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RESERVOIR COMPETENCE OF NORTH AMERICAN BIRD AND RODENT SPECIES FOR THE LYME DISEASE SPIROCHETE, BORRELIA BURGDORFERI

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To the Graduate Council:

I am submitting herewith a thesis written by DeLacy V. LeBlanc entitled "Reservoir Competence of North American Bird and Rodent Species for the Lyme Disease Spirochete, Borrelia burgdorferi." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with major in Biology.

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RESERVOIR COMPETENCE OF NORTH AMERICAN BIRD AND RODENT SPECIES FOR THE LYME DISEASE SPIROCHETE, *BORRELIA BURGDORFERI*

A Thesis

Presented to the College of Graduate Studies

In Partial Fulfillment of the Requirements for

Master's Degree

DeLacy V. LeBlanc

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Acknowledgments

I wish to sincerely thank several people who have aided me in the completion of my thesis. The amount of gratitude I have for my mentor, Dr. Chad Brooks, is immeasurable. His constant patience and energy motivated and lead me along this path. He made me realize that all of my goals are within my reach. Also, I could never have completed this thesis without the help of my research partner, Jon McMahan. His hard work and perseverance pushed me to work harder. Last, but certainly not least, the love and support from my friends and family have given me the strength and ability to continue to pursue my dreams.

Abstract

Lyme disease, transmitted by the spirochete *Borrelia burgdorferi*, is the most common vector-born disease in North America. *B. burgdorferi* is maintained in the environment through a complex enzootic life cycle involving the deer tick (*Ixodes scapularis*) and animal hosts. Historically, mice have been considered the most common reservoir for *B. burgdorferi* in nature but recent studies have proposed that birds may also play an important reservoir. The objectives of this study were to (i) survey Middle Tennessee for endemicity of *B. burgdorferi*, (ii) elucidate the organ preference of *B. burgdorferi* in wildlife, and (iii) investigate the competence of specific bird species as host reservoirs for *B. burgdorferi*. A total of 172 small mammals and 34 birds were sampled from seven counties in Middle Tennessee for Lyme disease. *Borrelia* infection was identified by two methods, typical microbiological culturing techniques and PCR-based assays. Competency of specific bird species was tested by culturing two different strains of *B. burgdorferi* in 40% bird sera followed by subsequent enumeration of viable spirochete by dark field microscopy. Analysis of the animals showed that 25% (43/172) of all small mammals tested PCR-positive for *B. burgdorferi* and 32% (55/172) of all small mammals tested PCR-positive for non-*B. burgdorferi*, *Borrelia* species. Analysis of the birds surveyed showed that 41% (14/34) of all birds caught tested PCR-positive for *B. burgdorferi*. Additionally, when individual mammal organs were analyzed (i.e., spleen, skin, bladder, heart, and liver), the bladder was shown to be most common and robust

PCR-positive result. However for birds, all organs analyzed (i.e., skin, heart, liver) were equally likely to test PCR-positive. Collectively, the organ data analysis has revealed that multiple tissues were required to accurately identify a *B. burgdorferi* infected animal. The results of the serum sensitivity assays indicated that the sera from certain bird species were lethal to *B. burgdorferi* organisms indicating avian host range is limited to particular species. In conclusion, this study has shown substantial *B. burgdorferi* infectivity among mammals and birds in Middle Tennessee. Additionally, this study suggests that future epidemiological surveys for *B. burgdorferi* should require more invasive molecular approaches other then ear-punch biopsies. Finally, this study provides the groundwork and reagents for future investigations regarding the molecular mechanisms responsible for the animal host range for *B. burgdorferi* in nature.

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Introduction

Lyme disease is transmitted by the pathogenic spirochete *Borrelia burgdorferi* and is the most commonly reported arthropod-borne disease in North America, Europe, and Asia (6, 18, 21). Lyme disease is a debilitating multi-symptom disease which can chronically infect patients for decades, having disease manifestations that include arthritis, carditis, and neuritis (20, 22). *B. burgdorferi* is maintained in nature by a complex enzootic life cycle involving the horizontal transmission of bacteria between *Ixodes* ticks and animals (14). Humans only become infected when they encroach into habitat infested with *Ixodes* ticks (i.e., potential carriers of Lyme disease) as a corollary of urban sprawl or outdoor recreation activities. Identification of Lyme disease is routinely diagnosed by the appearance of an erythema migrans rash or more commonly referred to as a "Bull's eye rash" (11). Once infected, an untreated person never develops high bacteremia anywhere within the body. In this regard, humans are considered accidental, dead-end hosts for *B. burgdorferi* infections as it is unlikely that sufficient bacteremia are present in surface dermal tissues for a naïve tick to acquire a *B. burgdorferi* infection. Prompt diagnosis results in the administration of appropriate antibiotics which results in alleviation of Lyme disease manifestations and clearance of infection (11). However, unfortunately, it is unclear as to why a very minute portion of Lyme disease patients continue to suffer from Lyme disease sequelae even after extensive therapy. Therefore, due to the high infection frequency of this arthropod-borne disease and the prospect that some Lyme disease cases can not be cured, Lyme disease is considered an

important human disease by the National Institutes of Health and a required reportable agent to the Centers for Disease Control and Prevention.

Although not officially recognized as a reportable disease in the United States until 1975, some symptoms of Lyme disease were described much earlier. In fact, the rash commonly associated with the onset of the disease, originally termed erythema migrans (EM), was first described by Arvid Afzelius in 1909 (4). In Europe, several medical professionals made notable observations concerning Lyme disease infections. For example, in 1922, Drs. Garin and Bujadoux described the first report of neurological complications that resulted from a tick bite (12). However, at the time, no one had yet linked the various symptoms to just one disease. In 1975, Dr. Allen Steere, a Yale medical professional, diagnosed a group of unrelated children in Lyme, Connecticut showing very similar arthritic symptoms. The arthritic disease was named "Lyme" disease to reflect the home town of the afflicted individuals (24). The causative agent of Lyme disease remained a mystery until 1982 when researchers Willy Burgdorfer and Jorge Benach serendipitously discovered a new spirochetal bacterium within the midguts of deer ticks (*Ixodes scapularis*) while surveying various tick species for rickettsial pathogens. The researchers noticed that there was an unusually high correlation between the frequencies of *Ixodes* ticks in areas where Lyme disease was commonly diagnosed (5). Soon after, it was determined that patients diagnosed with Lyme disease had antibodies specific for the new spirochete, confirming that it was the cause of the disease. The spirochete was named

Borrelia burgdorferi in honor of Willy Burgdorfer because of his significant contribution to the discovery of the bacterium.

