SURVEY OF MIDDLE TENNESSEE MAMMALIAN AND TICK POPULATIONS FOR TICK-BORNE DISEASES: BORRELIA BURGDORFERI, BORRELIA HERMSII, AND ANAPLASMA PHAGOCYTOPHILA

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Dr. Chad S. Brooks, Major Professor

We have read this thesis
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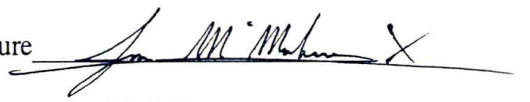
Signature 

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SURVEY OF MIDDLE TENNESSEE MAMMALIAN AND TICK POPULATIONS FOR TICK-BORNE DISEASES: *BORRELIA BURGDORFERI*, *BORRELIA HERMSII*, AND *ANAPLASMA PHAGOCYTOPHILA*

A Thesis

Presented to the College of Graduate Studies

In Partial Fulfillment of the Requirements for

Master of Science Degree

Jon L. McMahan

August 2007

DEDICATION

This thesis is dedicated to my family, the structured unit I was brought into this world in and will not survive its hardships without. My life's accomplishments are a result of their enduring love, support, and modeling.

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It is at this time that I would like to acknowledge and express appreciation to the people that have made this endeavor obtainable. First, I wish to thank my major professor, Dr. Chad S. Brooks, for his guidance and unyielding patience with me. His knowledge and talents within the science discipline is unmatched by any other human I have ever met. It has been an honor and privilege to serve with him.

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ABSTRACT

Lyme disease is the most common vector-borne disease in North America. Contraction of Lyme disease is attributed to an infection with *Borrelia burgdorferi,* a spirochete that is maintained in nature by a complex enzootic life cycle between ticks and mammalian hosts. Research generated in the summer of 2006 has determined that *B. burgdorferi*, historically perceived to not be prevalent within the Southeast region of the United States, to have a 17% (29/172) infectivity rate for Middle Tennessee's mammalian population. Also from this study, it was determined that the method of screening for *B. burgdorferi* in nature should expand beyond traditional techniques of analyzing just mammalian ear biopsies and include the analysis of tissues from the heart, liver, bladder, and spleen. Results from this novel technique of screening for *B. burgdorferi* showed that there would have been an 85% (45/53) identification rate if ear biopsies alone were analyzed. A related observation led to the suggestion that the random tissue distribution of *B. burgdorferi* infectivity could be attributed to the presence of another pathogenic entity, *Anaplasma phagocytophila*. *A. phagocytophila* has been attributed to cause anaplasmosis in humans. It was interesting to note that 6% (11/172) of the specimens were determined to harbor another spirochete organism. This indicates that there are other *Borrelia* related spirochetes present within the Middle Tennessee region. One such species of the *Borrelia* genus may include *Borrelia hermsii*, known to cause relapsing fever in humans. This project continues to screen mammalian populations, as was conducted in the preliminary study, while also incorporating the vectors associated with these pathogens. The

objectives of this study are to: (i) catch specific local small mammals to screen for *B. burgdorferi, B. hermsii,* and *A. phagocytophila* , (ii) collect, identify, and screen ticks from those collected mammals for *B. burgdorferi*, *B. hermsii,* and *A. phagocytophila*, (iii) analyze specimens from the 2006 survey study for *B. hermsii,* and *A. phagocytophila* , and (iv) compare the frequencies of tick infection with mammalian infection. PCR analysis for these pathogens reported no infection within the 38 mammals collected in 2007 from Montgomery and Robertson County, Tennessee. Similarly, analysis of samples from 2006 did not reveal any infectivity of *B. hermsii,* and *A. phagocytophila*. Analysis of tick-vectors from 2007 did, however, show that 36% of all collected ticks to harbor *B. burgdorferi*. It was significant to note that all these ticks were *Dermacentor variabilis*. This information was interesting to report because *D. variabilis* has not been considered a competent tick-vector for *B. burgdorferi*; *Ixodes scapularis* ticks have been the historically accepted competent tick-vector for *B. burgdorferi*. The data discussed herein, suggest that the diversity of vectors for *B. burgdorferi* may be more widespread than originally perceived.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figures Page

CHAPTER I

INTRODUCTION

Background. Lyme disease (LD) is caused by zoonotic infection with the pathogenic spirochete *Borrelia burgdorferi* and has been described as the most commonly reported vector-borne disease in North America, Europe, and Asia (7,14,16,18). Additionally, since its discovery in 1981 by Willy Burgdorfer and Jorge Benach, it has also been declared as the United States' most rapidly emergent human disease (4,7,14,16,18,27). LD is attributed to the human acquisition of a spirochete, *B. burgdorferi*, via bite of an infected tick (2,7,14,18,28). In most cases, transmission of *B. burgdorferi* is accomplished by a parasitic encounter of various *Ixodes* tick species (27). For the United States, the Western black-legged tick, *Ixodes pacificus*, is responsible for transmission of LD on the west coast while the black-legged tick or more commonly known as the deer tick, *Ixodes scapularis*, is responsible for transmission on the east coast (2,7,14,18,28). Human infection with *B. burgdorferi* is considered to be an accidental incident since it seems unlikely that *B. burgdorferi* will complete its complex life cycle from a human host. (1,18). The enzootic cycle which *B. burgdorferi* is perpetuated in involves the cyclic feeding of tick vectors, principally believed to be various *Ixodes* tick species, on a wide variety of *B. burgdorferi* infected vertebrate hosts. The spirochete is only maintained in the environment within these vertebrate hosts/reservoirs since transovarian transmission is not believed to occur (1,2,18). Due to urban sprawl though, human exposure to LD increases as we encroach

Due to urban sprawl though, human exposure to LD increases as we encroach upon the ecosystems that maintain this enzootic cycle (18). Therefore, due to the medical severity and increasing frequency of this vector-borne disease, LD has become a national, as well as worldwide, concern.

The rapid and frequent onset of LD attracts much attention from the medical community. The hallmark symptom and often diagnostic of LD is the manifestation of an erythema migrans rash or also known as a bull's eye rash. Other disease sequelae include post infection manifestations of the musculoskeletal, cardiovascular, and/or neurologic systems (7,11,27). These symptoms can be debilitating, chronic and/or deadly for the infected person dependent upon time of diagnosis and treatment (2,7,11,24,27). Still, even after treatment, particular symptoms from LD infection unexplainably persist for some individuals (23).

Several studies have detected the presence of *B. burgdorferi* so to better understand its world-wide distribution in order to make human risk assessments. Traditional survey methods involve analyzing human diagnosed cases of LD (17). This method is not ideal for establishing *B. burgdorferi's* distribution because it leaves out *B. burgdorferi*'s ecology and relies solely on medical interpretation. Additionally, the interpretation of LD is subject to a bias diagnosis since the symptoms of LD are similar to many other illnesses which, in theory, could lead to misdiagnosis. Therefore, many researchers and medical professionals doubt and/or deny the presence of *B. burgdorferi* within the Southeast region of the United States despite its numerous reported cases (17). Recent research by

DeLacy LeBlanc and Dr. Chad Brooks has shown evidence supporting the presence of *B. burgdorferi* in Middle Tennessee (unpublished).

History and Distribution. The causative agent of LD, *B. burgdorferi*, was discovered in 1981 by Willy Burgdorfer and Jorge Benach. However, the history of this disease dates much further back than just the last several decades. In fact, the first clinical reference to LD dates as far back as to 1909 during a dermatological meeting in Sweden where Arvid Afzelius correlated the erythema migrans (EM) skin lesions of patients with tick bites (5,10,16,27). Then, in 1922 and 1930 this disease was attributed to cause, respectively, neurological and psychiatric complications in patients that were bitten by ticks (10,13). It was not until 1970 that the United States had acquired its first reported case of EM and then in 1977 that this full symptom complex was named LD (5,10,16,27) . LD was named after a small town in Connecticut, Old Lyme, by Dr. Allen Steere after he discovered a correlation between the symptoms of LD and clustered cases of LD in unrelated children (5,10,16,26,27). Four years later, Willy Burgdorfer and Jorge Benach stumbled upon the causative agent of LD, *B. burgdorferi*, as they were examining the midguts of *Ixodes* tick species while looking for rickettsial pathogens (5,10,16,26,27). Since the discovery of the causative agent of LD, surveillance of LD has been conducted by the Center for Disease Control (CDC). Since the beginning of CDC's investigations, there have been increasing reports of LD events throughout the United States (7). LD has been found present in 49 of the 50 states of the United States, excluding Montana, while being mostly

concentrated in the Northeast, North-central, and Midwest regions of the country (2,7,18).

