTTCTGGCTCGGCTGCTACACATTASGCAATATGCACGCTATTACATTAGGCTATTAGACC  240
         |___________________________________________________________|
Sbjct 4303  GTTTTTCTGGCTCGGCTGCTACACATTASGCAATATGCACGCTATTACATTAGGCTATTAGACC  4244

Query 241  TATT  244
         ||||
Sbjct 4243  TATT  4240

**Figure 12.** Representative sequence alignment for PCR positive *Borrelia* samples amplified from raccoon blood samples. Lower case “t” within the query sequence represents a low complexity sequence that was filtered by BLAST to prevent any artificial hits between sequences that were not truly related.
DISCUSSION

Lyme disease cases from southeastern states have been reported to the CDC, and the presence of *B. burgdorferi sensu lato* has been documented in several southeastern states including North Carolina, South Carolina, Georgia, Tennessee, Florida, Virginia, and Kentucky (Duobinis-Gray et al., 1993; Kollars, 1993; Levine et al., 1993; Sonenshine et al., 1993; Norris et al., 1996; Ouellette et al., 1997; Lin et al., 2001; Clark et al., 2002; Clark, 2004; Oliver et al., 2008). Despite the potential for transmission of *Borrelia burgdorferi* to humans in the southeast U.S., challenges in determining the primary tick vectors and reservoir hosts, along with the presence of a Lyme-like illness, have made unequivocal confirmation of the presence of Lyme disease difficult. Most of what is known about the ecology and epidemiology of *B. burgdorferi sensu lato* in the eastern U.S. has been derived from studies conducted in the northeast where the majority of human cases have occurred. Therefore, there is an urgent need for additional studies of potential tick vectors and reservoir hosts of *B. burgdorferi* in the southeast U.S.

The purpose of the present study was to utilize PCR and DNA sequencing technologies to determine if *B. burgdorferi* is present in tick species that commonly parasitize raccoons and opossums in south-central Kentucky. The same technologies were also used to analyze blood samples removed from the raccoons and opossums in order to evaluate the potential for reservoir competence. In the present study, the presence of *B. burgdorferi* in ticks recovered from wild-trapped raccoons and opossums in south-central Kentucky was demonstrated by PCR and sequencing techniques. Of the raccoon blood samples taken, 8.7% were also positive for *B. burgdorferi* by PCR.

A wide variety of mammals are known to have been infected with *B. burgdorferi* including white-footed mice (*Peromyscus leucopus*), eastern chipmunks (*Tamias*
striatus), cotton mice (*Peromyscus gossypinus*), hispid cotton rats (*Sigmodon hispidus*), wood rats (*Neotoma floridana*), rice rats (*Oryzomys palustris*), house mice (*Mus musculus*), grey squirrels (*Sciurus carolinensis*), least shrews (*Cryptotis parva*), meadow voles (*Microtus pennsylvanicus*), cottontail rabbits (*Sylvilagus floridanus*), marsh rabbits (*Sylvilagus palustris*), striped skunks (*Mephitis mephitis*), raccoons (*Procyon lotor*), Virginia opossums (*Didelphis virginianus*), and white-tailed deer (*Odocoileus virginianus*) (Fish and Daniels, 1990; Duobinis-Gray et al., 1993; Kollars, 1993; Levine et al., 1993; Sonenshine et al., 1993; Norris et al., 1996; Ouellette et al., 1997; Lin et al., 2001; Clark et al., 2002; LoGiudice et al., 2003; Clark, 2004; Oliver et al., 2008).

*Borrelia burgdorferi* infections have also been found in birds (Carolina wren (*Thryotherus ludovicianus*) and rufous-sided towhee (*Pipilo erythrophthalmus*)) and lizards (six-lined race runner (*Cnemidophorus sexlineatus*) and ground skink (*Scincella lateralis*)) in the southeast U.S. (Levine et al., 1993; Oliver et al., 2008). In the southeast, *B. burgdorferi* infections and reservoir competence has been studied more extensively in raccoons than in any other medium-sized mammal in the U.S. (Fish and Daniels, 1990; Duobinis-Gray et al., 1993; Kollars, 1993; Levine et al., 1993; Sonenshine et al., 1993; Norris et al., 1996; Ouellette et al., 1997; Lin et al., 2001; Clark et al., 2002; LoGiudice et al., 2003; Clark, 2004; Oliver et al., 2008). This is most likely due to their abundance in both suburban and rural areas, their ubiquitous distribution throughout the U.S., and the relatively simple methods that can be used for trapping and handling. Opossums share similar geographic ranges and habitats with raccoons, and they are relatively easy to trap and handle, which makes them another popular medium-sized mammal for study.
In the present study, a majority of the raccoons, 52/75 (69.3%), and approximately half of the opossums, 25/52 (48.1%), examined were infested with ticks. The most common tick species collected was *D. variabilis* (59%), and it was the only tick species collected from opossums. This percentage is somewhat lower than previous studies in which *D. variabilis* accounted for 90% of the ticks collected from raccoons and opossums as well as gray squirrels, gray and red foxes, and coyotes (Kollars, 1993). It has been reported that the raccoon is the principal host for *D. variabilis* in Land Between the Lakes in Kentucky (Duobinis-Gray et al., 1993; Kollars, 1993). *Amblyomma americanum* was the second most abundant tick collected (35%) in the current study. Ninety-nine percent of the *A. americanum* recovered were nymphs. Ticks of the *Ixodes* genus accounted for only 6% of the total ticks collected with *Ixodes scapularis* accounting for 5.5%, followed by *I. texanus* (0.4%) and *I. cookei* (0.1%). The present results for *A. americanum* (35%) were very high in comparison to Kollars’ (1993) observation of 0.07% from raccoons and opossums. Results for *I. scapularis* were also high while the percentage of *I. texanus* and *I. cookei* were low as compared to Kollars’ (1993) observations. Kollars (1993) reported that *I. scapularis*, *I. texanus*, and *I. cookei* represented 0.11%, 5.26%, and 2.71% of the ticks collected respectively from raccoons and opossums. Differences in abundance of each tick species may have occurred due to variance in trapping periods and season in which trapping occurred. Another factor that plays a role in the low abundance of *Ixodes* ticks is that these ticks frequently feed on lizards in the southeast (Oliver et al., 1993; Clark et al., 2002). Pung et al. (1994) and Duobinis-Gray et al. (1993) found an abundance of ticks during the months of April through September except with *I. scapularis* which were more numerous during the
cooler months from October to March. This is consistent with our results in which *D. variabilis* and *A. americanum* were collected more commonly from April through October and the *Ixodes* were collected primarily during August, September, and January (See Figures 7 and 8).

