DISTRIBUTION OF TICKS OF MEDICAL AND VETERINARY IMPORTANCE ALONG THE CHISHOLM TRAIL AND DEVELOPMENT OF A MOLECULAR ASSAY TO DETECT *RICKETTSIA* SPP. IN FIELD-COLLECTED TICKS IN OKLAHOMA

By

JACLYN E MARTIN

Bachelor of Science in Entomology

Oklahoma State University

Stillwater, Oklahoma

2012

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2016

DISTRIBUTION OF TICKS OF MEDICAL AND VETERINARY IMPORTANCE ALONG THE CHISHOLM TRAIL AND DEVELOPMENT OF A MOLECULAR ASSAY TO DETECT *RICKETTSIA* SPP. IN FIELD-COLLECTED TICKS IN OKLAHOMA

Thesis Approved:

Dr. Bruce Noden

Thesis Adviser

Dr. Francisco Ochoa-Corona

Dr. Justin Talley

ACKNOWLEDGEMENTS

I want to thank my parents, Dr. John W. Martin and Cheryl Martin, APRN-CNP, for all their support before, during, and after graduate school. I also want to thank my cats Spooky and Banchee for keeping me company while I studied, wrote, and stressed.

I want to thank my thesis advisor Dr. Bruce Noden and my other committee members Dr. Francisco Ochoa-Corona and Dr. Justin Talley and the other members of the department, especially Drs. Jackie Lee, Kris Giles, Jack Dillwith, and Phil Mulder for all their help and support.

Finally, I want to thank all the members in my laboratory and those who helped with lab work: Jennifer Salazar, Faithful Williams, Krista Pike, Jes Mitcham, Sharon Andreason, Chris Timmons, and Trisha Dubie - you guys rock! Also, thanks to all my other school friends: Sulo, Kyle, Sunny, Shane, Theresa, Tanner, and Brandon - you guys are awesome!

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: JACLYN E MARTIN

Date of Degree: MAY, 2016

Title of Study: DISTRIBUTION OF TICKS OF MEDICAL AND VETERINARY IMPORTANCE ALONG THE CHISHOLM TRAIL AND DEVELOPMENT OF A MOLECULAR ASSAY TO DETECT *RICKETTSIA* SPP. IN FIELD-COLLECTED TICKS IN OKLAHOMA

Major Field: ENTOMOLOGY AND PLANT PATHOLOGY

Abstract: Cases of tick-borne rickettsial diseases are increasing in the Central United States, augmenting the need for updated distribution maps for primary tick vectors and the development of new surveillance tools to detect changing disease patterns. Throughout the summer of 2014, ticks were collected at state parks and other public use lands in counties surrounding the Chisholm Trail in central Oklahoma. Results demonstrated that established populations of *A. americanum* currently exist in 20 out of 21 sampled counties in eastern and central Oklahoma. Ticks were more prevalent in the eastern part of the state compared to the western side. These results augment previously published studies and demonstrate the importance of updating distribution maps. Loop-mediated isothermal amplification (LAMP) is a novel molecular tool which can be developed to detect arthropod-borne pathogens in field-collected arthropods. The aim of this part of the project was to design and validate a novel LAMP assay to detect *Rickettsia* spp. in field-collected ticks. All results were compared with a pan-specific PCR assay which targeted the 17 kDa gene of *Rickettsia* spp. The 802 field-collected ticks from various Oklahoma state parks during the summer of 2014 were tested using the two assays. Preliminary results indicated that the two tests correlated, signifying that LAMP assay is a promising molecular surveillance tool which can be used to effectively detect pathogens in field-collected ticks. This assay can then be further developed for use in resource-limited countries to assist with surveillance of tick-borne pathogens.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. LITERATURE REVIEW...5

Rocky Mountain spotted fever and other Spotted Fever Group Pathogens. 5
Ehrlichia chaffeensis 10
Ehrlichia ewingii 13
Francisella tularensis 16
West Nile Virus..19
Potential Emerging Diseases..21
Molecular Tests for Rickettsial Species..27
A Brief History of LAMP use with Arthropod-borne Pathogens 31

III. Tick Distribution along the Chisholm Trail in Oklahoma 34

Introduction..34
Materials and Methods...38
Results..41
Discussion 43

Chapter | Page

IV. Development of a Novel Assay to detect *Rickettsia* spp. in field-collected ticks .47

Introduction 47
Materials and Methods 49
Results 57
Discussion 75

V. CONCLUSION 81

REFERENCES 83

LIST OF TABLES

Table Page

1 PCR, qPCR, LAMP, and HDA comparative chart 30
2 Collection dates and locations by county along the Chisholm Trail 38
3 Ticks collected along the Chisholm Trail in summer 2014 42
4 Sequences and Thermodynamics of PCR and LAMP primers 57
5 Selected BLASTn results of sequences from 17kDA *Rickettsia* spp. after gel extractions 61
6 BLAST results from randomly chosen positive samples of *Rickettsia* 17 kDa primer set with State Park tick pools 65
7 BLAST results from randomly chosen positive results from 17 kDa *Rickettsia* PCR primers, sequences from 200 bp band 65
8 BLAST results from randomly chosen positive samples of *Ehrlichia chaffeensis* HE1 and HE3 nest primer set with state park tick pools 67
9 Prevalence of *Rickettsia* spp. And *Ehrlichia chaffeensis* in ticks collected from state parks in Oklahoma, summer 2014 74

LIST OF FIGURES

Figure Page
1 Map of Ehrlichiosis incidence in the United States in 2010 12
2 Map of Ehrlichiosis incidence in the United States in 2008-2012.......................15
3 West Nile Virus neuroinvasive disease incidence by state- US, 2013 20
4 Geographic range of lone star tick in the United States......................................25
5 Established and Reported Lone Star Tick Populations prior to summer 2014 as per Springer et al. (2014) with a line representing the historic Chisholm Trail running through Oklahoma 35
6 Rocky Mountain spotted fever incidence map 2000-2007 36
7 Map of dryer climate versus more humid climate of Oklahoma with Chisholm Trail (black line highlighted with yellow) (WebAtlas.com)................................36
8 Eco-Regions of Oklahoma with Chisholm Trail 37
9 Vegetation Flag 40
10 Carbon dioxide trap...40
11 Established and reported Lone Star Tick populations before and after Summer 2014 along the Chisholm Trail 42
12 Location of PCR and LAMP primers on Rickettsial gene..................................50
13 Rickettsia 17kDa 24° C Temperature Gradient 60
14 Rickettsia 17kDa 5° C Temperature Gradient 60
15 Rickettsia 17kDa Specificity Panel...61
16 Rickettsia 17 kDa Rickettsia Species Specificity Panel 61
17 17 kDa *Rickettsia* spp. PCR sensitivity of primer set 63
18 Verification end-point PCR for Tick DNA...64
19 17 kDa *Rickettsia* spp. PCR primers with Tick Pools 64
20 Phylogenetic relationship of PCR diagnostic sequences obtained by PCR from the 17 kDa gene from tested Rickettsia spp. 65
21 *Ehrlichia chaffeensis* ECC and ECB primer set Temperature Gradient.............66
22 *Ehrlichia chaffeensis* HE1 and HE3 nested primer verification 66
23 Ehrlichia PCR State Park Tick Pool samples, nested primers HE1 and HE3......67
24 Ehrlichia PCR State Park Tick Pool samples 67
25 Rickettsia LAMP F3 and B3 primer set at 5 μM concentration Temperature Gradient PCR with positive control *R. rickettsii* 68
26 Rickettsia LAMP Temperature Gradient 65-70° C, *R. rickettsii* as template......69
27 Rickettsia LAMP Temperature Gradient 62-67° C with *R. rickettsii* as template.. 69
28 LAMP *Rickettsia* spp. specificity panel..70
29 LAMP Rickettsia spp. Specificity...70
30 LAMP *Rickettsia* spp. sensitivity panel using *R. rickettsii* as template...............71