Ecology. The bacterium, *B. burgdorferi*, is maintained in nature by a complex enzootic life cycle. *B. burgdorferi* persists in the environment within vertebrate hosts and transmitted amongst host by tick vectors. A naive tick, being a tick that has yet to become infected with *B. burgdorferi*, will serve as a disease-vector only after it has acquired the spirochete from a parasitic encounter with an infected vertebrate host. The pathogen is then transmitted by the infected tick to other vertebrate hosts (18). As the tick feeds upon its host, the spirochetes localized in the tick's midgut, will migrate to the salivary glands where it is passed to the new host via tick-saliva (8). Since there is no vertical transmission of *B. burgdorferi* from adult ticks to tick-eggs, acquisition of the bacterial pathogen is solely dependent on blood meals from animal hosts which are infected with *B. burgdorferi* (8). Thus the vertebrate host serves as the reservoir for *B. burgdorferi* where its acquisition is repeatedly transmitted by the cyclic feeding of infected ticks (18). The white-footed mouse, *Peromyscus leucopus*, is the primary reservoir for *B. burgdorferi* within the United States (1). The primary vector for *B. burgdorferi*, in the Eastern portion of the United States, is the Black legged tick (a.k.a., Deer tick), *Ixodes scapularis*, which feeds on various mammalian, avian, and reptilian hosts during its larval, nymph, and adult developmental stages (1,18). As it relates to LD, if this enzootic cycle is interrupted by human

encroachment then *B. burgdorferi* infected ticks could potentially bite and infect people (2).

Symptoms, Diagnosis, and Treatment. LD is easily diagnosed by the erythema chronicum migrans which forms typically 3-30 days after a *B. burgdorferi*-infected tick bite (2,7,11). Transmission of *B. burgdorferi* by a tick bite requires an attachment period by the tick for at least 48 hours (2,7,11). Additional diagnosis of early LD infection include symptoms of muscle aches, fever, headaches, and lymphadenopathy (2,7). If treatment for LD is not sought, infection can lead to disease manifestations of the musculoskeletal, cardiovascular, and/or neurologic systems (7,11). These manisfestations could possibly result in cranial-nerve facial palsy, meningitis, radiculopathy, heart block, joint arthritis, encephalitis, and cognitive disorders (2,7). Success of treatment for LD is dependent upon time of diagnosis. Prevention has always been the best clinical tool against health disorders. In the case for LD though, preventative measures extend no further than being able to remove a feeding tick from a person's body within that 48 hours of initial attachment. This seems simple enough, but the nymph form of the *Ixodes* vector are small, up to 1 cm in size, and are easily overlooked. If tick bites are overlooked, then the transmission of *B. burgdorferi* will be allowed resulting in the contraction of LD. If infection does occur, treatment with doxycycline or tetracycline drugs are warranted and have proved to be highly successful in most cases (2,23). Unfortunately though, for poorly understood reasons, there are some cases where treatment with the

appropriate antibiotics have proven to be less than affective (22). In these cases symptoms of fatigue, musculoskeletal pain, and neurocognitive difficulties still persist for the diagnosed patients even after treatment (6). Thus, due to the implications and severity of LD research has been undertaken to give a better understanding of *B. burgdorferi*-ecology for LD human risk assessments.

LD surveys, for the majority, have involved analyzing human LD cases using molecular, culturing, and/or systematic symptom techniques (7,8). However, few studies analyze the ecological portion of *B. burgdorferi*'s existence. The ecological aspect of *B. burgdorferi* is just as important as the medical investigations being conducted for LD. With a better understanding of the ecological role of *B. burgdorferi*, a more conclusive and qualitative human risk assessment for LD can be made.

2006 Research. A study examining *B. burgdorferi*'s ecology took place in the Summer of 2006. A total of 172 small mammals were analyzed for *B. burgdorferi* infectivity. Collection efforts took place from March until September 2006 within seven counties of Middle Tennessee, including: Robertson, Dickson, Stewart, Williamson, Montgomery, Houston, and Lincoln County (Figure 1). Approximately, 84% (144/172) of the captured mammals were identified as the White-footed mouse, *P. leucopus*. Incidentally, the white-footed mouse has been historically viewed as the major reservoir for *B. burgdorferi* (Table 1). Other mammalian species were also captured including 11% (19/172) Prairie voles, *Microtus ochrogaster*, and 5% (9/172) Eastern Harvest Mice, *Reithrodontomys*

humulis (Table 1). For the 2006 mammalian samples, PCR analysis reported that 22% (37/172) of the sampled individuals tested PCR positive for a spirochete within the *Borrelia* genus. It is also interesting to note that when analyzed by *B. burgdorferi* specific primers, there was a 17% (29/172) infectivity rate amongst samples.

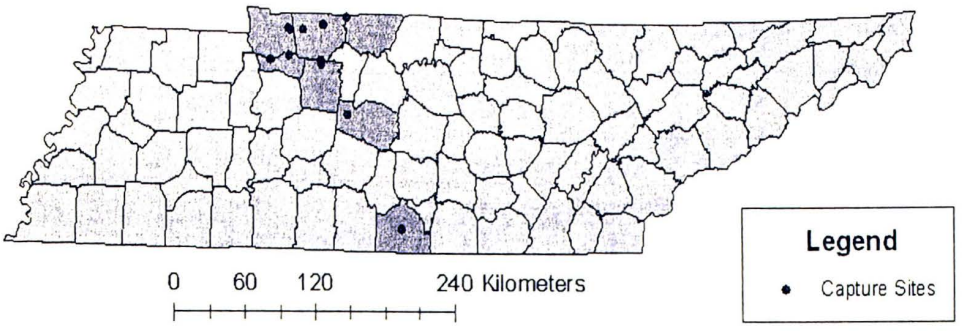

Figure 1. Tennessee map of animal collection sites from the summer of 2006.

Table 1. Infectivity of small mammal specimens for 2006 collections.

County	Species	Total	*Borrelia* species Positive[a]	*Borrelia burgdorferi* Positive[b]	Unknown
Robertson	*Peromyscus leucopus*	41	8	11	0
	Microtus ochrogaster	16	13	9	4
	Reithrodontomys humulis	4	0	0	0
Dickson	*Peromyscus leucopus*	29	1	1	0
	Microtus ochrogaster	2	1	1	0
Stewart	*Peromyscus leucopus*	21	5	2	3
Williamson	*Peromyscus leucopus*	13	1	1	0
	Reithrodontomys humulis	1	1	1	0
Montgomery	*Peromyscus leucopus*	21	2	1	1
Houston	*Peromyscus leucopus*	16	2	1	1
	Microtus ochrogaster	1	1	0	1
	Reithrodontomys humulis	5	1	0	1
Lincoln	*Peromyscus leucopus*	2	1	1	0
Total		172	37	29	11

a. Total number of specimens determined to be PCR positive by the FlaB primer set.
b. Total number of specimens determined to be PCR positive by the Tec1 primer set.

Screening Techniques. Traditional methods of determining *B. burgdorferi's* frequency in nature have been reliant upon several methods. One such method involves the collection and analysis of various tick species within a locale for the prevalence of the spirochete. This method has produced substantial data but of which may be biased since the tick could have fed from an infected animal two seasons prior. Additionally, such investigations may have inherently produced bias data due to the occurrence of a type II error. Reasons to explain the possibility of these errors are unfortunately due to the poorly understood obligate vector(s) for *B. burgdorferi*. Within the methods of such projects, tick drags were utilized in randomized locations to collect ticks of all species. From a tick drag, a large majority of the collected ticks vary in species, so the true vector for *B. burgdorferi*, *Ixodes scapularis*, may be outweighed by other species. In conjunction, it is debated within the scientific community whether or not other species than *Ixodes scapularis* has the ability to harbor *B. burgdorferi* (19). Due to this reasoning, reports on the frequency of *B. burgdorferi's* presence from random tick collections alone may unintentionally result in bias. In response, more accurate data on *B. burgdorferi's* frequency in nature would conceptually come from analyzing the ticks that are directly involved in the enzootic cycle with one of the spirochete's major reservoirs. By analyzing such parasitizing ticks, the effort towards detecting the spirochete in nature would become more focused rather than randomized by tick drags.

PCR is a fairly recent *B. burgdorferi* screening technique which has served for several surveys (14). This technique has proven to be specific and sensitive in

testing but even this method has its limits. There has to be a certain concentration of the targeted DNA present in the initial PCR reaction in order to be detected. If that amount of DNA is not substantial, but still present, then analysis from PCR may result as a negative where as it should have been a positive. In addition, it is interesting to note that the replication of the number of spirochetes in a tick's midgut dramatically increase due to cues produced once a tick has initiated parasitism. These spirochetes quickly replicate in order to increase their odds of survival as they migrate from the vector into the host. For this reason, it seems more feasible to analyze ticks that are actively parasitizing on a host as compared to analyzing non-feeding ticks (i.e., more *B. burgdorferi* DNA in the feeding ticks).