B. burgdorferi is perpetuated in the environment by a complex enzootic life cycle involving *Ixodes* ticks and animals (14). *Ixodes scapularis*, commonly known as the deer tick, is the American tick vector of *B. burgdorferi* and has a two year life cycle with three distinct morphological stages, each separated by a single blood meal (14, 23). Since there is no transovarial transmission of *B. burgdorferi*, naïve ticks can only acquire *B. burgdorferi* infection by feeding on infected animals and as such, only the nymph and adult stages have the capacity to infect other animals (1, 14). It is generally considered that due to its small size and the time of year, the nymphal stage is the most common tick stage which transmits *B. burgdorferi* infection to humans. Currently, mice are recognized as the major reservoir of the spirochete in nature, but recent research indicates that other animals, such as birds, may also serve as very important environmental reservoirs for *B. burgdorferi*.

In Europe, recent evidence points to different animal reservoirs which may serve as "preferred" animal hosts for distinct strains of *Borrelia*. This notion is best illustrated when comparing *B. burgdorferi* and *B. garinii*, two Lyme disease agents found in Europe. Several serological experiments by different laboratories of Marconi, Stevenson, Kraiczy, and Wallich have shown a differential susceptibility to serum-mediated killing between *B. burgdorferi* and *B.*

garinii which was dependent on using sera from different animal species. In particular, *B. garinii* organisms are killed within a few minutes in the presence of mouse, dog, horse, and human serum while *B. burgdorferi* appears unharmed. In contrast, *B. garinii* survives well in the presence of pheasant (bird) serum while *B. burgdorferi* suffers significant cell death, albeit, not total (2). However, it is important to note that only a few European ecological surveys have been performed and they indicate that *B. burgdorferi* does, in fact, infect several different bird species. Regardless, these data indicate that significant genomic disparities may exist between *B. burgdorferi* and *B. garinii*. Fortunately, both *B. burgdorferi* and *B. garinii* genomes are completely sequenced. In a recent study by Brooks and co-workers, comparative analysis of their genomes resulted in the identification of an important *B. burgdorferi* surface protein, identified as CRASP-1, which is capable of protecting *B. garinii* in *trans* from challenge with human serum (3). Additionally, mutant *B. burgdorferi* lacking *CRASP-1* expression are killed by human serum in less than one hour. However, intriguingly, this does not reduce the virulence or infectivity of the mutant in mice indicating that other important borrelial molecule(s) are involved (Brooks, unpublished data). This may be consistent with observations of other investigators regarding the large animal host range *Borrelia burgdorferi* infects. By identifying new animal reservoirs, we may gain significant insight to unique parasitic strategies and thereby, gain greater control of Lyme disease.

Due to the lack of marketable Lyme disease vaccines, research into the destruction of tick habitat has become important strategy in the prevention of Lyme disease. One aspect of the habitat supporting Lyme disease is the animal hosts that serve as reservoirs for *Ixodes* ticks to perpetuate *B. burgdorferi*. In this regard, mice have been widely accepted as the most common reservoir host for *B. burgdorferi* in the environment, however, in the last two decades, research into other potential reservoirs has shown that birds may also be important in the propagation of the bacterium. This casts significant doubt as to the efficacy of controlling Lyme disease infections at the environmental/animal host level.

Several European and Asian studies have surveyed different potential animal reservoirs for different borrelial species. A Slovakian study found that birds serve as reservoir hosts for *B. garinii* and *B. valaisiana* but not *B. afzelli* (9). Another study, albeit related, suggests that the uptake of avian blood by the tick kills *B. afzelli* (13). Other research examined a large variety of different avian species for *B. burgdorferi* infection resulting in the identification of many species from the order Passeriformes to be competent reservoir hosts (15, 17). Unfortunately, fewer studies testing this notion have been performed in North America. The competence of only a few select species of birds captured off the coast of New York was tested and showed moderate levels of competence for *B. burgdorferi* infection that varied dependent on the bird species tested (8). In a different study, Nevada birds in the yellow pine transition habitat might contribute to the spread of *B. burgdorferi* while rodents in the area did not seem to be

significant contributors (25). Canadian researchers tested many species of passerines and determined that ground-dwelling birds possibly play a role in dispersal of the ticks that carry the Lyme disease spirochete (16). Therefore, along these lines, these studies have suggested that host birds may carry infected ticks long distances, dispersing the ticks along their migratory pathways. Supporting this notion, Japanese research has shown that *B. garinii* may have been introduced to the nation via migratory birds from China (10). Understanding the role birds play in the propagation of *B. burgdorferi* in the environment is important to the study and development of potential strategies to combat the spread of Lyme disease.

In this study selected birds and rodents will be screened for *B. burgdorferi* infection to determine the reservoir competence of selected North American animal species. Knowing the "preferred" animal reservoirs of *B. burgdorferi* could provide further insight into the reasons spirochetes are able to live in certain hosts and not in others. The objectives of this study are to (i) catch specific local birds and rodents to screen for infection, (ii) investigate the competence of specific bird species as host reservoirs for *B. burgdorferi*, and (iii) elucidate the organ preference for *B. burgdorferi* in animals. This study hypothesized that North American birds serve as a natural reservoir host for *B. burgdorferi* in the environment.

Methods and Materials

Animal collection. Federal and State permits for animal collection were obtained before field work was begun. The field work in this study involved collecting birds and small rodents. The following counties in Tennessee were surveyed: Montgomery, Lincoln, Robertson, Stewart, Houston, Dickson, and Williamson. Sherman traps and snap traps were set up in fields, along fence rows, and various buildings (e.g., barns) to collect small animals such as mice and voles. Species caught included white-footed mice (*Peromyscus leucopus*), Eastern harvest mice (*Reithrodontomys humulis*), and prairie voles (*Microtus ochrogaster*). The traps were baited with a mixture of oatmeal and peanut butter and were checked every day. Birds were shot in the field with either a 0.177 caliber pellet rife with a 4.5x scope or a 12 gauge shotgun. Based on prior studies, species from the Order Passeriformese were preferentially collected. All animals were captured in the field and brought back to the lab for tissue collection. These tissues include the blood, bladder, spleen, heart, and dermis. The dead animals and extracted tissues were sealed in plastic bags or 1.5 ml eppendorf tubes and kept on ice until placed into a -80°C freezer on APSU campus. Each animal was tagged with tape which listed the animal's trap number, animal number, and date of capture. All collected animal carcasses will be disposed of as appropriate animal waste and incinerated according to the Tennessee Department of Health guidelines.

Bacterial cultivation. Approximately 25% of each excised tissue will be placed into sterile BSKH media (Sigma-Aldrich Chemical Company, St. Louis, MO) supplemented with 6% heat-inactivated rabbit serum and containing 50 µg/ml of rifampin and 25 µg/ml of amphotericin B (Sigma). Cultures will be checked daily for positive growth by dark field microscopy.