Figure Page

31 LAMP Rickettsia MgSO4/Betaine Concentration Gradient, *R. rickettsii* as template....71
32 LAMP *Rickettsia* MgSO4/Betaine Concentration Gradient, *R. rickettsii* as template....72
33 LAMP *Rickettsia* State Park Tick pools with *R. rickettsii* as positive control 73

CHAPTER I

INTRODUCTION

Ticks of the family Ixodidae comprise all of the hard ticks. Hard ticks are pests to humans, wildlife, livestock, and companion animals. Besides the annoyance and pain, tick bites can also lead to secondary infections and even paralysis. More importantly, ticks are vectors of several pathogens which cause disease. Ticks are a major problem in Oklahoma because of their wide geographic distribution across the state and their role as vectors for human and zoonotic pathogens. Little is currently known concerning the ecology and risk of specific environments in which tick-borne diseases can be encountered in the state. One group of tick-borne pathogens, the spotted fever group Rickettsiae (SFGR), is responsible for causing Rocky Mountain Spotted Fever (RMSF) and other similar diseases. In order to identify the 'hot zones' of RMSF transmission in the state and monitor the changes in tick presence throughout the state, it was necessary research the ecology and environmental factors responsible for the transmission of RMSF in the South-Central United States.

Several tick species of public health importance exist in Oklahoma. Major species include the Lone Star tick (*Amblyomma americanum*), the American dog tick (*Dermacentor variabilis*), the blacklegged tick (*Ixodes scapularis*), and the Gulf Coast tick (*A. maculatum*). *A. americanum* is a vector of the pathogens responsible for ehrlichiosis, tularemia, and southern tick associated rash illness (STARI) as well as various spotted fever group rickettsiae (SFGR). *D.*

variabilis ticks transmit the pathogens causing Rocky Mountain Spotted Fever (RMSF) and tularemia as well as other SFGR. *Ixodes scapularis* transmits the pathogens that cause Lyme disease, anaplasmosis, and babesiosis while *A. maculatum* vectors the SFGR, *R. parkeri* (CDC 2015). Ticks transmit a diverse array of pathogens and are found in different geographic regions and environments, have varying host preference, and exhibit seasonality.

The Lone Star tick (*A. americanum*) has historically been distributed in the eastern half of the United States, but recent surveillance studies have reported movement further west (Springer et al 2014; Barrett et al 2015). This species prefers wooded areas with dense underbrush and can be found on a vast number of host species (Bishopp and Trembley 1945). Besides a population along the California coast, the American dog tick (*D. variabilis*) is mainly distributed east of the Rocky Mountains (CDC 2015). The American dog tick prefers more humid environments and grassy areas with brush cover (Bishopp and Trembley 1945). The preferred hosts of immature stages of *D. variabilis* are small rodents whereas adults prefer dogs and raccoons (Bishopp and Trembley 1945). The Gulf Coast tick (*A. maculatum*) is distributed along the Gulf Coast, although populations have also been found further inwards from the coastal states (Teel et al. 2010; CDC 2015). The immature stages prefer ground-nesting birds and the adults prefer larger mammals such as cattle (Bishopp and Trembley 1945). The blacklegged tick (*I. scapularis*) maintains populations in the eastern half of the United States with a break in distribution along the Ohio River Valley (CDC 2015). Hosts of *I. scapularis* vary greatly, but immatures prefer small rodents or reptiles (Oliver et al 1993; Durden et al 2002). In Oklahoma, immatures feed on skinks which have an immune response to kill pathogens which is why Lyme disease does not occur widely in Oklahoma (Garvin et al 2015). The most common tick species to be encountered by the public in Oklahoma is the Lone Star tick (*A. americanum*) which increases the risk of exposure to tick borne pathogens. More field work in Oklahoma is needed to determine the current geographic range of the Lone Star tick and other species and their associated pathogens.

Several molecular tools exist to detect spotted fever group rickettsiae (SFGR) with the most commonly used being the Polymerase Chain Reaction (PCR). However, other molecular technologies have been developed which have expanded diagnostic options. A novel molecular assay, known as Loop-mediated isothermal Amplification (LAMP), emerged in 2000 which utilizes one temperature, instead of four cycling temperatures, and four or six primers, instead of two (Notomi et al 2000). LAMP has several benefits including: practicality, rapidity, simplicity, robustness, sensitivity, and specificity (Notomi et al 2000). Besides being a faster way to detect tick-borne pathogens from infected ticks, this tool could be modified into a cost effective assay for use in field settings. LAMP would be especially useful in areas in need of easy to use molecular methods such as Africa where vector-borne diseases are more common.

LAMP assays have been created to detect several vector-borne disease pathogens of public and veterinary importance transmitted by mosquitoes, flies, sandflies, and ticks. Most of the research using these assays have tested for the pathogen in blood samples and have not tested pathogen prevalence in the arthropod vector. Of significance, a LAMP assay has detected the ompB region of the SFGR genome from patient blood samples located in China (Pan et al 2012) while another LAMP assay detected *Borrelia burgdorferi*, causative agent of Lyme disease, in field collected ticks (Yang et al 2012). Because of their ease and adaptability, it is clearly advantageous to adapt LAMP assays for the testing of arthropods in order for this molecular method to become a viable tool for resource-limited areas.

Based on the information gathered for this project, the research objectives for this research project were as follows:

1. Determine established vs reported *Amblyomma americanum* tick population in counties along the historic Oklahoma Chisholm Trail.

- Hypothesis: Ecological and environmental conditions in counties west of the historic Chisholm Trail will reduce the likelihood of established Lone Star tick populations.

2. Develop a Loop-mediated isothermal Amplification (LAMP) diagnostics assay for the detection of Spotted Fever Group Rickettsiae (SFGR) in field collected ticks.