Another method of screening for *B. burgdorferi* in nature is by analyzing the mammalian hosts for infectivity. Typically, this procedure involves taking ear punches from captured mammals and analyzing those tissues with the above described DNA based assays. This technique, though less detrimental on the local mammalian population, is less efficient due to misdiagnosis (LeBlanc et al. unpublished data). The misdiagnosis of an individual mammal with LD is due in part on the fact that analyzing just an ear sample is not sufficient. Analysis of other organs beyond an ear sample is necessary because the spirochete has a tendency to reside in other tissues. Based on LeBlanc's study in 2006, in samples that tested positive for *B. burgdorferi*, only 15% (8/53) was found PCR-positive from ear tissues. This leaves 85% (45/53) of the PCR-positive samples which were later identified from non-ear tissues (Table 2). This underscores that the

traditional screening practices used in mammalian populations would have under-represented the true endemicity of *B. burgdorferi* in nature with an approximately 85% error rate. For this reason it is important to analyze other tissues such as the heart, liver, spleen, and bladder in conjunction with ear biopsies.

On interesting observation from the 2006 survey was that the different tissues were found differentially infected with *B. burgdorferi* while other were not. One explanation for this addresses the possibility that another pathogenic organisms could be competing with *B. burgdorferi* for residence. In this regard, one such organism was *Anaplasma phagocytophila*, a ricketsial parasite which causes anaplasmosis (12). It has been documented that *A. phagocytophila* has the tendency to compete with *B. burgorferi* within the tissues of a host, consequently forcing the spirochete to seek alternative residence (12). Analyzing for *B. burgdorferi* in several tissues rather than just the ear of mammals and in conjunction with the presence of *A. phagocytophila* would provide more accurate information on the prevalence of these pathogens.

Another observation from the 2006 survey noted that other non-*B. burgdorferi*, *Borrelia* organisms were present in some animals. PCR analysis from that study reported that 22% (37/172) of the sampled individuals tested PCR positive for a *Borrelia* related spirochete, 17% (29/172) tested PCR positive for specifically *B. burgdorferi*, and 6% (11/172) were found to be infected by some unidentified *Borrelia* species. In response to these unidentified samples, it was suspected that *Borrelia hermsii* may be the agent of uncertainty. *B. hermsii* was a

spirochete much like *B. burgdorferi* in that it is pathogenic to humans and is transmitted by tick bites (20). *B. hermsii* has been implicated to cause relapsing fever in humans and thus should be an agent of concern (20).

Table 2. Tissue specific infectivity for 2006 samples.

County	Specimens PCR Positive for *B. burgdorferi*	PCR Positive for Heart	PCR Positive for Liver	PCR Positive for Spleen	PCR Positive for Bladder	PCR Positive for Ear	Total Tissues PCR Positive for *B. burgdorferi*
Robertson	20	5	7	9	11	8	40
Dickson	2	0	0	1	1	0	2
Stewart	2	0	1	1	0	0	2
Williamson	2	2	0	2	1	0	5
Montgomery	1	0	1	0	1	0	2
Houston	1	0	0	0	1	0	1
Lincoln	1	0	0	0	1	0	1
Total	29	7	9	13	16	8	53

Objectives. This study focuses on determining if there is a correlation between the frequency of *B. burgdorferi* within tick species and with potentially infected mammalian hosts. Results of this study will provide the local community, counties of Middle Tennessee, with a realistic concept of the potential risk of human exposure to LD causing bacteria and other tick-borne infections. Additionally, the results of this study will provide more precise techniques within the scientific community to survey for *B. burgdorferi* in ecological settings. The objectives of this study are to (i) catch specific local small mammals to screen for *B. burgdorferi, B. hermsii,* and *A. phagocytophila* , (ii) collect, identify, and screen ticks from the collected mammalians for *B. burgdorferi*, *B. hermsii,* and *A. phagocytophila* and (iii) compare the frequencies of tick infection with mammalian infection. This study hypothesizes that there is a correlation between spirochete-infected ticks and mammalian infected hosts.

CHAPTER II

METHODS AND MATERIALS

Specimen Collection. Permits for animal collection were first obtained from both Federal and State agencies to ensure that any threatened mammalian species would not be further impacted. In accordance with these permits a report of animal collections will be generated to provide the number of each species removed, the location of their previous establishment, and detailed descriptions of their fate in laboratory research will be sent to both Federal and State Wildlife agencies. Procedural collections began in May of 2007 and extended through July of 2007. Sampling sites were situated on farm properties in either Montgomery or Robertson Counties, Tennessee. At each sampling site, approximately 40 Sherman traps were baited with a mixture of peanut butter and oatmeal and were deployed along fence lines, brush rows, and various structures (e.g., barns, old houses, and trash piles). Sherman traps have been designed for the live capture of small mammals and consequently were inspected on a 24 hour basis while being relocated and re-baited every 7 days. Upon successful capture, the species of each animal was identified and compared to the acquired Federal and State collection permits in order to follow designated guidelines. All captured animals that were permitted for collection, were immediately brought back to the laboratory for euthanasia and tissue harvesting. Specimens were sacrificed by carbon dioxide asphyxiation followed by cervical dislocation as listed in the IACUC guidebook. Heart, liver, spleen, bladder, and ear tissues were harvested from each animal. Each extracted tissue sample was appropriately labeled, placed in a 1.5 ml

eppendorf tube, and then frozen at -80°C for further analysis. The remaining carcass of each specimen was sealed in a correspondingly labeled plastic bag and stored in a -80° C freezer. Assuming some of the animal carcasses are not used as education models for future classes, all collected animal carcasses will be disposed of as appropriate animal/human waste according to the Tennessee Department of Health guidelines via a professional and licensed animal/human waste disposal company (already on contract with the university).

During tissue harvest, each specimen was screened for parasitizing ticks using a dissecting microscope. All ticks discovered were individually placed in 1.5 ml eppendorf tubes and labeled correspondingly with their animal host. Notes on the developmental stage and feeding status were recorded for each specimen. Additionally, when possible, the identification of each tick species was noted, however PCR analysis with species specific primer sets would verify these assumptions. All tick specimens were subsequently stored in a -80°C freezer for later analysis.

DNA Isolation from Animal Tissues. The extracted tissues from each specimen were enzymatically digested using approximately 0.5 g of each tissue and a two step digestion involving collagenase and proteinase K. Tissues were initially treated with 1 mg/ml collagenase suspended in phosphate buffered saline for 6 hours at 34°C. After incubation, tissue samples were triturated then exposed to proteinase K for 16 hours at 34°C. Upon successful digestion of each tissue, samples were thoroughly vortexed and then centrifuged at 14,000 x g for 5

minutes to pellet cellular debris. All aqueous components of the digested tissue samples were immediately transferred to a new 1.5 ml eppendorf tube and then exposed to 400 μl of phenol and 400 μl chloroform. This solution was then vortexed for approximately 15 seconds and allowed to sit at room temperature for 5 minutes. Samples were subsequently centrifuged at 14,000 x g for 5 minutes to produce two distinct layers within solution. The top aqueous layer, containing nucleic acid, was transferred to a new tube. The above described procedure was repeated until all samples appeared free of any protein contamination. After DNA purification was complete, each sample received approximately 100 μl of 3M sodium acetate and 500 μl of 100% isopropanol and mixed by inversion. Each sample was then chilled at -80°C for 5 minutes and then centrifuged at 14,000 x g for 10 minutes at 4°C to precipitate the DNA. The aqueous contents of the samples were then carefully removed so to not disturb the DNA pellet. Excess salts were removed by washing the pellet with 80% ethanol followed promptly by centrifuging at 14,000 x g for 5 minutes. The DNA pellets for each sample were then allowed to air dry and were subsequently resuspended in 50 μl of molecular grade water. All samples were then frozen at -80°C for later analysis by PCR.

DNA Isolation from Ticks. All tick specimens were subjected to DNA isolation. It was originally assumed that tick specimens could be used in PCR analysis without having to perform DNA isolation. It was found that a polysaccharide component within ticks was inhibitory for DNA amplification (25). Consequently, it was apparent that DNA purification was essential requiring the above described

phenol:chloroform isolation technique to be performed. During preliminary analysis, this method of isolating DNA did not prove as efficient as desired. Alternative techniques for isolating DNA were pursued. Another methodology of isolating DNA from such small arthropod entities would require the use of a specifically designed isolation kit. An isolation kit designed for the use of isolating DNA from such organisms was obtained from the Quiagen Company. The Quiagen isolation kit was tested with ticks irrelevant to this projects investigations before actual samples were used. Instructions for utilizing this isolation kit specified the requirement of complete breakdown of each tick's body. This was accomplished by freezing each tick in liquid nitrogen and then grinding the ticks with a mortar and pestle. The resulting tick debris was then enzymatically digested by proteinase K, provided within the isolation kit, for a 24 hour period at 56°C. After incubation, the digested tick solutions were passed through isolation columns as instructed by the kit's guidelines. All isolated tick DNA were suspended in 100 μl of molecular grade water and frozen away at -80°C for later analysis.

