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The Lyme Disease Spirochete in Tick Species Collected from Warren County, Kentucky

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THE LYME DISEASE SPIROCHETE IN TICK SPECIES COLLECTED FROM WARREN COUNTY, KENTUCKY

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

By

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* * * * *

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ABSTRACT

The overall goal of this present study was to determine the prevalence of the Lyme disease spirochete, *Borrelia burgdorferi*, in south central Kentucky. A survey of ticks collected from a single location in Warren County, Kentucky was conducted between the months of April and June, 2010. A total of 293 ticks were collected. Three species were identified: 264 (91.7 %) of the ticks were *Amblyoma americanum*, 18 (6.6%) were *Dermacentor variabilis*, and 1 (0.35%) was *Ixodes scapularis*. The ten remaining ticks were immature nymphs and could not be identified. After identifying the species and sex of each tick, DNA isolations were performed for each specimen. DNA concentrations ranged from 2.2 ng/µl to 90.9 ng/µl. A PCR-based assay was used to test for the presence of DNA from *Borrelia burgdorferi* into DNA. The results indicated that 31/293 (10.95%) of ticks tested positive for *Borrelia burgdorferi*.

Keywords: Capstone Experience, Thesis, B. Burgdorferi, Lyme Disease, Ticks, Kentucky

Dedicated to my family, friends, and advisors

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FIELD OF STUDY

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CHAPTER I

INTRODUCTION

Tick Classification

Ticks are considered the second most common vector for common infectious diseases (Parola and Raoult, 2001; Hill and Wikel, 2005). Ticks are classified in the order Ixodida, with two predominant tick families: the Ixodidae, or “hard ticks” and Argasidae, or “soft ticks”. The family Ixodidae is of greatest importance to humans because it includes the species that serve as medically relevant vectors (Oliver et. al, 2008; Parola and Raoult, 2001). Ticks can range in size from 2-30 mm (Parola and Raoult, 2001). The scutum, an important taxonomic characteristic, is a sclerotized dorsal plate which is present on Ixodid ticks, and can actually expand during feeding (Sonenshine et al.,1993; Parola and Raoult, 2001).