DNA isolation from animal tissues. Approximately 0.5 g of each extracted tissue were manually diced and digested by 1mg/ml collagenase (Sigma-Aldrich Chemical Company, St. Louis, MO) in phosphate buffered saline (PBS) for 4 h at 37°C and Pronase for 16h at 37°C. After incubation, the digested tissue sample were triturated to disrupt cellular matrixes and an equal volume of a 1:1 ratio of phenol:chloroform was added. The sample was vortexed thoroughly for 10 seconds and allowed to set at room temperature for approximately 5 minutes. The sample was then centrifuged at 14,000 x g for 5 minutes and the top, aqueous layer was moved to a new tube. An equal amount of phenol:chloroform was added to the tube followed by vortexing the sample for 10 seconds and centrifugation as described above. This step was repeated twice until the sample appeared free of protein contamination. Once the sample appears clear of protein contamination, 5 volumes of ethanol (95%-100%) or two volumes of isopropanol and 100 µl of 3M sodium acetate were added to the sample for DNA precipitation. The samples were allowed to sit at -80°C for 5 minutes and then centrifuged at 14,000 x g for 10 minutes. The resulting DNA pellet was washed

once in 80% ethanol and subsequently centrifuged at 14,000 x g for 5 minutes. The DNA pellet was air dried and resuspended in 50μl of molecular grade water.

Identification of *B. burgdorferi* infected animal tissues. Two primers were used for the identification of *B. burgdorferi* DNA in the samples. First a nonspecific *Borrelia* primer, Flab, was used. This primer recognizes the genes in all Borrelia spp. that codes for the flagella. The second primer, TEC1/LD2, is specific for *B. burgdorferi* DNA (7). DNA concentrations representing approximately 50% of each tissue, but no more than 4 μg served as the template for each PCR. PCR will be carried out in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μM each of the four dNTP, 1.25 units of Taq DNA polymerase and 25 pmol of each primer. Amplification will be carried out for 35 cycles at temperatures appropriate for the primers employed. The success of the PCR reactions will be verified by gel electrophoresis of the infected animal tissue amplicons and *B. burgdorferi* control DNA through a 0.8 % agarose gel in 1 x TAE buffer. Preliminary PCR on each animal caught was preformed by pooling 1μl of DNA from each organ from the mouse for the template DNA and using the two primer sets in duplex. Animals positive in the preliminary PCR were subsequently tested again with each organ being tested individually and with each primer set in a monoplex.

Host competency testing. Extracted blood will be centrifuged at 10,000 x g for 10 minutes to separate the cellular fraction from the serum. Incubation with

40% serum was used to access the bactericidal activity of each collected animal's serum on viable *B. burgdorferi*, *in vitro*. After 1, 4, and 16 h, bacterial concentrations were enumerated to determine serum-dependent cell death. Two strains of B. burgdorferi were used. Strain B31MI contains the full plasmid repertoire of the bacteria and strain B31cF contains approximately half of the plasmids found in the bacteria. The two different strains were used in order to determine if any of the plasmids missing from B31cF are necessary for host serum resistance. Controls were set up using serum that was heat-inactivated by boiling.

Results

Trapping took place from March until September 2006. The sample sites consisted of eleven sites in seven counties in Middle Tennessee (Figure 1). The sites are as follows: A farm in Roberson County served as the first sampled site and was sampled from March 19 through May 1. From this site, 61 small mammals were collected. Sampling in Montgomery County began towards the end of sampling in Robertson County, occurring from April 28 until May 24. A different part of this county was sampled again on July 25, overlapping with Stewart County sampling. Twenty-one animals were caught from the sites in Montgomery County. Stewart County was sampled between June 27 and August 5, in two different locations. From Stewart County, 21 small mammals were trapped and six birds comprising five different species were collected. Williamson County was sampled from June 13 though June 24 with 14 animals and 15 birds representing eight different species were collected. Dickson County was sampled along with Montgomery County from May 3 to June 10 with 31 animals being trapped. Lincoln County was sampled very briefly from May 26 to May 28. Because of the distance from APSU, Lincoln County could not be sampled multiple times and only two animals were trapped. Houston County was

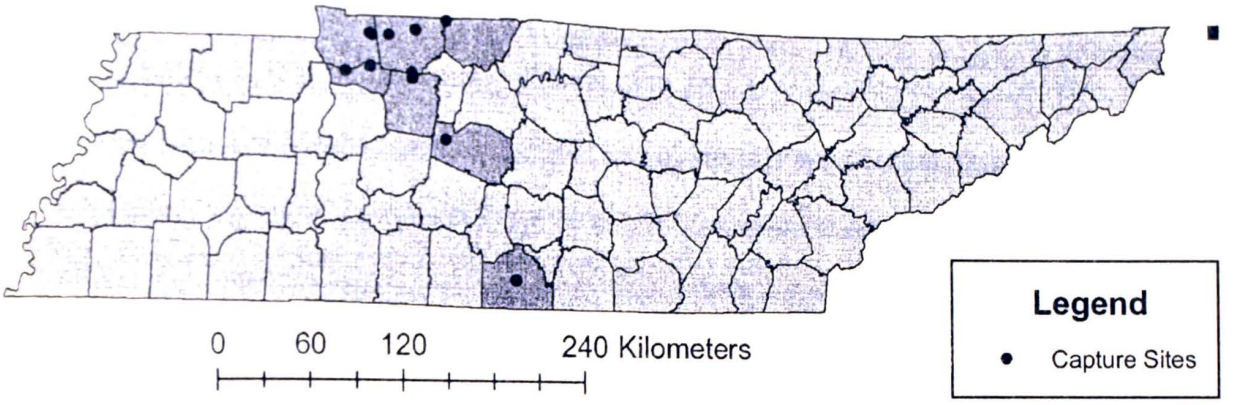

Figure 1. Tennessee map indicating counties and sites for animal collection.

sampled from August 8 to September 20. A total of 22 small mammals were captured.

In Robertson County, three species of small mammals were collected: white-footed mice (*Peromyscus leucopus*), prairie voles (*Microtus ochrogaster*), and Eastern harvest mice (*Reithrodontomys humulis*). PCR data from the small mammals in Robertson County showed that 34% (14/41) of white-footed mice, 56% (9/16) of prairie voles, and 25% (1/4) of the Eastern harvest mice were found to harbor *B. burgdorferi* (Table 1). This resulted in 39% (24/61) of all Robertson County mammals analyzed found PCR-positive for *B. burgdorferi*. Additionally, 4 animals were PCR-positive for only the FlaB primer set indicating infection with some type of *Borrelia* species. Direct culture of the collected mammalian tissues for *B. burgdorferi* showed spirochetes in 12% (5/41) of the white-footed mice and 0% in both Eastern harvest mice (0/4) and prairie voles (0/16) (Table 3).