   - Hypothesis: The LAMP test will perform better in all aspects than the end-point PCR assay to detect SFGR in field-collected ticks from Oklahoma state parks

CHAPTER II

LITERATURE REVIEW

This literature review will cover relevant research related to the research objectives. The first section will focus on a brief overview of important vector-borne diseases in Oklahoma. Each disease causing pathogen will be presented individually with sub-sections focusing on the history, epidemiology, occurrence in Oklahoma, signs and symptoms, arthropod vectors, and gaps in information. The second section will summarize the different molecular techniques used to test arthropods (mainly ticks) for *Rickettsia* spp.

Rocky Mountain spotted fever (RMSF) and other Spotted Fever Group Pathogens

History

Rocky Mountain Spotted Fever (RMSF) is associated with the bacteria *Rickettsia rickettsii* and other *Rickettsia* spp. These bacteria are gram negative and intracellular in the order Rickettsiales (Gage et al 1995), replicating inside host cells (Raoult and Roux 1997). Order Rickettsiales infects the endothelial cells thus causing the distinguishing rash associated with RMSF (Raoult and Roux 1997). Scientists believe the organelle, mitochondria, has its origins from the order Rickettsiales (Gray 2012). In particular, *R. rickettsii* belongs to the Spotted Fever Group (SFG) of the genus *Rickettsia* as opposed to the Typhus group which contains pathogens such as *R. typhi* which are vectored by fleas (Raoult and Roux 1997). The SFGR comprises of around 20 species (Parola et al 2013). The first clinical case of RMSF was described in Idaho in 1899. Later, in 1916, *R. rickettsii* was discovered in blood vessels of an infected patient (reviewed by Dantas-Torres 2007). Since then, RMSF has become one of the most important tick-borne diseases in the United States (CDC 2015). Interestingly, RMSF seems to pose a greater threat to American

Indians than other races. For example, RMSF cases among American Indians in Oklahoma (37.4 cases/million) were higher than those among other residents of Oklahoma (21.6 cases/million) (McQuiston et al 2000). The reason for this higher risk of infection among American Indians is thought to be a part of their occupational and recreational activities which put them at an increased risk for tick exposure (McQuiston et al 2000). In fact, between 1999 and 2007, American Indians had case fatality rate of 2.1% compared with 0.53% and 0.33% in White and African American populations, respectively (Dahlgren et al 2012). Additionally, recent studies are finding that more people have been exposed and might have had an infection with *R. rickettsii* than previously reported (Vaughn et al. 2014).

Rickettsia parkeri, a SFG rickettsiae, was first isolated from cows in Texas in 1937 with infection associated with *Amblyomma maculatum* ticks (Raoult and Roux 1997). The first human case of *Rickettsia parkeri* was detected in 2002 which established the species as pathogenic to humans (Amsden et al 2005). Although cattle were hypothesized to be reservoirs for *R. parkeri*, recent studies have demonstrated that cattle only transport pathogen-infected *A. maculatum* (Edwards et al 2011). Another study has demonstrated that cotton rats are potential reservoirs for *R. parkeri*, but bobwhite quail are resistant to infection (Moraru et al 2013).

Rickettsia amblyommii is another SFG rickettsiae species found in ticks (Jiang et al 2010). While the pathogenicity of this Rickettsiae species is unknown, several studies have indicated that it could be pathogenic to humans or dogs (Barrett et al 2014). In cases where *R. rickettsii* is clearly not present, the SFG rickettsiosis may be caused by another *Rickettsia* spp. and could potentially be *R. amblyommii* (Barrett et al 2014). In a study of RMSF patients from North Carolina, several patients seroconverted to *R. amblyommii* possibly implicating the species as pathogenic to humans (Vaughn et al, 2014). More research must be conducted to determine the extent to which *R. amblyommii* impacts public and veterinary health.

Many other SFGR species exist worldwide and are pathogenic to humans (Parola et al, 2013). Species and their associated pathogens include *R. africae* which causes African tick-bite fever and is distributed in sub-Saharan Africa and the West Indies, *R. conorii* which causes Mediterranean Spotted Fever and is located in the Mediterranean region as well as Africa, and several other pathogenic Rickettsial species (CDC, 2015).

Epidemiology

Spotted Fever Group rickettsiosis has been steadily increasing over the last 20 years in the United States with incidence of reported cases increasing from 1.7 million/persons in 2000 to 7 million/person in 2007 (Openshaw et al, 2010). One of the species responsible, *R. parkeri*, occurs in along the coast in the eastern and southern United States which corresponds with the geographic distribution of *A. maculatum* (CDC 2015). Other SFGR reports include *R. amblyommii* detected in *A. americanum* ticks from several states in the eastern half of the United States (Jiang et al 2010), blood samples from patients in North Carolina seroconverting to *R. amblyommii* and *R. parkeri* without any other evidence of another Rickettsia species (Vaughn et al 2014), and *R. montanensis* isolated from the blood of a six-year old from Georgia after mild symptoms of RMSF and a *D. variabilis* bite (McQuiston et al 2012).

This national trend has also been observed in Oklahoma. Between 2001 and -2005, 15.24% of the total RMSF cases reported to the CDC from the United States were from Oklahoma (Adjemian et al 2009), placing Oklahoma at second highest incidence for that time period. More recently, Oklahoma again had the second highest incidence for SFG rickettsiosis in the United States between 2008 and 2012 (Drexler et al 2016). The hospitalization rate for RMSF in Oklahoma between 1980 and 1996 was 16.9 cases/million, which was significantly higher than the rate for the entire United States (2.1 cases/million) (McQuiston et al 2000). The Indian Health Services (IHS) Oklahoma City region accounted for 69% of all RMSF reported to IHS between

2001 and 2008 with Native Americans in Oklahoma having a higher rate of RMSF compared to other populations in the state (Folkema et al 2012). In the realm of veterinary health, titers of *R. amblyommii* were detected from dogs with tick exposure in Payne County, OK but no clinical symptoms were recorded in those dogs (Barrett et al 2014). This is most likely due to the high prevalence (10%) of *R. amblyommii* in Lone star ticks in Payne County, Oklahoma (Mixson et al 2006).

Signs and Symptoms

The three hallmark symptoms of RMSF and most other diseases caused by SFGR are a sudden onset of fever accompanied by rash and headache (Gage et al 1995). Although high fever, rash, and headache are the characteristic traits of disease, only 40% of patients experience all three symptoms together (Amsden et al 2005). Gage et al (1995) also reported muscle pain and loss of appetite as commonly associated with disease with symptoms usually lasting 2-3 weeks. More severe forms of the disease can result in pulmonary and peripheral edema, renal failure, hemorrhagic purpura, hypovolemia, and low blood pressure or more serious neurological signs (Raoult and Roux 1997). The best form of treatment is with tetracycline antibiotics (Amsden et al 2005).