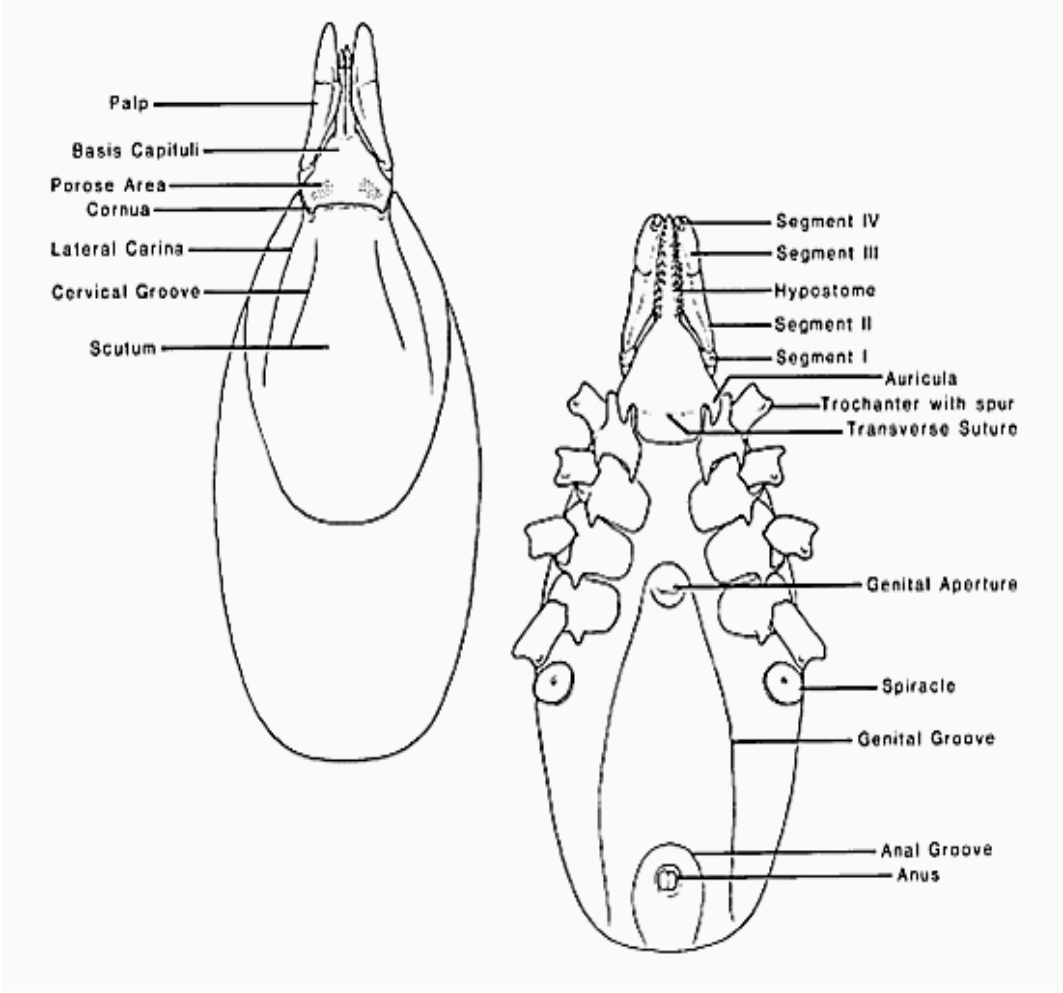

Figure 1.1 Diagram of hard tick characteristics (from Keirans and Litwak, 1989)**.**

Tick Life-Cycle

The typical tick life cycle is composed of egg, larva, nymph, and adult stages. This cycle is generally 2.5 years long, but the exact length depends on several factors including: temperature, relative humidity, and photoperiod (see Fig. 2; Oliver et. al, 2008; Parola and Raoult, 2001). Adults and nymphs possess 4 pairs of legs, whereas larvae only possess 3 pairs (Oliver et. al, 2008; Parola and

Raoult, 2001). All tick stages lack antennae and are different from insects in that they do not contain divided regions such as the head, thorax, and abdominal region (Parola and Raoult, 2001). Common reservoir hosts for ticks in the U.S.A. include dogs, opossums, raccoon, and deer. The distribution of the reservoir hosts explains why ticks are highly distributed in rural regions of the Southeast United States.