There were three different sample sites in Montgomery County for small mammal collections, with white-footed mice being the only species caught. One location was a small farm, one was the Austin Peay State University farm, and the other was public land by the Cumberland River. PCR analysis showed that 24% (5/21) of these white-footed mice were infected with some *Borrelia* species, with 14% (3/21) being infected with infected with *B. burgdorferi* specifically (Table 1). Interestingly, spirochetes were observed in 50% (9/18) of the cultured samples (Table 3). In addition to the mice, eight different species of birds were sampled from two different sites in this county. PCR analysis determined that

Table 1. Table of Infectivity for Small Mammals

County	Species	Common Name	Total Sampled	FlaB[a] Primer set	TEC1/LD2[b] Primer set	Both[c]
Robertson	*Peromyscus leucopus*	White-footed Mouse	41	13	14	12
	Microtus ochrogaster	Prairie Vole	16	13	9	10
	Reithrodontomys humulis	Eastern Harvest Mouse	4	0	1	0
Dickson	*Peromyscus leucopus*	White-footed Mouse	29	4	2	0
	Microtus ochrogaster	Prairie Vole	2	1	1	1
Stewart	*Peromyscus leucopus*	White-footed Mouse	21	9	3	3
Williamson	*Peromyscus leucopus*	White-footed Mouse	14	4	3	3
Montgomery	*Peromyscus leucopus*	White-footed Mouse	21	5	3	3
Houston	*Peromyscus leucopus*	White-footed Mouse	16	4	4	1
	Microtus ochrogaster	Prairie Vole	1	1	1	1
	Reithrodontomys humulis	Eastern Harvest Mouse	5	0	1	0
Lincoln	*Peromyscus leucopus*	White-footed Mouse	2	2	1	1

a. Total number of small mammals that testes positive for the FlaB primer set.

b. Total number of small mammals that tested positive for the TEC1/LD2 primer set.

c. Total number of small mammals that tested positive for both FlaB and TEC1/LD2 primer sets.

Table 2. Table of Infectivity for Bird Species

County	Species	Common Name	Total Sampled	FlaB[a] Primer set	TEC1/LD2 Primer set	Both[c]
Montgomery	*Seiurus aurocapillus*	Ovenbird	1	0	0	0
	Mimus polyglottos	Northern Mockingbird	2	1	1	1
	Cardinalis cardinalis	Northern Cardinal	1	1	1	1
	Sturnus vulgaris	European Starling	2	0	0	0
	Baeolophus bicolor	Tufted Titmouse	4	2	2	2
	Poecile carolinensis	Carolina Chickadee	1	0	0	0
	Sialia sialis	Eastern Bluebird	1	0	0	0
	Cyanocitta cristata	Blue Jay	1	0	0	0
Williamson	*Turdus migratorius*	American Robin	5	2	2	2
	Saynoris phoebe	Eastern Phoebe	1	1	1	1
	Sialia sialis	Eastern Bluebird	1	1	1	1
	Passerella iliaca	Fox Sparrow	1	0	0	0
	Cardinalis cardinalis	Northern Cardinal	2	1	1	1
	Cyanocitta cristata	Blue Jay	2	0	0	0
	Poecile carolinensis	Carolina Chickadee	2	2	2	2
	Thryothorus ludovicianus	Carolina Wren	1	1	1	1
Stewart	*Cardinalis cardinalis*	Northern Cardinal	1	0	0	0
	Carduelis tristis	American Goldfinch	1	0	1	0
	Spizella passerina	Chipping Sparrow	1	1	1	1
	Saynoris phoebe	Eastern Phoebe	2	0	0	0
	Zenaida macroura	Mourning Dove	1	0	0	0

a. Total number of each bird species that tested positive for the FlaB primer set.

b. Total number of each bird species that tested positive for the TEC1/LD2 primer set.

c. Total number of each bird species that tested positive for both the FlaB and TEC1/LD2 primer sets.

Table 3. . Table of Culture Analysis

County	Species	Common Name	Total Sampled	Total Tested [a]	Culture Positive [b]
Robertson	*Peromyscus leucopus*	White-footed Mouse	41	41	5
	Microtus ochrogaster	Prairie Vole	16	16	0
	Reithrodontomys humulis	Eastern Harvest Mouse	4	4	0
Dickson	*Peromyscus leucopus*	White-footed Mouse	29	26	8
	Microtus ochrogaster	Prairie Vole	2	2	0
Stewart	*Peromyscus leucopus*	White-footed Mouse	21	0	0
Williamson	*Peromyscus leucopus*	White-footed Mouse	14	0	0
Montgomery	*Peromyscus leucopus*	White-footed Mouse	21	18	9
Houston	*Peromyscus leucopus*	White-footed Mouse	16	0	0
	Microtus ochrogaster	Prairie Vole	1	0	0
	Reithrodontomys humulis	Eastern Harvest Mouse	5	0	0
Lincoln	*Peromyscus leucopus*	White-footed Mouse	2	2	1

a. Total number of each species of animal which were analyzed for infection by culturing spirochetes from each organ in BSK-II media.

b. Total number of each species of animal in which spirochetes were observed.

31% (4/13) of the birds sampled from Montgomery County were infected with *B. burgdorferi* (Table 2). No culture data were obtained for the birds.

In Stewart County, two different sites were sampled for small mammals. Both sites were wood-edge habitats near homes. White-footed mice were the only mammals caught. PCR analysis determined that 43% (9/21) of the mice were infected with some *Borrelia* species with 14% (3/21) were infected with specifically *B. burgdorferi* (Table 1). Birds were sampled from one site in this county with 13% (2/6) found PCR-positive for *B. burgdorferi* (Table 2). No culture data were obtained for this county.

Dickson County was sampled from two different sites, in which white-footed mice and prairie voles were trapped. Of the white-footed mice, 14% (4/29) were identified infected with a *Borrelia* and with 7% (2/29) being infected specifically with *B. burgdorferi* using PCR (Table 1). PCR analysis of the voles showed that 50% (1/2) were specifically infected with B. *burgdorferi*. Culture analysis of these the white-footed mice showed 31% (8/26) of the animals were infected with spirochetes (Table 3).

A single site in Williamson County was sample, with only white-footed mice being caught. The sample site was a large open field beside a river. At this location, 28% (3/14) of the mice tested PCR-positive for *Borrelia* and 21% (4/14) tested positive for *B. burgdorferi* (Table 1). Also at this location, eight different species of birds were caught (Table 2). Of these birds, 53% (8/15) tested PCR-positive for *B. burgdorferi*. No culture data was obtained for this county.

Houston County was sampled at two different sites, one of which was a business producing food for animals and the other was an old house used for storage. Three types of small mammals were caught, white-footed mice, prairie voles, and Eastern harvest mice. PCR analysis showed that 25% (4/16) of the white-footed mice, 20% (1/5) of the Eastern harvest mice, and 100% (1/1) prairie voles were infected with *B. burgdorferi* (Table 1). Birds were not sampled from this county. Additionally, *Borrelia* culture techniques were not preformed on tissues collected from Houston County.