Arthropod Vectors

Ticks from the family Ixodidae are the primary reservoirs for *R. rickettsii* and other SFG rickettsiae (Gage et al 1995; Raoult and Roux 1997). These include *Dermacentor andersoni*, *A. americanum*, *A. maculatum*, *Dermacentor occidentalis*, *I. scapularis*, *Ixodes pacificus*, and *Ixodes cookei* (Raoult and Roux 1997). Recently, *R. rickettsii* was isolated from *Rhipicephalus sanguineus* after an unexpected outbreak of RMSF in Eastern Arizona (Demma et al 2005). Even with this discovery, *D. andersoni* and *D. variabilis* remain the most important tick vectors of *R. rickettsii* (Gage et al 1995).

The biology between tick vectors and rickettsial organisms is complex as SFG rickettsiae multiply in most of the tick's organs (Raoult and Roux 1997). Ticks spread the bacteria either transovarially or transstadially (Gage et al 1995). When spread transovarially, the bacteria infect all stages of the tick (Raoult and Roux 1997). Rickettsiae are spread to mammalian hosts through the salivary glands during tick feeding (Raoult and Roux 1997). An interesting phenomenon called 'co-feeding' occurs when several ticks aggregate to feed on one spot of vertebrate host. During this feeding period, the rickettsiae spread between ticks without ever infecting the tick host (Raoult and Roux 1997). Transovarial transmission comes at a cost to the tick as *R. rickettsii* does not allow many larvae to emerge from the thousands of eggs laid by the female (Dumler and Walker 2005). The prevalence and abundance of infected ticks in addition to the infected tick's preference for human host all play a role in the risk of ticks spreading the disease among human and animal populations (Raoult and Roux 1997). Normally, this transmission takes 24 hours after attachment, but it can happen in as little as 6-8 hours (Amsden et al 2005).

Rickettsia parkeri is another pathogenic species important to public health which causes SFG rickettsiosis (CDC 2015). The only tick species found to conclusively harbor *R. parkeri* is *A. maculatum* (Raoult and Roux 1997) and not *D. variabilis* (Pagac et al 2014). More research is needed to determine whether other tick species may be involved in the transmission cycle of *R. parkeri*.

R. montanensis, another SFG rickettsiae species involved in SFG rickettsiosis in humans, has been isolated from *D. variabilis* ticks (Pagac et al 2014), as well as *R. amblyommii* (Fritzen et al 2011), *R. cooleyi* (Moncayo et al 2010), and *R. parkeri* (Pagac et al 2014). Carmichael & Fuerst (2010) detected three species of *Rickettsia* in a single *D. variabilis* specimen: *R. bellii*, *R. montanensis*, and *R. rickettsii*.

Finally, the primary tick host for *R. amblyommii* is *A. americanum*, in which the rickettsia plays an endosymbiotic role (Jiang et al 2010). *R. amblyommii* may interfere with *R. rickettsii* infection in ticks considering the lack of *R. rickettsii* infection in ticks with *R. amblyommii* infections (Stromdahl et al 2008). Transmission of *R. amblyommii* among ticks occurs both transtadially and transovarially and is found equally among male and female ticks (Jiang et al 2010). This species is the most commonly occurring SFGR found in *A. americanum* ticks (Jiang et al 2010).

Ecology of Disease

The primary hosts for ticks that transmit SFG rickettsiosis are domestic dogs and wild mammals (Dantas-Torres 2007). For example, ground nesting birds such as quail and wild turkeys as well as white-tailed deer are hosts for various stages of *Amblyomma americanum*, cattle are main hosts for adult *Amblyomma maculatum*, dogs and sometimes humans for hosts for adult *Dermacentor variabilis*, and small rodents are hosts for immature *D. variabilis* (Bishopp and Trembley 1945). Most of these vertebrate hosts are considered reservoirs for SFGR although the importance of each individual host in the maintenance SFGR in the wild is unknown (Dantas-Torres 2007). Humans are normally not infectious and are considered dead end hosts for SFGR (Dantas-Torres 2007). As in the case of *R. parkeri*, cattle are not reservoirs for the pathogen but are important in expanding the distribution of *A. maculatum* populations which transmit *R. parkeri* (Edwards et al 2011).

***Ehrlichia chaffeensis*- Human Monocytic Ehrlichiosis**

History

Ehrlichiosis was first discovered in humans in 1986 in Arkansas and was mistaken as *Ehrlichia canis* (Childs and Paddock 2003). Once realizing that the genus *Ehrlichia* could infect humans, research focused on the possibility of other *Ehrlichia* species. Through sequencing of the 16s rDNA from patients clinically positive for Human Ehrlichiosis, a new species was discovered and

named *Ehrlichia chaffeensis* (Anderson et al 1991). It was described as typical for *Ehrlichia* genus with cytoplasmic growth and “morula” in the leukocyte cells. In addition, the phylogenetic relationship was similar to other species in the genus and most related to *E. canis*.

Epidemiology

Ehrlichiosis has been demonstrated across much of the south and central regions of the United States. In a canine study conducted across the south and central regions of the United States, reported seroprevalence rates for *E. chaffeensis* in Arkansas, Louisiana, Mississippi, Oklahoma, and Texas were 21.4%, 0%, 1.3%, 1.0%, and 0.1%, respectively (Beall et al 2012). Interestingly, domestic dogs have been shown to be co-infected with *E. chaffeensis* and *E. ewingii* at a rate of 1.4 % (Beall et al 2012). More recently, the southeast United States was found to have the highest prevalence of canine exposure in the United States with 3.2% testing positive for *Ehrlichia* spp. (Little et al 2014). This demonstrated a low level endemicity for *Ehrlichia* spp in the southeast and south central parts of the United States. This is mirrored in human populations as well. The map below (Fig. 1) shows the combined human incidence of *E. chaffeensis* and *E. ewingii* across the United States in 2010 (CDC 2015). This national map highlights the high prevalence of Ehrlichiosis in Oklahoma in which pediatric exposure has been reported (Harkess et al 1991). One principle reservoir for *E. chaffeensis* in Oklahoma is the coyotes (Kocan et al 2000; Paras et al 2012).

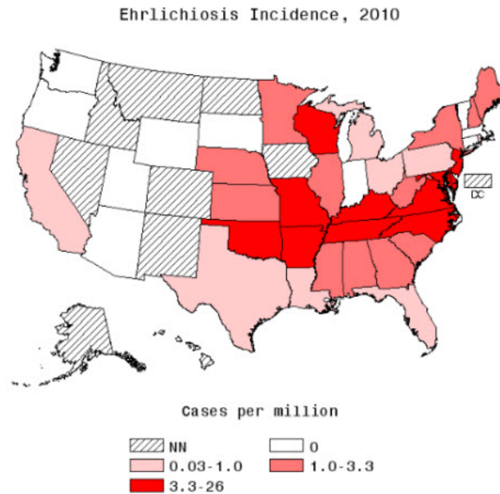

Figure 1. Map of Ehrlichiosis incidence in the United States in 2010 (CDC 2015).