A total of 163/642 (25%) tick samples were positive for *B. burgdorferi* by PCR with primers specific for a 307 bp region of the OspA gene. Prevalence of *B. burgdorferi* by tick species was 24.1% (141/577) in *D. variabilis*, 40.6% (13/32) in *A. americanum*, and 27.6% (8/29) in *I. scapularis*. The overall prevalence of positive samples (as well as the prevalence of positive *D. variabilis* ticks) is consistent with results reported in a 1993 study based in western Kentucky in which Duobinis-Gray et al. reported an overall prevalence of 15.6% for *B. burgdorferi* in ticks and 30.5% prevalence in *D. variabilis* ticks removed from *P. leucopus* (unpublished data). In contrast, a study performed by Ouellete et al. (1997) in North Carolina stated that only 1.9% of ticks removed from wild-trapped raccoons were positive for *B. burgdorferi* by IFA. The prevalence of positive *A. americanum* reported herein (35%), is much higher than the 10.3% reported by Duobinis-Gray et al. (1993), but this difference is most likely due to the relative abundance of nymphal *A. americanum* collected during the present study. Clark et al. (2002) reported no spirochete detection from *D. variabilis* or *A. americanum* in South Carolina. The infection rate of *I. scapularis* found in the present study (27%) is higher than reports from other studies performed in the southeast U.S. Sonenshine et al. (1993) reported that 15% of the *I. scapularis* collected in Virginia were positive for *B. burgdorferi* by IFA. Clark (2004) collected *I. scapularis* from small mammals in northern Florida and found 4.6% to be positive by PCR while only 1.3% were positive.
via culture. However, the results observed in this study are consistent with the lower infection rates observed for *I. scapularis* in the southeast as compared to the reported prevalence of 54% in the northeast U.S. (Oliver et al., 1993; Chang et al., 1998; Clark et al., 2002).

*Borrelia burgdorferi* has been detected in raccoons and opossums by hemoculture, serology, IFA, and dark-field microscopy (Fish and Daniels, 1990; Magnarelli et al., 1991; Norris et al., 1996; Ouellette et al., 1997; Clark et al., 2002; LoGiudice et al., 2003). However, Sonenshine et al. (1993) and Yabsley et al. (2003) were unable to detect *B. burgdorferi* in raccoons and opossums using serological or PCR methods. In the present study, 15% (8/53) of the total raccoon blood samples examined by PCR were positive for *B. burgdorferi*. This falls within the range of results found in other studies in the southeast where prevalence ranges from 0-26% in raccoons (Sonenshine et al., 1993; Ouellette et al., 1997; LoGiudice et al., 2003; Yabsley et al., 2003). None of the opossum blood samples were positive for *B. burgdorferi*, which is consistent with previous studies where prevalences were below 2% (Fish and Daniels, 1990; Sonenshine et al., 1993; LoGiudice et al, 2003). The low frequency of *B. burgdorferi* positive opossum blood samples is reportedly due to the ability of opossums to clear infection quickly.

While raccoons are often heavily parasitized by ticks, it is still unclear if they are efficient reservoirs. Previous studies indicate that raccoons may have a limited ability to infect attached ticks (Norris et al., 1996; Ouellette et al., 1997; Clark et al., 2002). However, laboratory studies may not reflect natural conditions in which the raccoons are repeatedly exposed to large numbers of ticks (Norris et al., 1996). In the present study
15% of raccoon blood samples were positive for *B. burgdorferi*, but only two of the positive blood samples corresponded to positive tick samples. However, blood samples were not obtained from 24 of the 75 raccoons trapped in this study. Therefore, reliable conclusions regarding correlations between PCR positive ticks and PCR positive blood samples cannot be reached.

The high percentages of *D. variabilis* and *A. americanum* infected with *B. burgdorferi* raise again the question of whether these ticks are able to transmit Lyme disease. Due to the continually increasing incidence of Lyme disease, additional experiments are urgently needed to investigate the vector competence of *D. variabilis* and *A. americanum* to transmit *B. burgdorferi sensu stricto* and other *B. burgdorferi sensu lato* genospecies that are more common in the southeast U.S. The high prevalence of *B. burgdorferi* infection in ticks common to raccoons and opossums observed in this study, as well as in a tick species that aggressively bites humans in the southeast U.S., creates concern that there are ample opportunities for people to come in contact with not only the infected animals but also the infected ticks. Future studies should also include analyses of questing ticks to determine the prevalence of *B. burgdorferi* in ticks that have fed on different hosts and will potentially attach to a human host. It will also be important to investigate the reservoir competence of raccoons and opossums using an approach that more closely represents natural conditions in which these animals would be repeatedly exposed to infected ticks. In addition, samples in this study were only collected from two counties in south-central Kentucky. Further studies will be required to determine the prevalence of *B. burgdorferi* in other areas of the state.
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