Of the two animals analyzed from Lincoln County, both came back positive; one was positive for some type of *Borrelia* and the other was PCR-positive specifically for *B. burgdorferi* (Table 1). Tissue culturing analysis revealed that spirochetes were in one of the mice from Lincoln County (Table 3).

Individual organ data were analyzed from all animals that were initially found PCR-positive during preliminary testing (Figures 2 -5). The organs were individually screened for *Borrelia* infection using both primer sets, FlaB and TEC1/LD2, in order to elucidate the organ preference for infecting spirochetes. Analysis of the all mammal species showed that the bladder was the organ that came up positive the most frequently with both FlaB and TEC1/LD2 primer sets (Figure 2 and 3). Additionally, individual organs were analyzed from the preliminarily positive birds (Figures 4 and 5). Of these tissues, the skin was found to be the most frequently infected organ for flab and TEC1/LD2.

Data analysis regarding gender revealed that the number of infected animals was not significantly different between males and females for infection

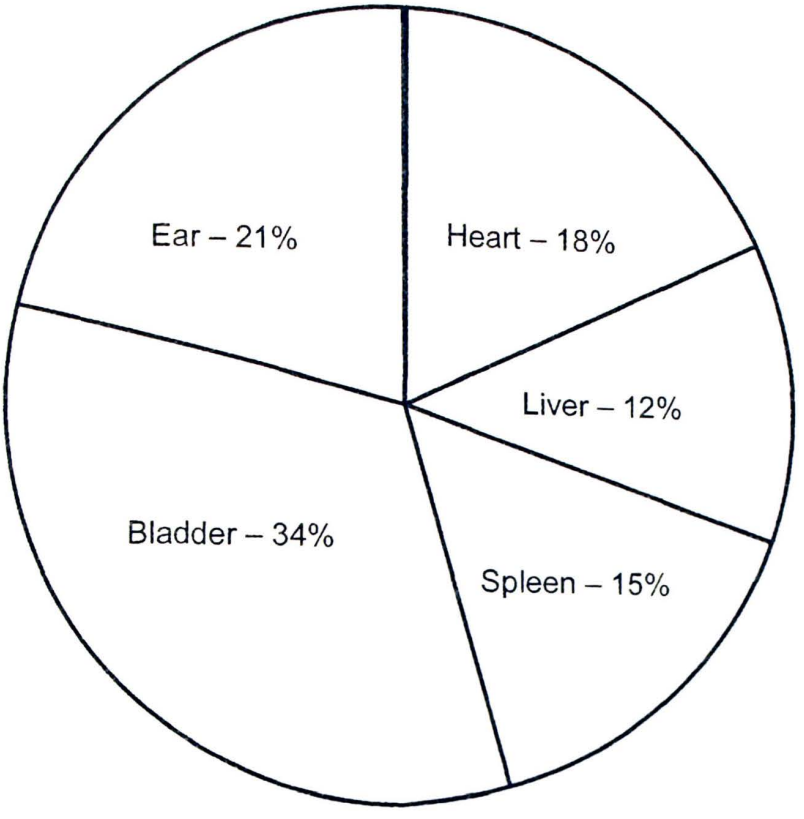

Figure 2. Small Mammal Individual Organ Analysis for FlaB

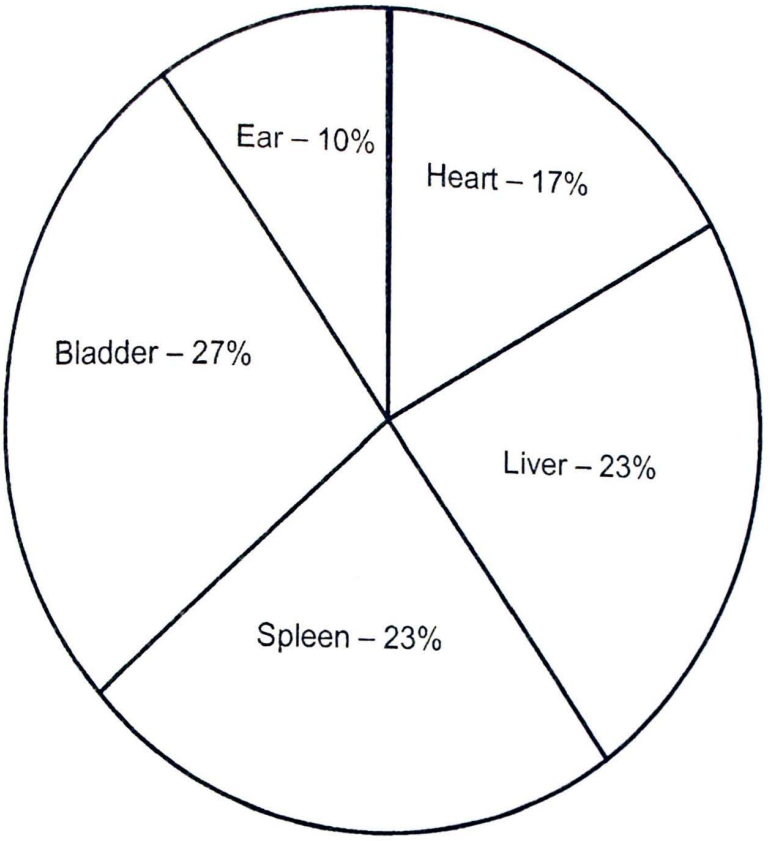

Figure 3. Small Mammal Individual Organ Analysis for TEC1/LD2 (*B. burgdorferi* specific primers).

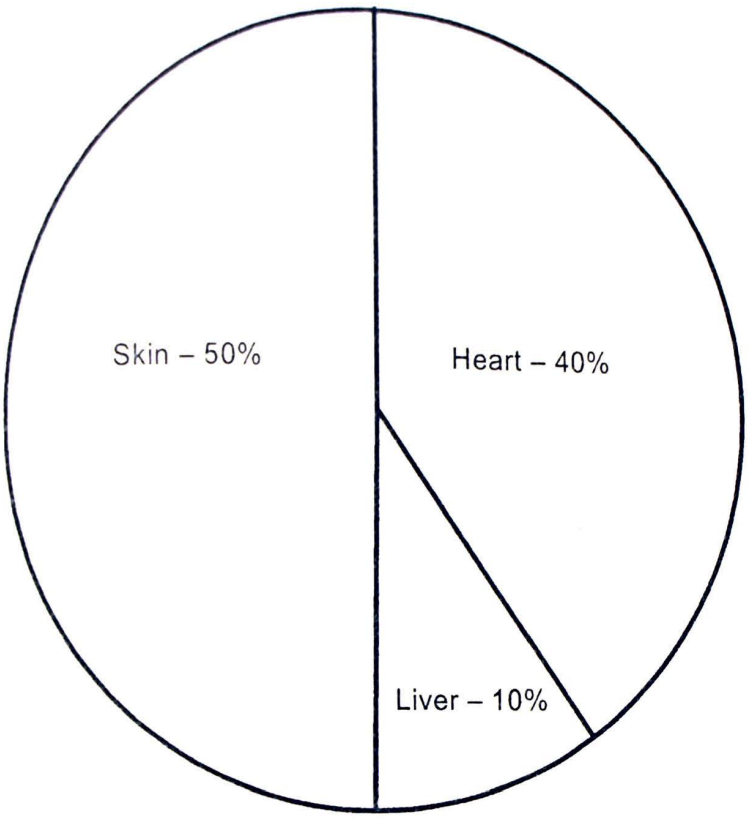

Figure 4. Individual Bird Organ Data for FlaB (*Borrelia* genus general primers).