Signs and Symptoms

Symptoms include fever and a light rash (Harkess et al 1991).

Arthropod Vectors

Ehrlichiosis in humans is normally preceded by a report of a tick bite in the weeks prior to infection (Harkess et al 1991) and the principle vector for *E. chaffeensis* is considered to be *A. americanum* (Anderson et al, 1993; CDC 2015). Confirming this fact, 3.3% of *A. americanum* collected and tested from Payne County, Oklahoma were positive for *E. chaffeensis* (Mixson et al 2006). *Dermacentor variabilis* has also been implicated as a potential vector for *E. chaffeensis* transmission (Amsden et al 2005).

Ecology of Disease

The hosts involved in the transmission cycle of *E. chaffeensis* include numerous mammals such as the white-tailed deer, raccoons, red and gray foxes, coyotes, and domestic dogs (Davidson et al 1991; Gage et al 1995; Kocan et al 2000; Beall et al 2012). White-tailed deer are considered as the ideal reservoir host (Varela-Stokes 2007). *E. chaffeensis* is maintained in nature by the white-

tail deer serving as both the reservoir for the pathogen and the blood-meal host for all three stages of *A. americanum* (Lockhart et al 1997). There is also the potential for a similar cycle to occur between Lone star ticks and coyotes in Oklahoma (Kocan et al 2000; Paras et al 2012).

Priority for Research

While *E. chaffeensis* has been reported in a wide distribution throughout the South-Central region of the United States, the white-tailed deer has been implicated as the main reservoir host (Lockhart et al 1997). However, there is a lack of knowledge about other potential reservoir hosts for this important pathogen (Lockhart et al 1997; Childs and Paddock 2003). *E. chaffeensis* has excellent research potential in Oklahoma, but more information needs to be ascertained about hosts and other essential aspects of the bacteria and its associated disease to study the epidemiological aspects of the disease.

***Ehrlichia ewingii*- Human Granulocytic Ehrlichiosis (HGE)**

History

Physicians diagnosed the first case of human ehrlichiosis in the United States in 1986, mistaking the pathogen as *Ehrlichia canis* (Childs and Paddock 2003). After much research, a new species named *Ehrlichia chaffeensis* was identified as a causative agent for human Ehrlichia infections (Anderson et al 1991). Reports soon began circulating in the early 1990s of a granulocytic form of ehrlichiosis (Gage et al 1995). Originally only thought to only infect dogs, *Ehrlichia ewingii* became the probable pathogen responsible in humans as well (Buller et al 1999). The similarities between the two pathogens made discovery process difficult. *Ehrlichia ewingii* and *E. chaffeensis* show no difference by direct immunofluorescence assay, but are distinctly different on Western blot analysis (Buller et al 1999). The serological evidence along with other factors

like morulae in the granulocytes instead of the mononuclear cells of patients provided a strong backing for adding *E. ewingii* as a pathogen present in humans (Buller et al 1999). Subsequent study identified a seroprevelence rate of 7% of Missouri patients' blood tested positive for *E. ewingii* (Buller et al 1999).

Epidemiology

Studies have demonstrated that Ehrlichiosis caused by *E. ewingii* is mainly found in the southern United States. Canine samples from Arkansas, Louisiana, Mississippi, and Texas had seroprevalence rates for *E. ewingii* of 36.9%, 0.7%, 6.0%, and 0.6%, respectively (Beall et al 2012). More recently, dogs from the southeast region of the United States had the highest seroprevelence for *E. ewingii* (3.2%) in the United States at (Little et al 2014). It is obvious that the exposure to this pathogen varies greatly, depending on location. The northeast US had the lowest positive percentage at 0.9% followed by the Midwest at 1.0% and the West at 1.3% (Little et al 2014). This study demonstrated that Oklahoma and surrounding states have the highest prevalence of *Ehrlichia* exposure in the United States. Shockingly, one county in Eastern Arkansas had a canine prevalence rate for *E. ewingii* of 59.0% (Little et al 2010). Depending on where the dogs are coming into contact with infected ticks, this high seroprevalence can come from a relatively low prevalence in the local tick populations. For example, compared with that county in Arkansas, only 6% of *A. americanum* ticks in Mississippi were PCR positive for *E. ewingii* (Castellaw et al 2010). While canines appear to be more at risk for *E. ewingii* than humans, the map below (Fig. 2) demonstrates the incidence of both Ehrlichia in humans in the United States between 2008 and 2012. Note the high incidence rates in eastern Oklahoma (Nichols Heitman et al 2016).

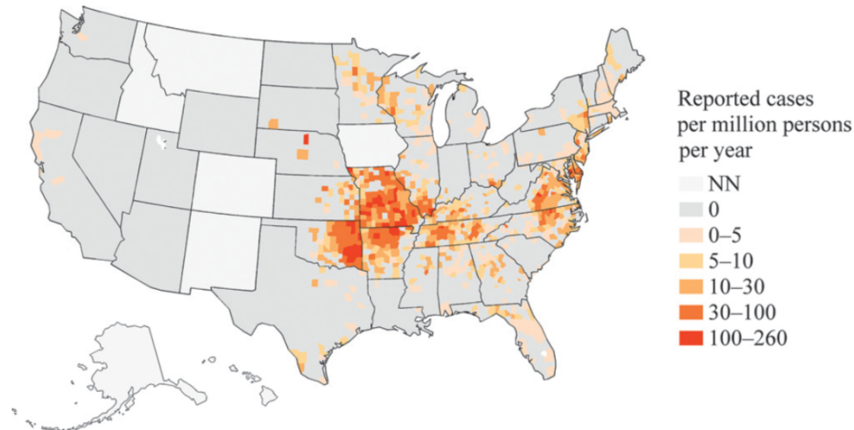

Figure 2. Map of Ehrlichiosis incidence in the United States in 2010 (Nichols Heitman et al 2016).

As the map above demonstrates, there is an increasing incidence of Ehrlichiosis occurring in Oklahoma and this has to be due to increased exposure of human populations to infected ticks. While none of the 25 *Amblyomma americanum* ticks collected in 2003 from Payne County, OK, were infected with *E. ewingii* (Mixson et al 2006), the presence of this pathogen in central Oklahoma is increased dramatically since that time, even in ticks collected from inside the Oklahoma City metropolitan area (Noden, unpublished data). Canine studies have reported a seroprevelence rate for *E. ewingii* in two Oklahoma counties of 55.1% and 25.5% (Little et al 2010), an overall infection rate of 7.2% (Beall et al 2012), counties in east and central Oklahoma with seroprevalence rates of over 2.0% (Little et al 2014) and a high seroconversion and infection rate when dogs were exposed to infected ticks in the lab and natural settings (Starkey et al 2014; 2015).

Signs and Symptoms

In humans, initial symptoms were described as fever, headache, and muscle ache (Gage et al 1995). Interestingly, some sort of immunosuppressive disorder was associated with 75% of positive *E. ewingii* patients (Buller et al 1999). Doxycycline is the treatment of choice (Buller et al 1999).