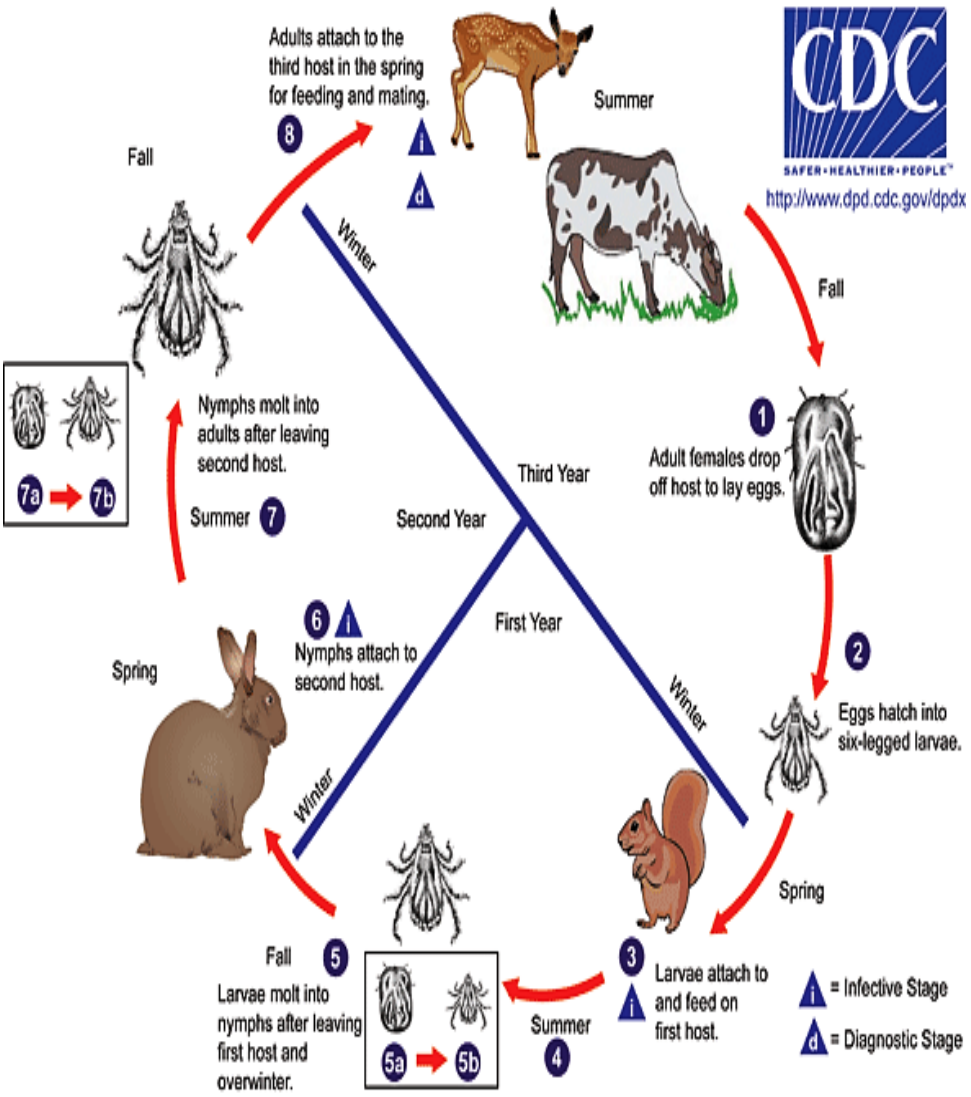

Figure 1.2 Three host life cycle of Ixodid ticks (from http://www.dpd.cdc.gov/dpdx).

Ticks of Southeast U.S.A.

In recent years, tick-borne infections such as Lyme disease, southern tick associated rash infection (STARI), human ehrlichiosis, anaplasmosis, and Rocky Mountain spotted fever (RMSF) have become emerging concerns for the southeastern United States. The most common tick vectors for these diseases are *Dermacentor variabilis* (the American dog tick or wood tick; see Figure 1.3)*, Amblyomma americanum* (the lone star tick; Figure 1.4)*, and Ixodes scapularis* (the deer tick; Figure 1.5). *Dermacentor variabilis* is found primarily east of the Rocky Mountains. The males and females can be distinguished by viewing the dorsal sections. Females are typically larger if blood-fed with a small scutum located right behind their mouth-parts while the male scutum covers the majority of the dorsal side of the tick.

Amblyomma americanum is known as the lone star tick because of the single white spot on the female's dorsal side. The male contains disconnecting white marks around the posterior area. *Ixodes scapularis* lacks white markings on its dorsal side (the black-legged tick; Figure 1.5). Female *Ixodes* have an orange-red colored scutum, whereas the males' dorsal side is black in color with a light brown ring around the scutum.

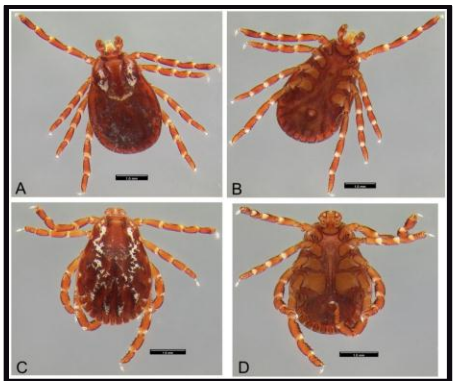

Figure 1.3. ***Demacentor variabilis***
A: Adult Female Dorsal B: Adult Female Ventral
C: Adult Male Dorsal D: Adult Male Ventral

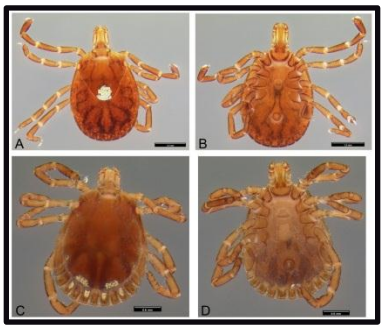

Figure 1.4. ***Amblyomma americanum***
A: Adult Female Dorsal B: Adult Female Ventral
C: Adult Male Dorsal D: Adult Male Ventral