Identification of Tick Species. In regard to the predominant tick species associated with the locality of specimen collection efforts, three species were of initial concern: *Ixodes scapularis, Amblyomma americanum, and Dermacentor variabilis*. Prior published data supported the use of tick specific primers which could only amplify its intended tick specie (25). Primer sets have been designed to amplify a gene sequence specific and unique for each of these tick species. In

addition to acquiring these primer sets representative samples of each species were collected by tick drags and then analyzed. Analysis involved the isolation of DNA for ticks, as described above and cross PCR analysis with each primer set so as to ensure that they amplified its intended complimentary gene sequence. This preliminary step would provide the following information: 1) that each primer set has the ability to amplify its appropriate gene sequence for identification purposes, 2) that each primer set would not accidentally amplify any erroneous DNA from another lineage of tick species to prevent misidentification, and 3) that there would be a species specific positive control sample available for gel electrophoresis analysis and comparison.

After preliminary assurance of the capabilities of each primer set was assessed, the isolated DNA of each tick specimen was analyzed for identification purposes. Analysis involved a simplexed PCR reaction of the isolated tick DNA with each primer set. PCR was carried out in a 20 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 50 mM KCl, 100 μM each of the four dNTPs, 1 unit of RedTaq DNA polymerase, and 25 pmol of primer. Isolated tick DNA was combined in this PCR mixture in various quantities so as to not exceed 4 μg of DNA as determined by spectrophotometry. Amplification took place in a thermocycler for 35 cycles at the following set temperatures: Melting temperature set at 94°C, Extension temperature set at 72°C, and Annealing temperature set at ~56-58°C (dependent upon each primer set).

Following PCR, success of amplification was verified by gel electrophoresis. Gels were composed of 0.8 g agarose solubilized in 100 ml of

TAE buffer and 7 μl ethidium bromide . Gel electrophoresis was allowed to proceed for 45 minutes at 140 volts. Verification of species was concluded by UV light exposure and the comparison of each PCR reaction to a 1,000 base pair ladder and positive controls.

Identification of *B. burgdorferi* and *hermsii*. Assortments of primers have been designed to identify the presence of *B. burgdorferi* and *hermsii* spirochetes. From this assortment three primer sets have been selected for utilization in this study. One primer set, termed FlaB, has been designed to recognize a gene that encodes flagella protein found within all *Borrelia* spirochete species (3). FlaB will thus provide general information indicating if any *Borrelia* spirochetes are present by PCR analysis. The second primer set, selected to identify *B. burgdorferi* in this study, is termed TEC1/LD2 (9). This primer set is specific for amplifying a gene unique only to *B. burgdorferi*. The third primer set that was used in this study, called Bh, has been designed to amplify a gene unique only to *B. hermsii* (21).

Preliminary PCR reactions of isolated mammalian tissue and tick DNA were performed by using the FlaB primer set. Each tissue sample from a single specimen was individually tested against FlaB. Tick samples were also analyzed by separate PCR reactions with this primer set. PCR reactions were performed and analyzed in the same manner as described above. Any samples that reported positive data from FlaB amplification were then subjected to additional PCR to identify presence of *B. burgdorferi* and/or *hermsii*.

B. burgdorferi and *hermsii* analysis utilized the TEC1/LD2 and Bh primer sets respectively in PCR reactions that reported FlaB positive. Simplex PCR reactions with these primer sets were performed and analyzed in the same manner as conducted for FlaB.

Identification of *Anaplasma phagocytophila*. A nested primer set has been designed to amplify a gene sequence unique only to *A. phagocytophila* (15). The design of a nested primer set is ideal in the detection of organisms such as *A. phagocytophila* because it utilizes a double screening process. The nested primer set is actually comprised of two primer sets. The primary set is used to amplify a unique gene only to *A. phagocytophila*. The secondary primer set amplifies a nucleotide sequence found within the amplified template produced from the primary primer. The benefit of this design has been shown in various other studies where just the primary primer set was not sufficient to produce a DNA band when analyzed on an electrophoresis gel even though there was complimentary template in the PCR reaction (15). By using the secondary primer set a segment of that originally amplified template was again amplified. This would result in a more obvious DNA band when analyzed by gel electrophoresis.

The above described procedure was utilized in the detection of *A. phagocytophila* in this study. PCR reactions of both individual mammalian tissues and ticks were performed with the primary primers of the nested set. The PCR reactions were analyzed on an electrophoresis gel. In addition, 2 μl of the resulting PCR product was utilized in a second PCR reaction with the secondary

primers of the nested set. This second PCR reaction was performed and analyzed in the same manner as all other PCR reactions previously described. All primers used in this study are listed (table 3)

Table 3. Primers used for PCR analysis of tick species identification and specimen infectivity for *B. burgdorferi, B. hermsii*, and *A. phagocytophila*.

Primer Reference	Specimen Identification	Primer type	Genetic Sequence
FlaB	*Borrelia* genus	Forward	5'-AGAGCTTGGAATGCAGCCT-3'
		Reverse	5'-GGGAACTTGATTAGCCTGCG-3'
Tec1	*B. burgdorferi*	Forward	5'-CTGGGGAGTATGCTCGCAAGA-3'
		Reverse	5'-GACTTATCACCGGCAGTCTTA-3'
Bh	*B. hermsii*	Forward	5'-TAGAAGTTCGCCTTCGCCTCTG-3'
		Reverse	5'-TACAGGTGCTGCATGGTTGTCG-3'
Nested Primary	*Anaplsama phagocytophila*	Forward	5'-CACATGCAAGTCGAACGGATTATTC-3'
		Reverse	5'-TTCCGTTAAGAAGGATCTAATCTCC-3'
Nested Secondary	*Anaplsama phagocytophila*	Forward	5'-AACGGATTATTCTTTATAGCTTGCT-3'
		Reverse	5'-GGCAGTATTAAAAGCAGCTCCAGG-3'
T16s	General tick DNA	Forward	5'-CTGCTCAATGATTTTTTAAATTGCTGTGGT-3'
		Reverse	5'-CCGGTCTGAACTCAGATCAAGTAGGA-3'
Deer Tick	*Ixodes scapularis*	Forward	5'-TGCGTTTTCTTTGAGCAAATGCACGAG-3'
		Reverse	5'-GTACGGGATTTTCCACAAACGGTATCCA-3'
American Dog Tick	*Dermacentor variabilis*	Forward	5'-CTGAAGATTCTTTGCGAGGAGCGG-3'
		Reverse	5'-GCGTCAGCTCGGCCAAC-3'
Lonestar Tick	*Amblyomma americanum*	Forward	5'-AAGCCCGCGCTCCAAGC-3'
		Reverse	5'-GCAGCAGTTCGGCTACACGTA-3'

CHAPTER III

RESULTS

Specimen Collection. Trapping efforts took place during the period of May through July of 2007. Sampling sites consisted of two locations situated in Montgomery County and Robertson County, Tennessee (figure 2). During this time period 38 mammals were live captured using Sherman traps. Trapping specifically took place in Robertson County on May 6 and continued through June 1 resulting in 28 live captured mammals. On June 2 collection efforts were relocated into Montgomery County and continued through June 21 with 9 captured mammals. Traps were again relocated to the original trapping location in Robertson County on June 22 and trapping continued though July 13 with only one mammal collected. Collection efforts were terminated after July 13 to allow for laboratory processing. Each captured mammal was identified as a white-footed mouse, *Peromyscus leucopus*.

In addition to this collection there were a total of 33 ticks obtained from the mammalian specimens. Not every mouse specimen was found to harbor tick(s) but for those that did one or more ticks were removed. Approximately 37% (14/38) of the mammalian specimens harbored ticks (table 4). The majority of ticks, 97% (32/33), came from specimens captured within Robertson County while only 3% (1/33) of ticks originated from Montgomery County specimens. Notes on the developmental stages and feeding status of each tick were made (table 4). The majority of ticks collected were in the nymph developmental stage, 82% (27/33), while the rest were in larval development, 18% (6/33). Additionally,

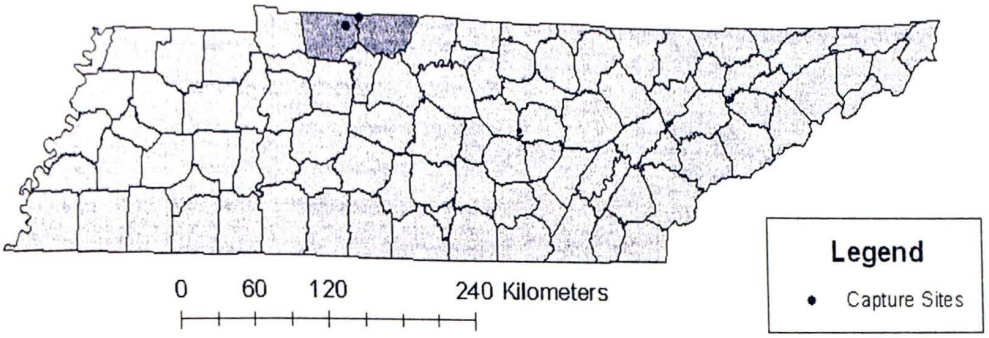

Figure 2. Tennessee map of animal collection sites from the summer of 2007.