Arthropod Vectors

Originally, researchers hypothesized that *Ixodes scapularis* was the vector of the granulocytic form of Ehrlichiosis (Chen et al 1994) but it has since been mainly linked to Lone star ticks (Mixson et al 2006; Castellaw et al 2010). Occupational and recreational exposures to ticks were cited as risk factors for infection for humans (Gage et al 1995). All patients testing positive for *E. ewingii* reported exposure to ticks and dogs; one patient's dog tested positive for *E. ewingii*, showing dogs are competent reservoir hosts (Buller et al 1999).

Ecology of Disease

Although the primary reservoir host for *E. ewingii* is considered to be the white-tailed deer, there are other potential tick vectors and animal reservoirs that need to be tested (Childs and Paddock 2003).

Priority for Research

Little is known about how *E. ewingii* maintains itself in nature outside of domestic dogs (Starkey et al 2014; Starkey et al 2015) as it has only recently been discovered as a pathogen in humans and it's pathogenicity in canines is still being worked out. We are aware that southern states, particularly Arkansas and Oklahoma, are affected (Little et al 2014), co-infection with *E. chaffeensis* is common and that often, immunosuppressed individuals may suffer more severely with the disease.

Francisella tularensis- Human Tularemia

History

Tularemia is a bacterial infection caused by *Francisella tularensis* (Taylor et al 1991). First reported in humans in 1914 (Amsden et al 2005), the Jellison Type A, the more virulent form of

the pathogen, is transmitted to humans by arthropods or through exposure to an infected animal (Taylor et al 1991). Historically, the incidence of tularemia was relatively high across the United States until the 1960s when case numbers dropped except in Arkansas and Missouri (Eisen 2007). This change was due mainly to decreased human exposure to infected animals, especially rabbits (Eisen 2007). Most cases from Arkansas and Missouri continue to be linked to tick exposure and subsequent infection (Eisen 2007).

Epidemiology

Tularemia is found throughout the United States but is concentrated mainly in the south-eastern and south-central states, particularly Arkansas, Missouri, and Oklahoma (Taylor et al 1991; CDC 2013). Between 1985 and 1987, New Mexico and Arkansas both averaged over 2.0 cases per one million people while Texas, Louisiana, and Mississippi had under 0.1 case per million population (Taylor et al 1991). Between 1990 and 2003, the incidence of Tularemia was high throughout Arkansas (Eisen et al 2008).

The average annual incidence of Tularemia between 1981 and 1987 in Oklahoma was 4.0 to 15.0 cases per million population (Taylor et al 1991). Historically, eastern Oklahoma has had high incidence rates and recent studies confirm this epidemiology with no expansion in the endemic focus (Taylor et al 1991). One notable aspect in Oklahoma is the higher risk for Tularemia infection among Native Americans (CDC 2013). This stems from their cultural practices, specifically rabbit skin collection for ceremonial practices (unpublished data, Dr. Bradley, OK Department of Health).

Signs and Symptoms

Common symptoms associated with Tularemia include fever, headache, and muscle aches and the case fatality ratio appears to rise with increasing age. The presence of ulcers has correlated with tick-borne Tularemia infections in Oklahoma (Taylor et al 1991).

Arthropod Vectors

As the link with ticks was discovered in Arkansas in the 1940s (Childs and Paddock 2003), *Francisella tularensis* has been identified in many tick species from the genera *Amblyomma*, *Dermacentor*, *Haemaphysalis*, and *Ixodes* (Hopla 1960). Eisen (2007) reported the three main tick vectors are *A. americanum* nymphs, *A. americanum* adults, and *D. variabilis* adults. Transovarial transmission of *F. tularensis* has been shown in multiple tick vectors but further investigation is needed to confirm this information (Hopla 1960). The bacteria appear to maintain different infectivity depending on the species of tick. *A. americanum* remain infected *with F. tularensis* even after feeding on resistant animals (Hopla 1960) while *D. variabilis* transmit *F. tularensis* transstadially with a low mortality rate between molts but transovarial transmission of *F. tularensis* does not occur (Mani et al 2012). While we know that ticks can play a role in the transmission of tularemia, finding an infected tick collected from the field is very rare. In a study of *Amblyomma americanum* ticks in Mississippi, none were found infected with *F. tularensis* (Castellaw et al 2010).

Ecology of Disease

Francisella tularensis lives as many different serotypes. Two principle types exist in the United States. Jellison Type A is found in rodents and lagomorphs and is highly virulent to lagomorphs and humans while Jellison Type B usually is found in water (Taylor et al 1991). One method of transmission for Type A involves direct contact with infected animals such as lagomorphs (Taylor et al 1991). Arthropods transmit *F. tularensis* usually through the bite of a tick (Taylor et al 1991), biting flies, or through handling dead carcasses (Gage et al 1995). Raccoons, opossums, and cats have been implicated as reservoir hosts in the transmission cycle of *F. tularensis* (Taylor et al 1991). Between 1981 and 1987, 76% of the reported cases of Tularemia in Arkansas involved an attached tick and 10% accounting exposure to rabbits (Taylor et al 1991). This was

similar in Oklahoma where 51% of cases involved an attached tick while 38% of cases reported exposure to rabbits (Taylor et al 1991).

Priority for Research

Tularemia poses a threat to public and animal health in the Southwestern United States. Although present in Oklahoma, states with higher risks include Arkansas and Missouri (CDC 2013). Additionally, there are more Tularemia cases in Oklahoma are linked to rabbit exposure compared to tick exposure (Taylor et al 1991). There is potential for research to develop around the socio-cultural practices which are putting persons in the region as risk for this dangerous disease.

West Nile Virus

History

West Nile Virus is a mosquito transmitted virus originating in Africa (Anderson et al 1999). A Flavivirus belonging to the Japanese Encephalitis serocomplex (Lanciotti et al 1999), West Nile Virus was first isolated in the United States in 1999 (Anderson et al 1999).

Epidemiology

The virus was discovered In New York City in 1999 when a surprising outbreak of human encephalitis occurred (Lanciotti et al 1999). The virus was isolated in Connecticut in American crows and a Cooper’s hawk (Anderson et al 1999). This strain was closely related to a serotype from Israel and was hypothesized to have been brought over by travelers from Israel to New York (Dobson and Foufopoulos 2001). By 2002, the virus had spread to the Midwestern portion of the United States and was recorded on to the West Coast in 2003 (Weaver and Reisen 2010).

First reports of the virus in Oklahoma were in 2002 in birds, humans and horses (CDC 2015). Three major outbreaks of West Nile Virus have occurred in Oklahoma since that time: the first in 2003, followed by one in 2007, and a most recently in 2012 (Johnson et al 2015). No significant seasonal or climatic correlations were drawn among these outbreaks which highlights the unpredictable nature of the virus (Johnson et al 2015). A map from the CDC's website is shown below (Fig. 3), outlining the neuroinvasive disease incidence by state.