Figure 1.5 Ixodes scapularis
A: Adult Female Dorsal, B: Adult Female Ventral

Tick-borne Infections in Southeast U.S.A

The tick-borne disease known as Lyme disease is caused by the spirochete bacterium, *Borrelia burgdorferi* which is transmitted by the tick vector *Ixodes scapularis* (Fraser et al., 1997, Sonenshine et al., 1993). After an incubation period of approximately one week, patients typically experience a "bulls-eye" rash at the inoculation site, fever, headache, and muscle or joint pain. It is also possible for flu-like systems to occur without rash. It is noted that approximately 24,000 cases of Lyme disease were confirmed in the U.S.A. in 2011. The symptoms of southern tick associated rash infection (STARI) are similar to Lyme disease following a bite from a lone star tick. However, the bacterium that causes STARI is believed to be *Borrelia lonestari*, nonetheless, additional research is required to confirm this hypothesis (Varela, et. al 2004). Ehrlichiosis is a general name describing bacterial diseases caused by bacteria in the genus *Ehrlichia*. According to the Center for Disease Control and Prevention (CDC), approximately 600 cases of ehrlichiosis were reported in 2006 with a gradual increase in cases per year, except for 2010. The *Ehrlichia* species responsible for the greatest number of cases of ehrlichiosis in the U.S.A. is *E. chaffensis*, which is transmitted by the lone star tick, *Amblyomma americanum*. Symptoms of this disease include fever, chills, headache, and myalgia. Anaplasmosis is a disease caused by the bacterial pathogen, *Anaplasma phagocytophilu*m (Dumler et al., 2005). Symptoms of this disease are very similar to influenza and include headaches, fever, chills, and muscle aches. The black-legged tick species *Ixodes scapularis* is believed to be responsible for

transmitting Anaplasmosis and *Borrelia burgdorferi* to humans in the upper Midwest and eastern part of the U.S.A. Statistics show approximately 1,761 cases were reported to the CDC in 2010. Finally, the most serious tick-borne infection in the U.S.A. is Rocky Mountain spotted fever (RMSF). The disease is caused by the bacterium *Rickettsia rickettsii* which is transmitted by, *Dermacentor variabilis*. Initial symptoms for RMSF occur in one to two weeks following the tick bite and include: fever, severe headache, and muscle pain. Later symptoms are more severe and include; nausea, vomiting, maculopapular rash, abdominal pain, joint pain, and diarrhea. The CDC statistics show that approximately 250-1200 cases of Rocky Mountain spotted fever have been reported each year since 1920 until 2008. Additionally, a colleague of mine reported the high prevalence of *D. variabilis* in his research conducted in 2010.

The goal of this study was to determine the prevalence of the Lyme disease spirochete *Borrelia burgdorferi* in ticks from south central Kentucky. A collection of ticks from a single location in Warren County, Kentucky was conducted between the months of April and June, 2010. *Amblyomma* and *Dermacentor* species are vital organisms to study because they are the most abundant tick species in the sample. Furthermore, the large numbers of ticks are liable to carry and transmit a pathogen to a host.

CHAPTER II

MATERIALS AND METHODS

Tick Collection and Identification

Ticks were collected from a single female canine in south-central Kentucky in the Anna community of Warren County (Figure 2.1) between the months of April and June, 2010. Ticks were removed with forceps and placed directly into vials of 70% ethanol. Individual ticks were identified using Keiran and Litwak's "Pictorial Key to the Adults of Hard Ticks" (1989), and Ruedisueli and Manship's "Tick Identification Key". Identified ticks were placed into microcentrifuge tubes containing fresh 70% ethanol and stored at room temperature prior to DNA extraction. The pictures displayed in Figures 1.3?-1.5 were taken with a Syncroscopy unit microscope; and represent the tick species collected in this study (photos courtesy of Kristina Tackett, 2009).

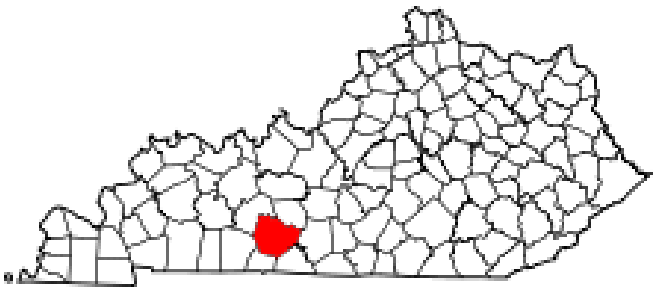

Figure 2.1 Map of Kentucky with Warren County highlighted.

DNA Isolation

A QIAamp DNA mini kit (Qiagen Corporation), catalog number 51304, was used to extract genomic DNA from individual ticks by following the manufacturer's suggested supplemental protocol for the purification of total DNA from ticks. The DNA concentration of each sample was determined using a Thermo-Scientific Nanodrop ND-100 Spectrophotometer. The spectrophotometer was blanked with sterile nano-pure water after each reading. DNA samples were stored at -20°C until further use.