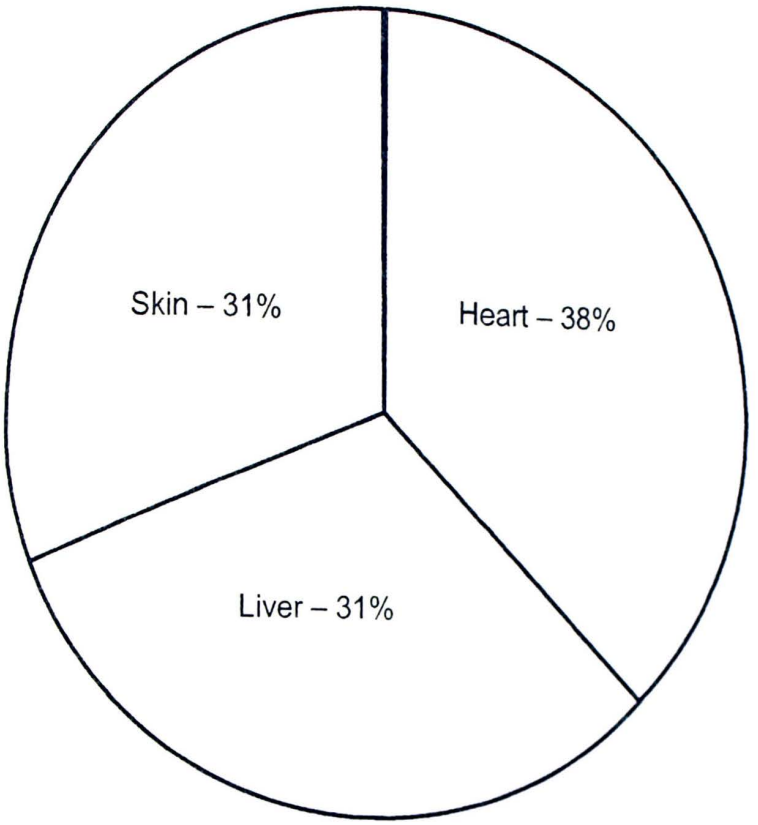

Figure 5. Individual Bird Organ Data for TEC1/LD2 (*B. burgdorferi* specific primers)

with any *Borrelia* species (p=0.8) including *B. burgdorferi* specifically (p=0.9) (Tables 4 and 5). The data was analyzed using a two-tailed, unpaired t-test.

Host competency was tested using sera from four bird species: American Robin (*Turdus migratorius*), European Starling (*Sturnus vulgaris*), Blue Jay (*Cyanocitta cristata*), and Mourning Dove (*Zenaida macroura*). Analysis of the American Robin and European Starling showed nearly identical results with no detectable B31cF viability after one hour of incubation (Figures 6 and 7). However, detectable viability was observed after 16 h. This indicated that either the presence of a subpopulation of B31cF was able to survive the sera or more probable, a sub-lethal amount of sera was used. Regardless, B31cF apparently lacks the necessary plasmid content which allows survival in Robin and European Starlings.

Due to limited resources, only Mourning Doves and Blue Jays were tested with both B31MI and B31cF strains of *B. burgdorferi.* Both strains of *B. burgdorferi* were cultivated in the presence of 40% Mourning Dove serum (Figure 8). Although agglutination was observed by dark field microscopy, the bird was shown to be PCR-negative for any type of *Borrelia* infection (data not shown). Therefore, it could be conclude that either the bird was never i) the bird was infected and we failed to detect it, ii) the bird was never infected with *B. burgdorferi* or iii) the bird has been infected and has since cleared the infection but specific anti-*B. burgdorferi* antibody remains. Additionally, the viability for both *B. burgdorferi* strains decreased over time (Figure 8). Similarly, *B. burgdorferi* strains showed significant cell death in the presence of 40% Blue Jay

Table 4. Table of Infectivity for Male Small Mammals

County	Species	Common Name	Total Sampled	Total Male	FlaB[a] Primer set	TEC1/LD2[b] Primer set	Both[c]
Robertson	*Peromyscus leucopus*	White-footed Mouse	41	19	5	5	5
	Microtus ochrogaster	Prairie Vole	16	3	3	3	3
	Reithrodontomys humulis	Eastern Harvest Mouse	4	1	0	0	0
Dickson	*Peromyscus leucopus*	White-footed Mouse	29	13	1	0	0
	Microtus ochrogaster	Prairie Vole	2	1	1	1	1
Stewart	*Peromyscus leucopus*	White-footed Mouse	21	15	5	1	1
Williamson	*Peromyscus leucopus*	White-footed Mouse	14	11	3	2	2
Montgomery	*Peromyscus leucopus*	White-footed Mouse	21	15	4	3	3
Houston	*Peromyscus leucopus*	White-footed Mouse	16	10	3	1	1
	Microtus ochrogaster	Prairie Vole	1	0	0	0	0
	Reithrodontomys humulis	Eastern Harvest Mouse	5	3	1	1	1
Lincoln	*Peromyscus leucopus*	White-footed Mouse	2	1	1	1	1

a. Total number of males from each species that tested positive for the FlaB primer set.

b. Total number of males from each species that tested positive for the TEC1/LD2 primer set.

c. Total number of males from each species that tested positive for both primer sets.

Table 5. Table of Infectivity for Female Small Mammals

County	Species	Common Name	Total Sampled	Total Female	FlaB[a] Primer set	TEC1/LD2[b] Primer set	Both[c]
Robertson	*Peromyscus leucopus*	White-footed Mouse	41	21	8	9	8
	Microtus ochrogaster	Prairie Vole	16	13	10	6	6
	Reithrodontomys humulis	Eastern Harvest Mouse	4	3	0	1	0
Dickson	*Peromyscus leucopus*	White-footed Mouse	29	16	3	2	0
	Microtus ochrogaster	Prairie Vole	2	1	0	0	0
Stewart	*Peromyscus leucopus*	White-footed Mouse	21	6	4	2	2
Williamson	*Peromyscus leucopus*	White-footed Mouse	14	3	1	1	1
Montgomery	*Peromyscus leucopus*	White-footed Mouse	21	6	1	0	0
Houston	*Peromyscus leucopus*	White-footed Mouse	16	7	0	2	0
	Microtus ochrogaster	Prairie Vole	1	1	1	0	0
	Reithrodontomys humulis	Eastern Harvest Mouse	5	2	0	2	0
Lincoln	*Peromyscus leucopus*	White-footed Mouse	2	1	1	0	0

a. Total number of females from each species that tested positive for the FlaB primer set.

b Total number of females from each species that tested positive for the TEC1/LD2 primer set.

c. Total number of females of each species that tested positive for both primer sets.