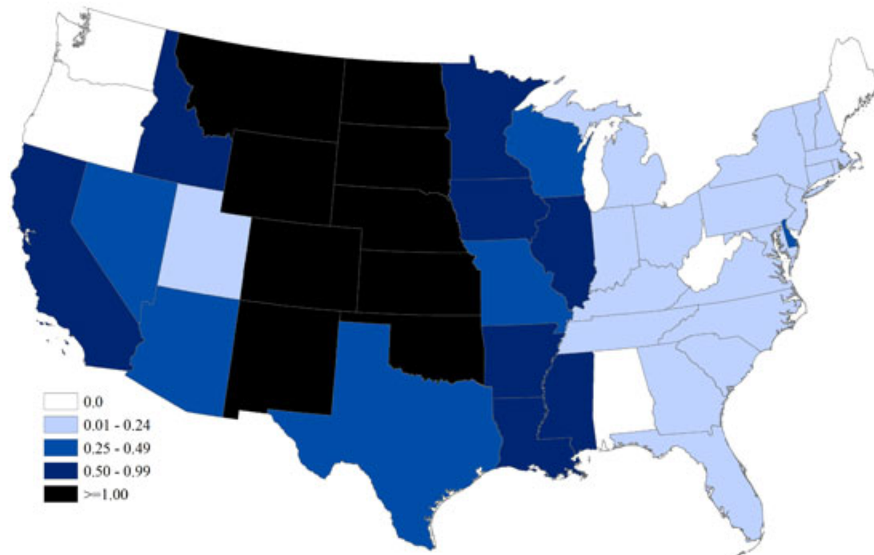

Figure 3. West Nile Virus neuroinvasive disease incidence by state – U.S., 2013 (CDC, 2013)

Signs and Symptoms

According to the Center for Disease Control (CDC 2013), most people with the virus are asymptomatic. The most common symptom is fever with a small portion of infected individuals experiencing serious neurological symptoms, sometimes resulting in death.

Arthropod Vectors

Many mosquito vectors have been implicated in the spreading of the virus (CDC 2012) but there are no known tick vectors (Weaver and Reisen 2010).

Ecology of Disease

The Japanese Encephalitis serocomplex, which includes West Nile virus, utilizes mosquitoes as vectors and avian reservoir hosts (Lanciotti et al 1999). Humans and equids are regarded as accidental hosts (Lanciotti et al 1999). The first mosquito vectors identified in the United States included *Culex pipiens* and *Aedes vexans* (Anderson et al 1999). After the initial epidemic, New York City built a large mosquito control program (Dobson and Foufopoulos 2001). This control program highlights how vector ecology can be utilized to create successful vector and therefore pathogen control efforts. After the outbreak in the United States, scientists developed a vaccine for horses and see no critical need for a human one (Weaver and Reisen 2010).

Priority for Research

The percentage of human deaths caused by West Nile Virus has decreased from 1999-2012 from 11% to 5% with a peak death percentage of 15% in 2001 (CDC, 2013). It is important for regions with potential risk to continue surveillance and control programs. Although there was an outbreak of the virus in Oklahoma in 2012, the research potential for West Nile Virus is low.

Potential Emerging Diseases

Heartland Virus

Heartland Virus is a newly described phlebovirus discovered in two patients from Missouri (McMullan et al 2012). It is closely related to thrombocytopenia syndrome virus (SFTSV) out of China. Both patients reported tick bites prior to infection and had severe symptoms including fever, fatigue, anorexia, and diarrhea (McMullan et al 2012). Since then, the virus has been identified in patients from Missouri and Tennessee (CDC 2015). In May 2014, a 55-year-old man from Delaware County in northeastern Oklahoma died from the virus (CBS News, 2014). Although not all possible vectors are confirmed as of yet, the virus was isolated from field

collected *A. americanum* in Missouri implicating the Lone Star tick as a potential vector of this disease (Savage et al 2013). These two viruses are the only tick-borne phleboviruses known to cause disease in humans (McMullan et al 2012). Much more needs to be done to identify the virus's epidemiological and ecological impact.

Bourbon Virus

Another novel tick-borne virus was identified in the United States in 2015 (Kosoy et al 2015). This virus, termed the Bourbon Virus, was isolated from a farmer under the age of fifty in Bourbon County, KS. The man reported a history of tick bites along with symptoms such as nausea, weakness, diarrhea, fever, anorexia, chills, headache, and muscle aches. The patient died shortly after becoming ill in February 2015. All other tick-borne diseases were ruled out and next generation sequencing determined the illness to be caused by a novel virus of the Thogotovirus group. This viral group contains around six viruses worldwide, one of which has been found in the United States from a sea-bird on the Texas coast (Kosoy et al 2015). The virus has also been confirmed in a man from Payne County, OK (Hulstine 2015). This man fully recovered from his illness which began in 2014. More research must be conducted to determine ecological information and possible tick vectors.

Anaplasmosis

Anaplasmosis is a bacterial tick-borne illness found in humans and cattle. The pathogens responsible are the bacteria *Anaplasma phagocytophilum* in humans and *A. marginale* in cattle. Principle symptoms in humans include fever, headache, chills, and muscle ache (CDC 2015). If present in Oklahoma, the primary tick vector in Oklahoma for *A. phagocytophilum* would be *I. scapularis* (CDC 2015). In 2010, Oklahoma reported an incidence rate of 0.7-3.1 cases per million persons (CDC 2015). In cattle, a positive seroprevalence to *A. marginale* was found of up to 17.6% between the years 1977 and 1991 (Rodgers et al 1994) with seroprevalence rates in

herds from throughout the state ranging from 30-96% (Logan et al 1985). *A. marginale* is endemic to the eastern half of the state (Logan et al 1985), but an outbreak occurred in the western part of the state in 1995 which may have involved *D. albipictus* (Ewing et al 1997).