Polymerase Chain Reaction (PCR)

The *Borrelia burgdorferi* specific oligonucleotide primers are SL1 5'-ATAGGTCTAATAATAGCCTTAATAGC-3') and SL2 (5'-CTAGTGTTTTGCCATCTTCTTTGAAAA-3'). The primers are specific for the OspA gene of *B. burgdorferi* (Demaerschalck et al., 1995; Sparagano et al., 1999). The PCR reaction consisted of 12µl of 5X Green *GoTaq®MasterMix,* 1.0 µl (2 µM) of each primer, 2.5 µl *B. burgdorferi* genomic DNA control or sample DNA, and sterile nanopure water in a final volume of 25 µl. A negative control (no DNA template) was included with each PCR analysis. The B*. burgdorferi* genomic DNA was purchased from the American Type Culture Collection. All reaction mixtures were then placed into an automated DNA thermal cycler for 40 cycles under the amplification conditions (shown in table 2.1):

Temperature (°C)	Time (minute)
95	1
95	1
57	1
72	1
72	5
4	forever

Table 2.1. Thermo-cycler conditions for PCR.

Gel Electrophoretic Analysis

After the cycles were complete, reactions were stored at -20°C until further analysis by agarose gel electrophoresis. The PCR products were separated on 1.2% agarose gels at 180 Volts for 120 minutes and stained with ethidium bromide. To minimize contamination during PCR, pipetting was performed in an area reserved for PCR using designated pipettes and pipette tips with aerosol barriers. The aersol barrier tips were purchased through Thermo Scientific , part 2749. Following agarose gel electrophoresis, gels were examined under UV light to view amplification products.

CHAPTER III

RESULTS

A total of 293 ticks were collected between the months of April and June, 2010. Three species were identified; 264 of the ticks were *Amblyomma americanum*, 18 were *Dermacentor variabilis*, and 1 was *Ixodes scapularis*. The ten remaining ticks were immature and could not be identified. The results of tick species and sex determination are shown in Table 2.

	A. americanum	D. variabilis	I. scapularis	Unknown	Total
Male	**116**	**4**	**0**	**-**	**123**
Female	**135**	**12**	**0**	**-**	**147**
Nymph	**10**	**2**	**1**	**-**	**13**
Total	**264 (90.1%)**	**18(6.1%)**	**1 (0.4%)**	**10 (3.4%)**	**293**

Table 3.1 Number of male and female ticks identified for each species.

DNA Isolation

Genomic DNA samples were isolated from individual ticks using the Qiagen QIAamp DNA mini kit protocol. Sample concentrations ranged from 3.3 ng/µl to 123.7 ng/µl with an average of 37.3 ng/µl.

PCR

Optimization of the PCR amplification reaction was completed using primers SL 1 and SL 2, using genomic *B. burgdorferi* as a positive control. Amplification resulted in a PCR product of approximately 307 base-pairs.

A total of 31 out of 293 tick DNA samples tested positive for *B. burgdorferi*. Table 3.2. representative results of PCR analysis are shown in Figure 3.1, which displays the results of electrophoretic analyses of the PCR samples conducted on November 4, 2012. Table 3.3 describes the samples used in the PCR assay in Figure 3.1.

Tick Species	Positive Results Females	Positive Results Males	Percent Positive
A. americanum	18	12	10.6%
D. variablis	1	0	0.34%
I. scapularis	0	0	0.00%
Total	19	12	10.95%

Table 3.2 Details of positive PCR samples for male and female ticks of *A. americanum, D. variablis, I. scapularis.*