Table 4. Origin, developmental, and feeding status of each tick specimen.

Specimen Identification	County Of Origin	Nomenclature	Developmental Stage		Feeding Status	
			Larvae	Nymph	Unfed	Engorged
3A	Robertson	*Dermacentor variabilis*		Nymph	X	
3B	Robertson	*Dermacentor variabilis*	Larvae			X
4A	Robertson	*Dermacentor variabilis*		Nymph		X
4B	Robertson	*Dermacentor variabilis*		Nymph		X
5A	Robertson	*Dermacentor variabilis*		Nymph		X
5B	Robertson	*Dermacentor variabilis*		Nymph		X
5C	Robertson	*Dermacentor variabilis*		Nymph	X	
7A	Robertson	*Dermacentor variabilis*		Nymph		X
7B	Robertson	*Dermacentor variabilis*		Nymph		X
7C	Robertson	*Dermacentor variabilis*	Larvae		X	
9A	Robertson	*Dermacentor variabilis*		Nymph		X
9B	Robertson	*Dermacentor variabilis*		Nymph	X	
9C	Robertson	*Dermacentor variabilis*		Nymph		X
9D	Robertson	*Dermacentor variabilis*		Nymph	X	
10A	Robertson	*Dermacentor variabilis*		Nymph	X	
10B	Robertson	*Dermacentor variabilis*		Nymph	X	
15A	Robertson	*Dermacentor variabilis*		Nymph	X	
15B	Robertson	*Dermacentor variabilis*	Larvae		X	
16A	Robertson	*Dermacentor variabilis*		Nymph		X
16B	Robertson	*Dermacentor variabilis*		Nymph	X	
17A	Robertson	*Dermacentor variabilis*		Nymph		X
17B	Robertson	*Dermacentor variabilis*		Nymph		X
22A	Robertson	*Dermacentor variabilis*		Nymph	X	
22B	Robertson	*Dermacentor variabilis*		Nymph	X	
22C	Robertson	*Dermacentor variabilis*		Nymph		X
22D	Robertson	*Dermacentor variabilis*		Nymph		X
22E	Robertson	*Dermacentor variabilis*		Nymph		X
22F	Robertson	*Dermacentor variabilis*		Nymph		X
22G	Robertson	*Dermacentor variabilis*		Nymph		X
23A	Robertson	*Dermacentor variabilis*	Larvae		X	
24A	Robertson	*Dermacentor variabilis*	Larvae		X	
26A	Robertson	*Dermacentor variabilis*	Larvae		X	
37A	Montgomery	*Dermacentor variabilis*		Nymph		X
Total	2 counties	1 species	6	27	15	18

55% (18/33) of the collected ticks were engorged with a blood meal from its host while the remaining 45% (15/33) of tick specimens appeared otherwise.

Identification of Tick Species. The identification of tick species proved difficult based on analyzing anatomical structures. Identification of adult ticks has been historically accomplished with ease but determining the species of nymph and larval ticks demands higher expertise. All collected tick specimens for this study were either in the larval or nymph developmental stage (table 1) of their life cycle so species identification relied upon DNA analysis (i.e., PCR) (25).

A control test was conducted to ensure that the primers utilized in the identification of tick species would all properly function (figure 3). It was determined that each species specific primer set was capable of only amplifying the DNA matched with its appropriate template and no other (figure 3). This control test also served as the basis of comparison when the tick specimens were analyzed by gel electrophoresis. PCR analysis was performed on each specimen with all three primer sets to deduce species. Of the 35 tick specimens, 100% (35/35) were identified as belonging to the American Dog tick species, *Dermacentor variabilis* (figure 4). This information is useful since there is a large discrepancy regarding the knowledge of the true long term vectors for *Borrelia* spp. spirochetes. Furthermore, it has been argued by the scientific community that *I. scapularis* may not be the only competent vector for *B. burgdorferi* but may also include such other tick species as *D. variabilis*. It has also been suggested that

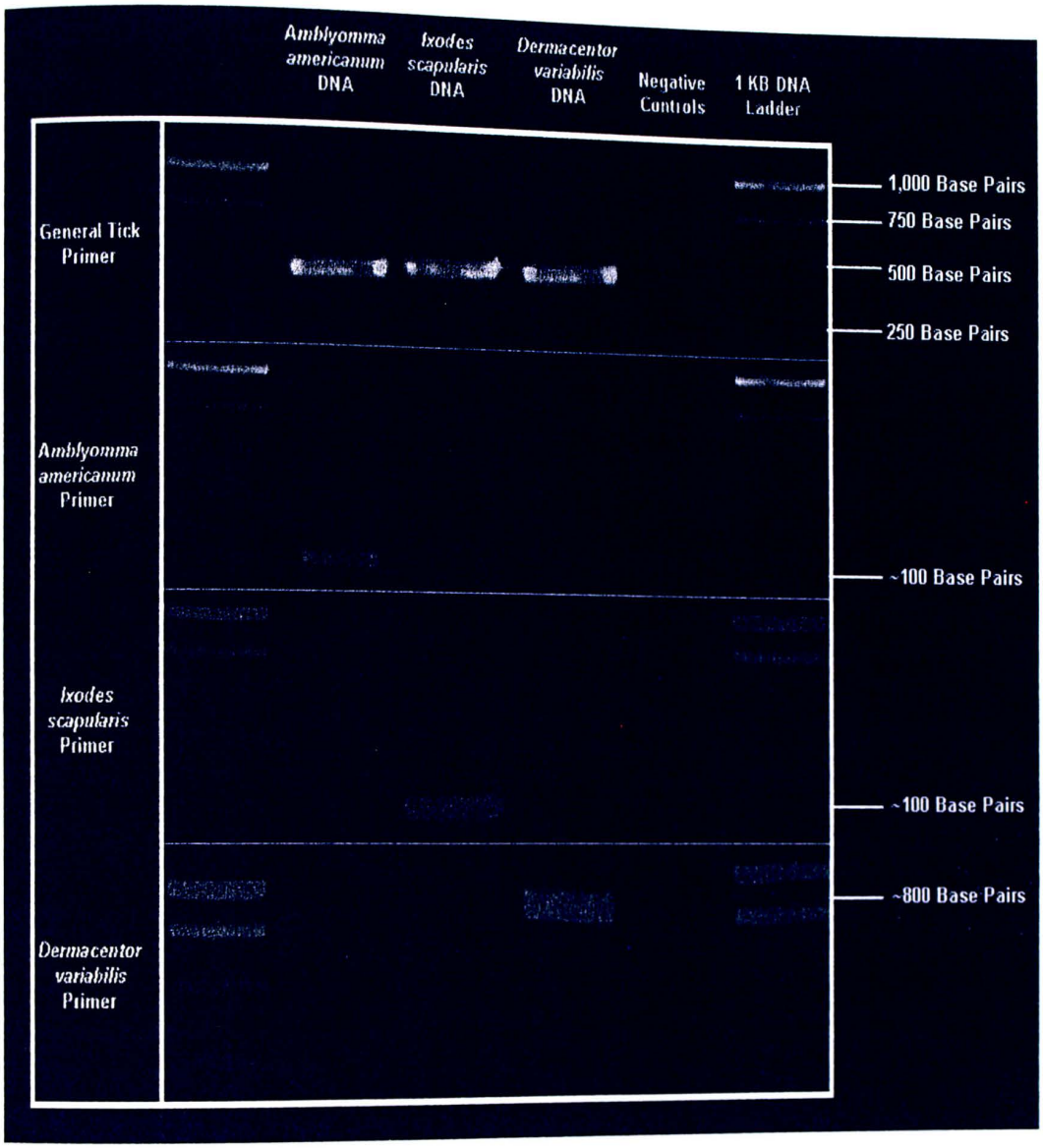

Figure 3. Electrophoresis gel demonstrating the proper amplification of tick DNA by the various species specific primer sets.