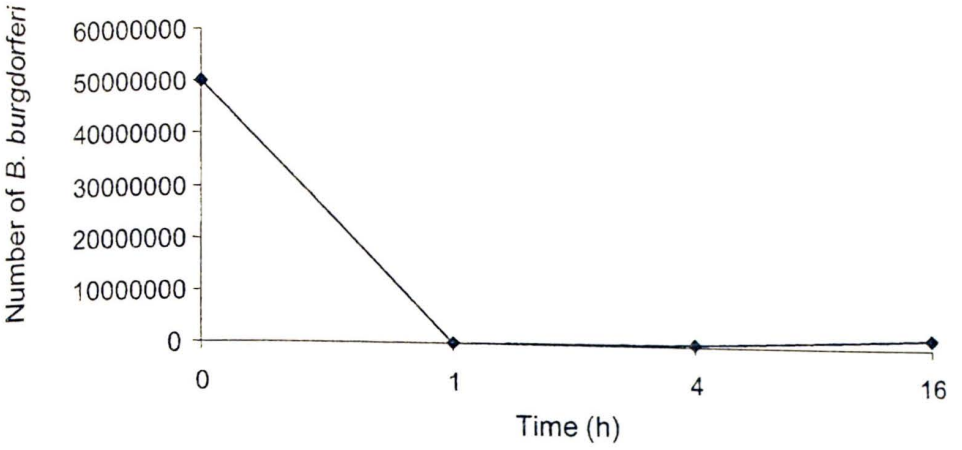

Figure 6. Serum Sensitivity Assay with European Starling Sera. B31cF organisms were incubated with 40% European Starling sera and the bacterial viability was enumerated by dark field microscopy at 1, 4 and 16 h.

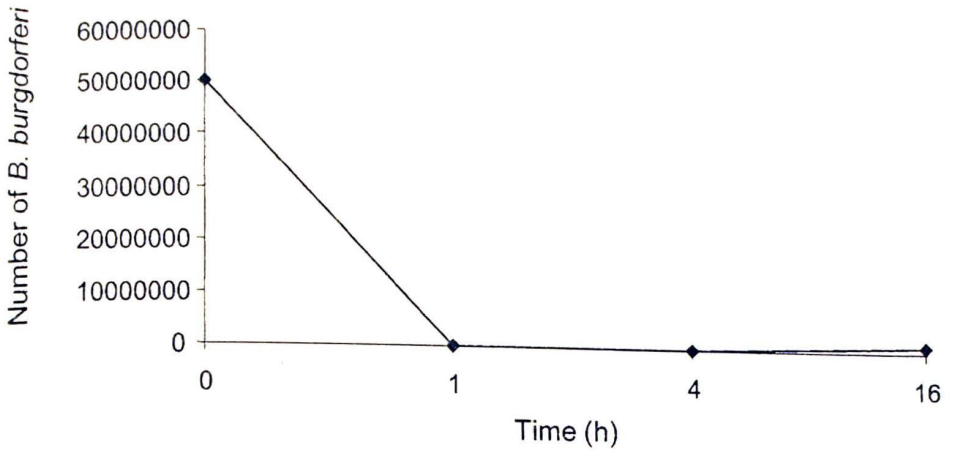

Figure 7. . Serum Sensitivity Assay with American Robin Serum. B31cF organisms were incubated with 40% American Robin sera and the bacterial viability was enumerated by dark field microscopy at 1, 4 and 16 h.

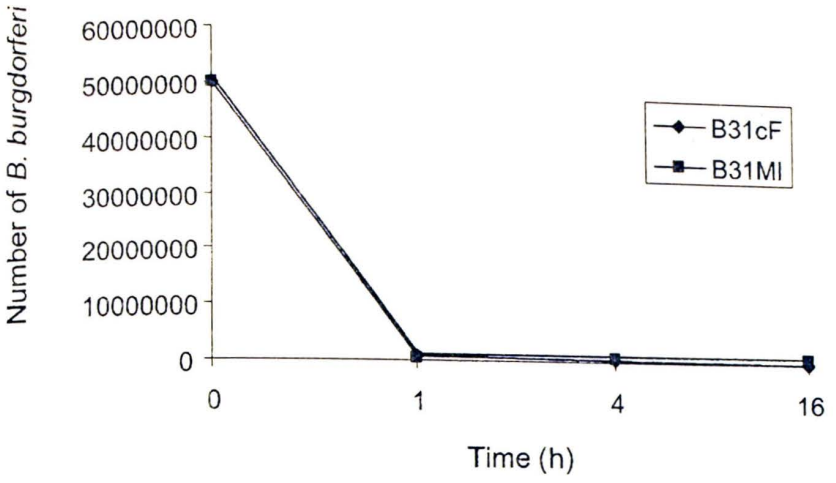

Figure 8. Serum Sensitivity Assay with Mourning Dove Serum. B31cF and B31MI organisms were incubated with 40% Mourning Dove sera and the bacterial viability was enumerated by dark field microscopy at 1, 4 and 16 h.

serum (Figure 9). However, *B. burgdorferi* viability decreased more rapidly for B31MI. These results indicated that neither Mourning Doves (with the exceptions mentioned above) nor Blue Jays may be competent hosts for *B. burgdorferi*.

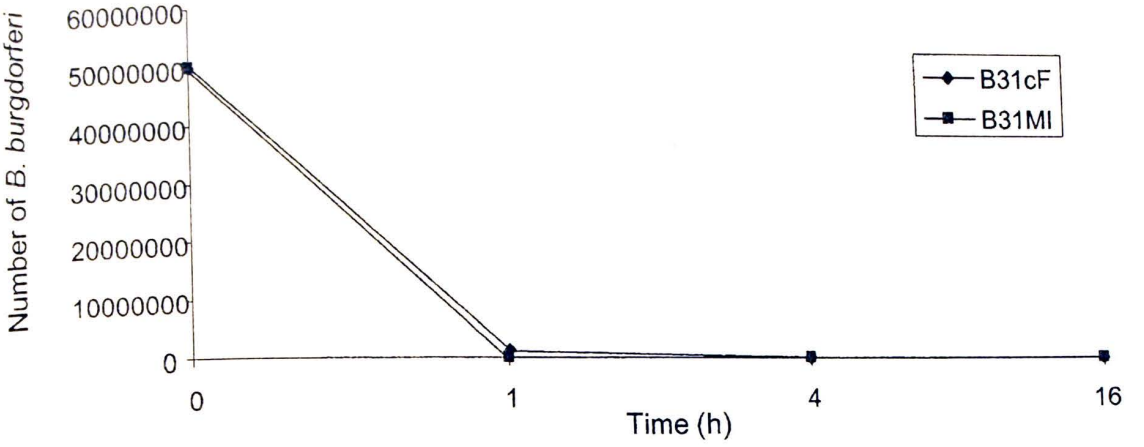

Figure 9. Serum Sensitivity Assay with Blue Jay Serum. B31cF and B31MI organisms were incubated with 40% Blue Jay sera and the bacterial viability was enumerated by dark field microscopy at 1, 4 and 16 h.