1 Kb ladder—

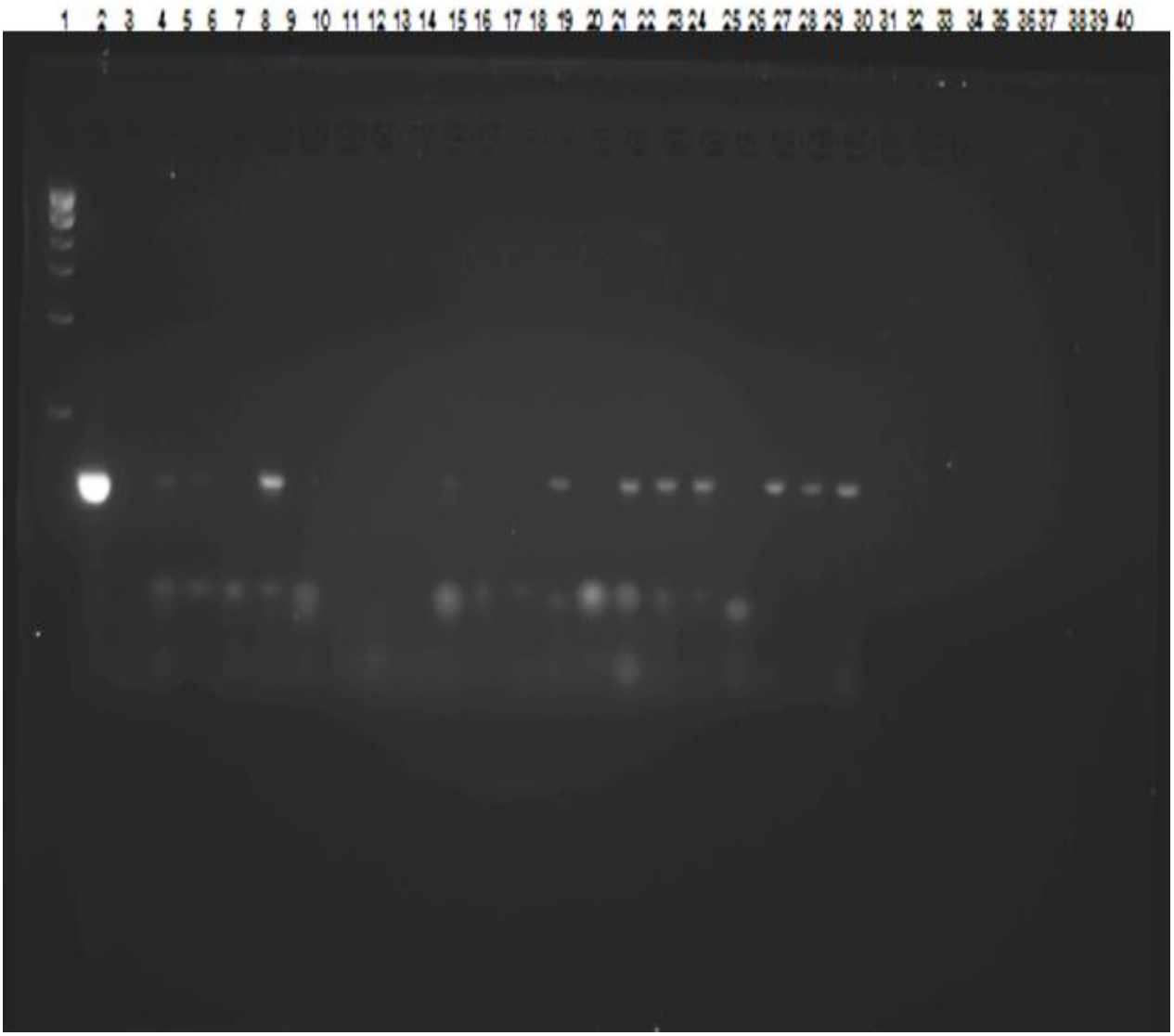

Figure 3.1. **Agarose gel electrophoretic separation of *B. burgdorferi* DNA amplified from the ticks collected in south-central Kentucky in the Anna community of Warren County (representative result shown). The lane 1 is a 100 bp ladder. Lane 2 is the positive control of genomic *B. burgdorferi* DNA. Lane 3 is the no template negative control. Lanes 4-40 represent the samples tested using PCR. Lanes 4, 5, 8, 15, 19, 21-23, and 27-29 show amplified DNA from the individual ticks samples collected from the single Warren county canine reservoir.**

LANE	SAMPLE ID	LANE	SAMPLE ID
1	Ladder	21	06
2	+ *B. burg* control	22	10
3	- control	23	07
4	45	24	11
5	84	25	09
6	00	26	13
7	76	27	12
8	85	28	15
9	38	29	14
10	91	30	16
11	43	31	17
12	92	32	18
13	17	33	19
14	95	34	20
15	41	35	21
16	47	36	08
17	54	37	09
18	44	38	25
19	01	39	24
20	05	40	27

Table 3.3. Key to tick DNA samples utilized for PCR and gel electrophoretic analysis as shown in Figure 3.1.