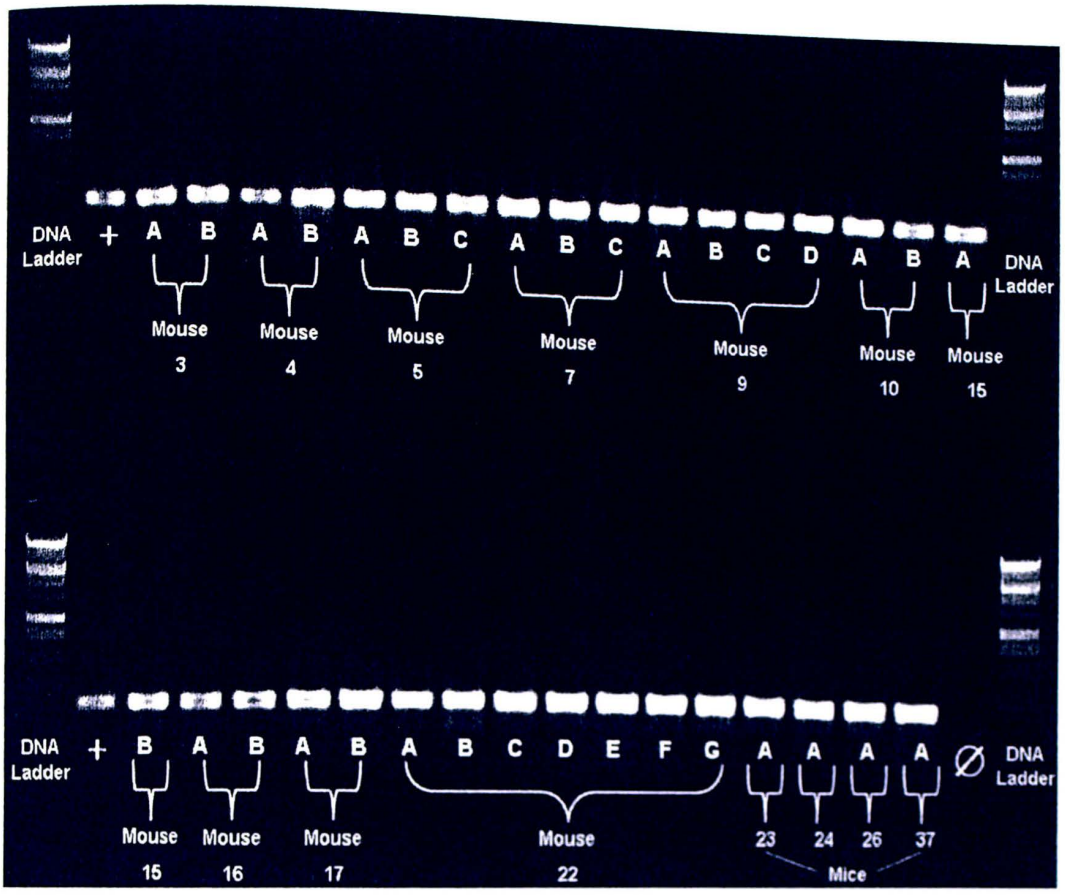

Figure 4. Electrophoresis gel portraying the identification of tick samples as American Dog Ticks, *Dermacentor variabilis*.

+ Denotes the positive control, *Dermacentor variabilis* specific primers and *D. variabilis* DNA.

∅ Denotes a negative control, where *Dermacentor variabilis* specific primers are present but there is no complimentary DNA template to be amplified

Ticks are identified by the mouse they were collected from and also are assigned a letter in order to differentiate amongst them.

D. variabilis might have the capability to facilitate other pathogens such as *B. hermsii* and *A. phagocytophila* (25).

Identification of *Anaplasma phagocytophila*. It has been a traditional methodology to screen for *B. burgdorferi* within wild mammalian populations by analyzing only ear biopsies. From the preliminary studies that took place in the summer of 2006 discrepancies were found concerning this original concept. For example, in all specimens that tested PCR positive for *B. burgdorferi*, there was only a 27% (8/30) infection identification rate using ear tissues. The other 73% (22/30) of infectivity was discovered in combination of ear tissues as well as various other tissues for the specimens as follows: 24% (7/29) infectivity within the heart, 31% (9/29) infectivity within the liver, 45% (13/29) infectivity within the spleen, and 55% (16/29) infectivity within the bladder. Traditional screening practices, in this case, would have misdiagnosed the infectivity of *B. burgdorferi* and greatly underestimate its true endemicity. Reasoning for this phenomenon could possibly be due in part of the presence of another pathogenic organism (AA). It has been proposed that the organism *A. phagocytophila,* during situations of co-infection, will compete for residence within various tissues, driving organisms such as *B. burgdorferi* to other alternative tissues (AA). If *A.* phagocytophila is discovered in these samples it would explain the random distribution of *B. burgdorferi* infectivity. Samples from the summer of 2006 and 2007 were analyzed for *A. phagocytophila* by utilizing nested PCR. The primary primers of the nested set were not successful in adequately amplifying enough

complimentary template to visualize a DNA band under UV light. This report of negative data does not necessarily mean that there was not any infectivity of *A. phagocytophila*. The secondary primers of the nested set would conclude *A. phagocytophila* infectivity and were subsequently utilized. Results from this testing also reported no infectivity of *A. phagocytophila* for all samples from the summer collection periods of 2006 and 2007. These findings leave the random tissue infectivity of *B. burgdorferi* as a remaining unexplained phenomena.

Identification of *Borrelia hermsii*. From the preliminary studies that took place in the summer of 2006 a total of 172 small mammals were collected. From the 2006 mammalian samples PCR analysis reported that 22% (37/172) of the sampled individuals tested positive by the FlaB primer set, 17% (29/172) to test positive by the Tec1 primer set, and 6% (11/172) were found to be infected by some unidentified *Borrelia* species. All tissue samples from the summer of 2006, that tested positive by the FlaB primer set (53 in total), were analyzed for B. *hermsii* in hopes of identifying these unknown infected specimens. PCR analysis with *B. hermsii* specific primers indicated that *B. hermsii* was not present in samples from 2006. In total the collection efforts from the summer of 2007 yielded 38 mammalian samples. In these samples no *Borrelia* species were detected but suprisingly there was detection of *Borrelia* spirochetes within the ticks collected from these mammals. There was a 36% (12/33) infectivity rate of *Borrelia* related organisms within tick samples. Upon analysis with Bh primers it

was determined that tick samples were harboring some other species of *Borrelia* other than that of *B. hermsii*.

Identification of *Borrelia burgdorferi*. The summer sampling efforts from 2006 gave rise to some very alarming data. The efforts in that study reported a 17% (29/172) infectivity rate of *B. burgdorferi* within seven counties of Middle Tennessee that were not originally perceived to harbor such a pathogen (Table 5). This current study is in essence a partial continuation of the 2006 project. One of the goals in this study was to continue to examine the rate of infectivity of *B. burgdorferi* within the small mammal populations of Montgomery and Robertson counties while also investigating the rate of infectivity associated with its vectors. Subsequently, trapping efforts of these two counties yielded 38 mammalian specimens identified as the white-footed mouse, *P. leucopus*. PCR analysis of these specimens was conducted by the use of the FlaB and Tec1 primers resulting in the discovery of no infectivity. The same procedures utilized in 2006 and, in most cases, the same locations for trapping did not report any presence of *B. burgdorferi* for 2007. This is very perplexing because the study the year prior resulted in *B. burgdorferi* infectivity rates of 33% (20/61) and 5% (1/21) for Robertson and Montgomery counties respectively (Table 5). Admittedly, the sample size for 2006 studies was much larger than that for this project, but it is very intriguing to not have encountered any infected mammals with *B. burgdorferi* or any *Borrelia* related species at all.

Table 5. Infectivity of *B. burgdorferi*, *B. hermsii*, and *A. phagocytophila* for 2006 Samples.

County	Species	Total	*Borrelia* species Positive[a]	*Borrelia burgdorferi* Positive[b]	*Borrelia hermsii* Positive[c]	*Anaplasma phagocytophila* Positive[d]	Unknown
Robertson	*Peromyscus leucopus*	41	8	11	0	0	0
	Microtus ochrogaster	16	13	9	0	0	4
	Reithrodontomys humulis	4	0	0	0	0	0
Dickson	*Peromyscus leucopus*	29	1	1	0	0	0
	Microtus ochrogaster	2	1	1	0	0	0
Stewart	*Peromyscus leucopus*	21	5	2	0	0	3
Williamson	*Peromyscus leucopus*	13	1	0	0	0	1
	Reithrodontomys humulis	1	1	1	0	0	0
Montgomery	*Peromyscus leucopus*	21	2	1	0	0	1
Houston	*Peromyscus leucopus*	16	2	1	0	0	1
	Microtus ochrogaster	1	1	0	0	0	1
	Reithrodontomys humulis	5	1	0	0	0	1
Lincoln	*Peromyscus leucopus*	2	1	1	0	0	0
Total		172	37	29	0	0	11

a. Total number of specimens determined to be PCR positive by the FlaB primer set.
b. Total number of specimens determined to be PCR positive by the Tec1 primer set.
c. Total number of specimens determined to be PCR positive by the Bh primer set.
d. Total number of specimens determined to be PCR positive by the nested *Anaplasma* primer.

Though the mammalian samples did not report any infectivity of *B. burgdorferi*, PCR analysis of tick samples with FlaB and Tec1primers did conclude its presence. There were at total of 33 American dog ticks, *D. variabilis*, collected from the captured mammals. Of the tick samples, 36% (12/33) reported PCR positive with some *Borrelia* species of spirochete and 12% (4/33) reported PCR positive with specifically *B. burgdorferi* (Table 6).

Table 6. Infectivity of *B. burgdorferi*, *B. hermsii*, and *A. phagocytophila* for 2007 Samples.