Discussion

Over the course of the study, 172 small mammals and 34 birds were collected from seven counties in Middle Tennessee. Of the small mammals, 25% (43/172) tested PCR-positive for *B. burgdorferi* infection. This clearly refutes the common belief held by many medical professionals locally that Lyme disease simply does not exist in Middle Tennessee. For this reason and others, Lyme disease often goes undiagnosed or misdiagnosed in this area. It is important to identify the true ecological range of *B. burgdorferi* across the United States in order to fully understand and estimate the risk associated with different areas for Lyme disease infections. It was also interesting to note that 32% (55/172) of all small mammals tested were PCR-positive for some type of spirochete in the *Borrelia* genus. This indicated that more research needs to be performed into other possible *Borrelia* species infecting Middle Tennessee wildlife as they may be agents of disease.

In regards to risk assessment, it was interesting to observe that 39% (24/61) of the mammals collected from Robertson County were found to harbor *B. burgdorferi*. Robertson County proved to have the highest incidence of *B. burgdorferi* infection among the wildlife analyzed (Table 1). Houston County ranked second in risk assessment with 27% (6/22) of the mammals identified as PCR-positive. Excluding Lincoln County since only two animals were analyzed, the counties examined showed animal infectivity rates of 10% to 39% for all mammals analyzed. This information dramatically underscores the importance

for a heightened awareness of Lyme disease risk to the medical community and for local Middle Tennessee residents to be mindful of Lyme disease risk in their area.

As evidence to the difficult nature of *in vitro B. burgdorferi* cultivation from wild animals, very few animal tissues resulted in positive spirochete growth as viewed by dark field microscopy (Table 3). Most common was for the animal tissue sample to become contaminated with other bacteria which precluded visualization of any spirochetes. However, organs from four counties were successfully analyzed by *in vitro* cultivation in BSK-II media. Spirochetes were observed in 26% (23/87) of all culture samples surveyed. This alternative and distinctly different approach supports the more substantial PCR evidence.

Three counties in Middle Tennessee were sampled for birds. A total of 34 birds were caught representing 15 different avian species. All species caught were locally abundant species and several foraged the ground for food, making them likely targets for *B. burgdorferi* infected ticks. In total, 41% (14/34) were PCR-positive for specifically *B. burgdorferi*, indicating that birds may serve as an important reservoir host for *B. burgdorferi* in Middle Tennessee. Several bird species showed higher levels of endemic infectivity, such as the Northern Cardinal, Carolina Wren, and Tufted Titmouse. All of these bird species belong to the Order Passeriformeses and typically utilize ground foraging behaviors to

find food which, in theory, would be consistent with probable contact with *B. burgdorferi* infected ticks.

After preliminary PCR analysis using pooled tissue-specific DNA, PCR-analysis was repeated on the previously PCR-positive animal tissues but using the DNA isolated from single organs. Historically, most *B. burgdorferi* surveys rely on ear-punch biopsies for cultivation and PCR-analysis to identify infected animals. However, this study reveals that the former approach is inadequate to truly survey the endemic nature of *B. burgdorferi*. The animals analyzed in this study showed that the bladder was the most consistent PCR-positive for mammals (Figures 2 and 3) and the skin for birds (Figures 4 and 5). Based on these findings, the most reliable method of screening animals using PCR-based methods was to pool DNA together from many organs prior to PCR-analysis.

The findings of this study support previous research that suggested that birds are important to the ecology of *B. burgdorferi* in North America (8,16, 25). Of the different species captured in this study, many appeared to be susceptible to *B. burgdorferi* infection while some other species seemed resistant based on the number of birds determine to be infected with *B. burgdorferi* (Table 2). Specifically, none of the European Starlings or Blue Jays sampled yielded PCR-positive results. This provided a good basis for testing the host-competency of these bird species in order to determine whether or not these species could ever be infected. This could provide valuable insight into the molecular mechanisms

within *B. burgdorferi's* genome responsible for conveying resistance to the serum of certain bird species.

Host competency results showed that both tested strains of *B. burgdorferi* were unable to survive in selected bird species tested, although two American Robins tested PCR-positive for *B. burgdorferi*. In this regard, it is important to note that when analyzing American Robin host competency, only B31cF was utilized and it lacks many borrelial plasmids which may be necessary for survival in the American Robin host. Consistent with this notion were previous reports showing that the American Robin was a competent animal host. Interestingly, the sera from the Morning Dove and the Blue Jay killed both strains of *B. burgdorferi* in less than one hour of incubation. Additionally, none of these birds were found PCR-positive for *B. burgdorferi* infection, indicating that these animals may be poor, if not incompetent, hosts for *B. burgdorferi*.

In conclusion, many more studies still need to be performed to fully understand the true ecology of *B. burgdorferi* in Middle Tennessee. The analyzed data in this study suggested that other non-*B. burgdorferi*, *Borrelia* species were present in Middle Tennessee wildlife. These data necessitate further investigation as a possible source of disease not only to humans but also to local wildlife. Additionally, this study warrants further investigation into the genetic factors regulating host-specificity (e.g., animal host range) for *B. burgdorferi* in wild animals. In this regard, valuable insight can be gain using

collected animal sera and plasmid-limited *B. burgdorferi* strains which could be used in concerted to elucidate key borrelial genetic elements required for animal host-specific infectivity and better appreciate *B. burgdorferi* ecology. Finally, the methodologies presented in this study could reveal the presence of other, possibly novel *Borrelia* species could be identified which may serve the foundation for new investigations.

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Vita

DeLacy V. LeBlanc graduated from Lincoln County High School in 2000 and began her undergraduate degree at Austin Peay State University in August 2000. She was the recipient of four scholarships from APSU, which she maintained for the four years of her undergraduate studies. In her senior year she began doing research and made the decision to continue her education at APSU. After graduation, DeLacy began her graduate work in August 2004. While getting her Master's degree in Biology, she worked as a teaching assistant for two years. For the last semester of her graduate degree, she was an adjunct instructor of General Biology and Microbiology classes and labs. After completion of her Masters degree, DeLacy intends to continue furthering her education in a PhD program.