CHAPTER IV

DISCUSSION

Very few comprehensive surveys have been performed to determine the prevalence of ticks and tick borne pathogens in the southern United States. According to the CDC, the prevalence of reported cases of Lyme disease and other tick borne infections in humans is on the rise in the southern U.S.A. However, there are many who have expressed concern that Lyme disease may be under-diagnosed and under-reported in this region (Little et al., 2010; Diuk-Wasser et al., 2012). Lyme disease has been called “The New Great Imitator”, because of the array of multisystem clinical manifestations that may occur (Fallon et al., 1998). Consequently, Lyme disease can be difficult to diagnose, particularly in regions where the infection is regarded as “rare”. Improved surveillance for tick vectors and their pathogens should be an important priority in the southeastern U.S.A. to better inform physicians, public health officials, and the public about the actual disease risks.

In the present study, tick vectors and the Borrelia burgdorferi spirochete were studied by using DNA extraction and PCR analysis. All ticks were collected from a single location in Warren County, Kentucky from one domestic canine. This canine was taking preventative tick medication, so the majority of ticks were

on the dog, but were not firmly attached or engorged, so the risk of infection of B. burgdorferi in the dog was reduced or eliminated. The majority of the ticks collected from the dog were Amblyomma americanum (90.1%). The second most prominent species was D. variabilis (6.1%). Only 1 tick was identified as Ixodes scapularis (0.4%). The sample pool also contained approximately 10 nymphs of various species that were not identified. The difference in the number of tick species can be explained in part due to the season that ticks were collected. A. americanum and D. variabilis are usually collected from April through September. Additionally, the low prevalence of adult Ixodes ticks was most likely due to the fact they are typically more abundant from October to March (Tackett, 2009).

Approximately 31/283 tick samples tested positive for B. burgdorferi by PCR analysis. This was determined by utilizing the SL1 and SL2 primers which are specific for the OspA gene of B. burgdorferi (Demaerschalck et al., 1995; Sparagano et al., 1999). The OspA gene encodes a major surface protein of B. burgdorferi. The frequency of B. burgdorferi positives by tick species was 10.6% (30/283) for A. americanum and 0.34% (1/283) for D. variabilis. In a 2009 study conducted on ticks removed from wild-trapped raccoons in Warren and Barren Counties of Kentucky, Tackett reported 25% of ticks collected were positive for B. burgdorferi as determined by PCR. Our results were closer to those of a 1993 study conducted at Land Between the Lakes in Western Kentucky that showed an overall prevalence of 15.6% B. burgdorderi positive ticks (Duobinis-Gray et al., 1993; Kollars, 1993).).

B. burgdorferi is thought to be transmitted exclusively by ticks in the genus Ixodes. Only one specimen of Ixodes scapularis was collected in our study, and that tick that was reported negative for B. burgdorferi by PCR analysis. However, an unexpected result in this study was the amplification of B. burgdorferi DNA from the two most abundant tick species in our collection, A. americanum and D. variabilis, since neither of these species are considered to be competent vectors in Lyme disease transmission. Although the prevalence of B. burgdorferi in these ticks was not as high as reported in the 2009 study by Tackett, our results confirm her conclusion that these ticks are undoubtedly feeding on B. burdorferi positive reservoir mammals in the same habitat.

The high percentages of A. americanum carrying B. burgdorferi DNA detected in this study, and in Tackett's 2009 study, raise concerns about the overall prevalence of B. burgdorferi in this region, and the potential risk of transmission of this disease to humans. According to the CDC, the number of human Lyme disease cases continues to increase, and additional studies are critical for increasing public awareness and for accurately evaluating disease risk. It will also be important to determine the prevalence of B. burgdorferi in potential reservoir hosts in the area such as raccoons, rodents, and deer. In addition, it will be necessary to sequence the positive PCR products generated in the present study in follow-up studies to confirm that the detected species is in fact B. burgdorferi sensu stricto, the genospecies that has been most frequently cultured from patients with Lyme disease. Nevertheless, our results suggest that the risk of contracting Lyme disease in south central Kentucky is likely greater

than the public (and public health officials) may presume. It is imperative that additional surveillance studies are initiated soon to accurately estimate the prevalence of the Lyme disease spirochete in tick vectors as well as reservoir hosts in Kentucky and in other states across the southeastern U.S.A.

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