	County	Species	Total	*Borrelia* species Positive[a]	*Borrelia burgdorferi* Positive[b]	*Borrelia hermsii* Positive[c]	*Anaplasma phagocytophila* Positive[d]	Unknown
Small Mammals	Montgomery	*Peromyscus leucopus*	9	0	0	0	0	0
	Robertson	*Peromyscus leucopus*	29	0	0	0	0	0
	Total		38	0	0	0	0	0
Ticks	Montgomery	*Dermacentor variabilis*	1	0	0	0	0	0
	Robertson	*Dermacentor variabilis*	32	12	4	0	0	8
	Total		33	12	4	0	0	8

a. Total number of specimens determined to be PCR positive by the FlaB primer set.
b. Total number of specimens determined to be PCR positive by the Tec1 primer set.
c. Total number of specimens determined to be PCR positive by the Bh primer set.
d. Total number of specimens determined to be PCR positive by the nested *Anaplasma* primer set.

CHAPTER IV

DISCUSSION

From the 2006 study, there were a total of 172 small mammals collected within seven counties of Middle Tennessee, including: Robertson, Dickson, Stewart, Williamson, Montgomery, Houston, and Lincoln County. PCR analysis reported that 22% (37/172) of the sampled individuals tested PCR positive for the presence of a spirochete within the *Borrelia* genus. It was also determined that there was a 17% (29/172) infectivity rate for specifically *B. burgdorferi*. This information supports many people's contention that *B. burgdorferi* is a real threat to public health in Middle Tennessee. Many medical practitioners have been reluctant to diagnose LD within the southeastern region of the United States because LD has not historically been diagnosed within this region. This study will hopefully encourage and support community awareness of human risk to LD exposure.

The 2006 *B. burgdorferi* survey also laid significant foundation data to warrant further investigation in Middle Tennessee. In this regard, the 2007 project (this thesis) makes efforts to expand upon some of those observations. First, the screening for *B. burgdorferi's* presence within the wild was continued. The *B. burgdorferi* survey continued for Montgomery and Robertson County, two counties of which portrayed a high infectivity rate, by collecting small mammal samples as performed the year before. In addition to analyzing mammalian populations for *B. burgdorferi*, ticks were collected and screened for *B. burgdorferi*. Since ticks are essential components for the perpetuation of LD in

nature, ticks should be continually analyzed for *B. burgdorferi* infection. The animal-collection and *B. burgdorferi*-PCR analysis of Montgomery and Robertson County's mammalian populations (38 samples) reported no infectivity of *B. burgdorferi* (Table 5). In contrast, tick collections obtained from the captured mammals did reveal *B. burgdorferi's* presence within Robertson County (Table 6). A total of 33 ticks were collected from the captured mammals from the two counties. From the tick samples, 97% (32/33) of those ticks originated from Robertson County and 13% (4/32) of those specimens revealed by PCR analysis to harbor *B. burgdorferi*. This data provided evidence that the spirochete *B. burgdorferi* was still present within the local environment as it was in 2006. It is also interesting to note that the ticks that were PCR positive for *B. burgdorferi* were each in their nymph developmental stage, meaning that those ticks had received the spirochete by either two methods: 1) each tick, at their point in development, could either receive the spirochete by parasitizing upon a host in 2007 that was already infected or 2) by parasitizing upon an infected host in their larval stage of development from 2006.

Some controversy exists in the literature concerning what are the true competent tick-vectors for *B. burgdorferi*. It was originally perceived that the Deer tick, *Ixodes scapularis*, was the obligate vector for the maintenance and transmission of this spirochete. However, this study has revealed that *B. burgdorferi* was found within another tick species, the American Dog tick, *Dermacentor variabilis*. The American Dog tick has been suggested to be a possible competent vector for *B. burgdorferi* (Y). Our data provide additional

evidence to support the concept that the diversity of competent tick-vectors for *B. burgdorferi* is broader than originally perceived. Additionally, the results from this project could be easily dismissed, concerning the issue of a possible new competent vector, if the mammalian host that the sampled ticks were removed from were found to be infected with the spirochete. If this was true, then the PCR analysis of *D. variabilis* ticks harboring *B. burgdorferi* would be simply explained by the recent acquisition of the spirochete from its most previous parasitic encounter. Results from PCR analysis for mammalian samples were all negative. Therefore, the PCR-positive ticks had to acquire the spirochete from a prior infected animal. Tick behavior involves a cyclic feeding process where one blood meal is obtained from a host before they undergo molting into their next developmental stage. Before developing into a nymph, being the case of the PCR-positive ticks in this project, ticks must mature from their larval form. All larval ticks are considered to be naive of *B. burgdorferi* considering that the spirochete can not be passed from adult ticks to larval offspring. Thus, the only opportunity that the PCR-positive ticks have had to acquire the spirochete was from previously parasitizing upon an infected host from 2006 or early 2007. Regardless of when the ticks had acquired the spirochete though, this evidence supports the concept of other ticks species as competent vectors for *B. burgdorferi*. Additional research on this topic is necessary though to conclude this story.

In addition to the surveillance for *B. burgdorferi*, other *Borrelia* related organisms became an issue of concern from the 2006 study. From the 2006 study, PCR analysis showed that 22% (37/172) of the sampled individuals tested

positive for the presence of a spirochete within the *Borrelia* genus. As mentioned before, approximately 17% (29/172) of the sampled mammals from 2006 were infected (PCR positive) with *B. burgdorferi.* From these reports, 12% (7/55) of the analyzed specimens still remain unidentified beyond establishing that they were in the *Borrelia* genus. Additionally, after the analysis of last year's results, the *Borrelia* genus PCR-positive samples were screened with another primer set specific for *B. lonestarii,* another pathogenic spirochete which causes Southern Tick-borne Associated Rash Illness (STARI) but no *B. lonestarii* were detected in our study. Attempts were made to identify those unknown *Borrelia* related organisms by screening the 2006 and 2007 collected specimens for *B. hermsii*. The 2007 collected specimens were analyzed with the FlaB primer set, being the same primer set used in the preliminary study, which would determine the presence of any organisms from the *Borrelia* genus. The 2007 collected specimens were PCR negative when tested with this primer set indicating that the samples were not infected with any *Borrelia*. The 2006 collected specimens consisted of 55 individuals that reported PCR-positive by the *Borrelia* genus primer set. These individuals were analyzed by *B. hermsii* specific primers and all tested PCR negative for *B. hermsii*. These efforts still resulted in the unknown identity of 12% (7/55) of last year's *Borrelia* related organisms. The combined efforts of this project and last year's studies have eliminated the possibility of infection and/or co-infection of such spirochetes as *B. burgdorferi*, *B. hermsii* and *B. lonestarii* within the unknown samples. Other *Borrelia* related organisms such as *B. andersonii* should be investigated to help identify these samples.

Another aspect of this project addressed an issue that was also generated from last year's preliminary study. It was noted that of the 29 *B. burgdorferi*-positive samples, there was a 27% (8/29) infection rate of just ear tissues. The other 73% (22/30) of positive mammals was discovered in combination of ear tissues as well as various other tissues for the specimens as follows: 27% (8/30) infectivity within the heart, 30% (9/30) infectivity within the liver, 43% (13/30) infectivity within the spleen, and 50% (15/30) infectivity within the bladder. Traditional screening practices, in this case, would have misdiagnosed the infectivity of *B. burgdorferi* in many of the specimens. Reasoning for this phenomenon could possibly be due in part of the presence of another pathogenic organism, *A. phagocytophila* . Previous research has indicated that during situations of co-infection between *A. phagocytophila* and *B. burgdorferi*, tissue residence becomes competitive (12). It is theoretical that *A. phagocytophila* has the capacity to dominate a particular tissue, such as the dermis layers of an ear, and drive *B. burgdorferi* away (12). This possibility was examined by screening all *B. burgdorferi* PCR-positive samples with primers specific for *A. phagocytophila.* The presence of *A. phagocytophila* could thus partially explain the random distribution of *B. burgdorferi* amongst host tissues. PCR analysis did not result in the amplification of *A. phagocytophila's* DNA. So, the reason for *B. burgdorferi's* random tissue infectivity still remains elusive.

In conclusion, more research still needs to be performed to address some of the issues left unsolved by this project. It is perplexing to note that last years efforts in determining *B. burgdorferi's* presence was much more successful than

that of this project. Reasons to explain this occurrence could possibly be due in part to a smaller sample size. Reasons for a smaller sample size for this project could be due to shorter collection periods and also atypical weather conditions. The general area of Middle Tennessee experienced a significant drought during the time of collection efforts which could have altered the behaviors of the small mammals or ticks. Future investigations of *B. burgdorferi* and other pathogens harbored by wildlife discussed in this project will provide additional insight necessary to further develop human risk assessments.

LITERATURE CITED

1. **Anderson, J.M., K.I. Swanson, T.R. Schwartz, G.E. Glass, and D.E. Norris.** 2006. Mammal diversity and infection prevalence in the maintenance of enzootic Borrelia burgdorferi along the western Coastal Plains of Maryland. Vector Borne Zoonotic Diseases. 6(4): 411-22.

2. **Bratton, R.L. and G.R. Corey.** 2005. Tick Borne Disease. American Family Physician. 71(12) .

3. **Brooks, C.S., S.R. Vuppala, A.M. Jett, A. Alitalo, S. Meri, and D.R. Akins.** 2005. Complement regulator-acquiring surface protein 1 imparts resistance to human serum in Borrelia burgdorferi. Journal of Immunology. 175(5): 3299-3308.

4. **Burgdorfer, W.** 1991. Lyme borreliosis: ten years after discovery of the etiologic agent, Borrelia burgdorferi. Infection. 19(4): 257-62.

5. **Burgdorfer, W.** 1996. Lyme disease. Emergency Medicine Service, Stanford University Medical Center. 5: 631-4.

6. **Cairns, V., and J. Godwin.** 2005. Post-Lyme borreliosis syndrome: a meta-analysis of reported symptoms. International Journal of Epidemiology. 34(6):1340-1345.

7. **CDC (Centers for Disease Control and Prevention).** 2002. Lyme Disease-United States, 2001-2002. MMWR Weekly. 53(17): 365-369.

8. **Crippa, M., O. Rais, and L. Gern.** 2002. Investigations on the mode and dynamics of transmission and infectivity of Borrelia burgdorferi sensu stricto and Borrelia afzelii in Ixodes ricinus ticks. Vector Borne Zoonotic Diseases. 2(1): 3-9.

9. **CYR, T.L., M.C. Jenkins, R.D. Hall, E.J. Masters, and G.A. McDonald.** 2005. Improving the specificity of 16S rDNA-based polymerase chain reaction for detecting Borrelia burgdorferi sensu lato-causative agents of human Lyme disease. Journal of Applied Microbiology. 98(4): 962-970.

10 . **Development of Vaccines to Infectious Diseases.** 2007. Lyme Disease. http://www.brown.edu/Courses/Bio_160/Projects2005/lyme_disease/history.htm.

11. **Hedayati, H.** 1992. Lyme disease. The Journal of the American Osteopathic Association. (6):755-60, 763-5.

12. **Holden, K., E. Hodzic, S. Feng, K. J. Freet , R. B. Lefebvre, and S.W. Barthold.** 2005. Coinfection with Anaplasma phagocytophilum alters

Borrelia burgdorferi population distribution in C3H/HeN mice. Infection and Immunity. 73(6): 3440-4.

13. **Kristoferitsch, W.** 1989. Lyme borreliosis in Europe. Neurologic disorders. Rheumatic Diseases Clinics of North America. 15(4): 767-74.

14. **Lebech, A.M.** 2002. Polymerase chain reaction in diagnosis of Borrelia burgdorferi infections and studies on taxonomic classification. APMIS Supplementum. 105: 1-40.

15. **Massung R.F., K. Slater, J.H. Owens, W.L. Nicholson, T.N. Mather, V.B. Solberg, and J.G. Olson.** 1998. Nested PCR assay for detection of Granulocytic Ehrlichiae. Journal of Clinical Microbiology. 36(4): 1090-1095.

16. **Masuzawa, T.** 2004. Terrestrial distribution of the Lyme borreliosis agent Borrelia gurgdorferi Sensu Lato in East Asia. Journal of Infectious Diseases. 57: 229-235.

17. **Oliver, J.H.** 1996. Lyme borreliosis in the southern United States: a review. Journal of Parasitology. 82(6):926-35.

18. **Ostfeld, R.S., C.D. Canham, K. Oggenfuss, R.J. Winchcombe, and F. Keesing**. 2006. Climate, Deer, Rodents, and Acorns as Determinants of Variation in Lyme-Disease Risk. PLOS Biology. 4(6): 145.

19. **Piesman, J. and C.M. Happ.** 1997. Ability of the Lyme disease spirochete Borrelia burgdorferi to infect rodents and three species of human-biting ticks (blacklegged tick, American dog tick, lone star tick) (Acari:Ixodidae). Journal of Medical Entomology. 34(4): 451-6.

20. **Schwan, T.G., P.F. Policastro, Z. Miller, R.L. Thompson, T. Damrow, and J.E. Keirans**. 2003. Tick-borne relapsing fever caused by Borrelia hermsii, Montana. Emerging Infectious Diseases. 9(9): 1151-4.

21. **Schwan, T.G., S.J. Raffel, M.E. Schrumpf, P.F. Policastro, J.A. Rawlings, R.S. Lane, E.B. Breitschwerdt, and S.F. Porcella.** 2005. Phylogenetic analysis of the spirochete Borrelia parkeri and Borrelia turicatae and the potential for tick-borne relapsing fever in Florida. Journal of Clinical Microbiology. 43(8): 3851-3859.

22. **Shadick, N.A., C.B. Phillips, E.L. Logigian, A.C. Steere, R.F. Kaplan, V.P. Berardi, P.H. Duay, M.G. Larson, E.A. Wright, K.S. Ginsburg, J.N. Katz,and M.H. Liang.** 1994. The Long-Term Clinical Outcomes of Lyme Disease: A Population-based Retrospective Cohort Study. Annals of Internal Medicine. 121(8): 560-567.

23. **Shadick, N.A., C.B. Phillips, O. Sangha, E.L. Logigian, R.F. Kaplan, E.A.Wright, A.H. Fossel, K. Fossel, V.Berardi, R.A. Lew, and M.H. Liang.** 1999. Musculoskeletal and neurologic outcomes in patients with previously treated Lyme disease. Annals of Internal Medicine. 131(12): 919-26.

24. **Shadick, N.A., C.B. Phillips, O. Sangha, E.L. Logigian, R.F. Kaplan, E.A. Wright, A.H. Fossel, K.Fossel, V.Berardi, R.A. Lew, and M. H. Liang.** 1999. Musculoskeletal and neurologic outcomes in patients with previously treated Lyme disease. Annals of Internal Medicine. 131(12): 919-926.

25. **Shone S.M., H.J. Dillon, S.S. Hom, and N. Delgado.** 2006. A novel real-time PCR assay for the speciation of medically important ticks. Vector Borne and Zoonotic Diseases. 6(2): 152-160.

26. **Steere, A.C., S.E. Malawista, D.R. Snydman, R.E. Shope, W.A. Andiman, M.R. Ross, and F.M. Steele.** 1977. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. Arthritis and Rheumatism. 20(1):7-17.

27. **Wikipedia.** 2007. Lyme Disease. http://en.wikipedia.org/wiki/Lyme_disease [Lyme disease.Wikipedia, the free encyclopedia.htm.].

VITA

Jonathan Lindsay McMahan was born in Clarksville, Tennessee on June 11, 1983 to Steven Hoyt and Rebecca Slayden-McMahan of Clarksville, Tennessee. He has one sibling, Christopher Steven McMahan. He graduated from Montgomery Central High School in Clarksville, Tennessee in May 2001. After graduation he attended Austin Peay State University where he completed a B.S. Degree in Biology and Agriculture. During this time he served as a research assistant assuming responsibility for assisting the ecology-based research projects of both professors and graduate students of the Biology Department and the Center of Excellence for Field Biology.

Jonathan graduated from Austin Peay State University with an undergraduate degree in May 2005 and received a graduate research assistantship in the Biology Department of Austin Peay State University. The graduate research assistantship involved duties and responsibilities in both teaching and research. Primary teaching responsibilities included undergraduate labs for general biology and human anatomy and physiology classes. Responsibilities also included both personal and collaborative research, providing the opportunity to pursue various study areas and become involved in multiple research projects with professors who are leaders in their respective fields.

The assistantship additionally afforded the opportunity of collaborating with peers involved in various areas of investigative study and representing the university at various state and regional conferences supported by the professional organizations of the respective fields. A presentation on Chytrid fungus was

delivered at the Second College of Science and Mathematics Forum at Austin Peay State University in Spring 2006 and at the 2006 Tennessee Academy of Science. Other research activities have included ecology-based research projects of both professors and graduate students of the Biology Department and the Center for Field Biology of Austin Peay State University. These studies included conservation efforts in land restoration, stream habitat restoration, water quality testing, plant and animal surveying, and water microbial analysis. An additional research activity included stream assessment and surveying utilizing GIS and GPS technologies. Cooperative research efforts were conducted with the following organizations: the Tennessee Wildlife Resource Agency, Middle Tennessee State University Biology Department, Red River Watershed Association, and a Yale University grant funded project.

Jonathan completed a M.S. in Biology in August 2007. His thesis was: SURVEY OF MIDDLE TENNESSEE MAMMALIAN AND TICK POPULATIONS FOR TICK-BORNE DISEASES: *BORRELIA BURGDORFERI*, *BORRELIA HERMSII*, AND *ANAPLASMA PHAGOCYTOPHILA*. He plans to pursue a doctorate in ecology from the University of Florida following the completion of an internship with the Environmental Protection Agency.