Lyme Disease

The causative agent of Lyme disease is the bacterial spirochete, *Borrelia burgdorferi*. Scientists discovered *B. burgdorferi* as the etiological agent of Lyme disease and named the novel pathogen in the early 1980s based on Lyme, CT where it was first identified (Johnson et al 1984). Lyme disease symptoms include fever, headache, fatigue, and the characteristic "bullseye" rash (CDC 2015). Although suspected as the vector of the disease, researchers did not isolate *B. burgdorferi* from ticks until 1981. The most important tick vectors are *I. scapularis* in the eastern United States, including Oklahoma, and *I. pacificus* in the western United States (Bowman et al 2009). Reported cases of Lyme disease concentrate in the upper Midwest and the Northeast regions of the United States (CDC 2015). The geographic variations of this disease stems from differences in host feeding preferences for immature ticks in the southern United States. In the northeast and north central part of the country, immature ticks feed on the white-footed mouse which is an excellent reservoir for the pathogen (Oliver et al 1993). However, in the southern United States, immature ticks feed on reptiles which have a natural anti-bacterial component in their system (Durden et al 2002). Between 2004 and 2013, Oklahoma reported fourteen cases of Lyme disease, which is less than 0.1 confirmed case per 100,000 persons each year (CDC 2015). When one compares that incidence rate to the 107.6 incidence rate in Vermont in 2013, it is apparent that Lyme disease is not a problem in Oklahoma (CDC 2015). The pathogen can also infect dogs with the most prevalent regions also involving the upper Midwest and the northeast (Bowman et al 2009). Reports of *B. burgdorferi* in Oklahoma include: infection in partially fed field collected *I. scapularis* and *D. albipictus* adults in the early 1990s (Kocan et al 1992), a 0.2% infection rate in dogs from Oklahoma based on serology (Bowman et al 2009), an 11.7% infection rate in dogs,

mainly in central Oklahoma based on serology (Mukolwe et al 1992), and a 4.5% infection rate based on serology in white-tailed deer concentrated mainly in the eastern half of the state (Mukolwe et al 1992). The serological basis of all these confirmations bring questions that other cross-reacting *Borrelia* spp. may be involved, such as *Borrelia* spp. responsible for relapsing fever or *B. lonestari*, and it may not actually reflect *B. burgdorferi* prevalence. While Lyme disease remains something to look out for in Oklahoma, the low incidence rates combined with lack of a proper reservoir for immature ticks to feed make this disease a low priority for research in the state.

Southern Tick Associated Rash Illness (STARI)

A new spirochete related to *Borrelia* species was discovered in the mid-1990s (Armstrong et al 1996). After confirmation of a new species, the bacterium was named *Borrelia lonestari* (Barbour et al 1996). The disease linked to *B. lonestari* is called Southern Tick Associated Rash Illness (STARI). STARI is described as being "Lyme-like" because of the characteristic rash patients present (James et al 2001). This new spirochete has been isolated from *Amblyomma americanum* (Castellaw et al 2010). Because of *A. americanum*'s wide geographic range (Fig. 4), STARI is considered an emerging disease with far reaching implications for public health. The map below illustrates the geographic distribution of *A. americanum* which is correlated with that of STARI (CDC 2015). There are still many unanswered questions about this newly emerging disease.

Figure 4. Geographic range of Lone Star tick in the United States (CDC, 2015)

Chagas Disease

Chagas disease is caused by the protozoan hemoflagellate *Trypanosoma cruzi* (Bradley et al 2000). It is spread by insect vectors, particularly the Triatomine bugs, and is sustained in mammalian hosts (Bradley et al 2000). When evaluated in Oklahoma, 3.6% of tested dogs had serological evidence of exposure to *T. cruzi* (Bradley et al 2000). Because of the disease's highly infectious and enzootic nature, *T. cruzi* should be considered an emerging pathogen that could invade Oklahoma through Texas (Kjos et al 2009).

Cutaneous Leishmania

Cutaneous leishmania comprises one of three types of the complex parasitic diseases known as the leishmaniases (Clarke et al 2013). Canine leishmania was first reported in the north-central region of Oklahoma in 1979 from a private kennel of 16 dogs (Anderson et al 1980). In 2003 and 2005, a physician reported two cases of cutaneous leishmaniasis in southeastern Oklahoma (Clarke et al 2013). Although not confirmed by molecular assay, the likely parasite for these cases was *Leishmania mexicana* and was linked to the drought experienced in southeastern Oklahoma during that time period (Clarke et al 2013). Both the rodent hosts and sand fly vectors

can be found in Oklahoma (Clarke et al 2013). This disease has not been previously reported in the state, but the potential to spread is something to be considered.

Dog Heartworm

The agent of dog heartworm, *Dirofilaria immitis*, is continues to be more prevalent in Oklahoma. In one study, 6.5% of coyotes tested positive for *D. immitis* infection (Paras et al 2012). A recent study showed more heartworm vectors, including *Aedes albopictus*, are found in urban areas compared to rural ones in Payne County, Oklahoma (Paras et al 2014). Although dog heartworm poses no serious threat to humans, the nematode can still infect humans (Simon et al 2012). The infection results in a rapid immune response in humans, contrasting the slow one seen in dogs, and forms a granuloma around the adolescent worm (Prioleau et al 1976). The granuloma can be mistaken as carcinogenic and could result in an unnecessary surgery (Prioleau et al 1976). Since canine heartworm is so prevalent in Oklahoma, local physicians should be aware of its appearance in humans before jumping to drastic surgical measures.

Zika Virus

The Zika Virus is transmitted primarily by *Aedes aegypti* and *A. albopictus* mosquitoes and from infected blood or sexual fluids and rarely from mother to child (CDC 2016). There are no known tick vectors. Symptoms include fever and muscle aches, although four in five patients are asymptomatic. There is a possible association between Zika virus infected pregnant women and microcephaly in their newborns, but more research needs to be done to confirm this. No locally acquired cases have been reported in the continental United States, although travel-associated cases have been reported from several states including Texas and Arkansas (CDC 2016). This new virus to the Americas should be monitored by public health officials.

<u>Molecular Tests for Rickettsial Species</u>

Several molecular tools exist for detecting specific genes sequences which can be used for the detection and diagnosis of certain genus and species of pathogens (Renvoise et al 2012; Solanki 2012). The gold standard in today's ever-changing technological world is still the Polymerase Chain Reaction (PCR) (Garibyan and Avashia 2013). The use of similar methods, including quantitative PCR (qPCR) and other recent developments, have allowed the development of many assays for the detection of arthropod-borne pathogens (Table 1).

PCR

The Polymerase Chain Reaction, PCR, is considered the standard molecular tool to test DNA. PCR detects and amplifies DNA with the aid of primers, nucleotides, and DNA polymerase (Garibyan and Avashia 2013) (Table 1). PCR works by amplifying a target sequence of DNA exponentially with nucleotide building blocks attaching to primers and using the template DNA strand to construct a new DNA strand on the targeted segment of a gene (Solanki 2012). The first step of PCR, denaturation, heats up the DNA to separate it into two strands followed by the second step, annealing. During this step, primers bind (anneal) to these complementary sequences in the targeting genomic region. Subsequently, the polymerase synthesizes two new strands of DNA starting from the annealed primers using separated strands as template and the multicycle steps of PCR extension results in an exponential increase of DNA (Solanki 2012). PCR has revolutionized diagnostics and the way that we interact with pathogens in arthropods. At the same time, there are a couple of drawbacks which have needed to be improved (Francois et al 2011). First of all, PCR is susceptible to contamination from host or vector cells (Aonuma et al 2008). Additionally, it takes time for the reaction to occur, necessitates technical skills and requires monetary cost to run the test, and requires specific types of facilities in which to run the test (La Scola and Raoult 1997; Francois et al 2011). Based on these observations, while very

helpful, PCR may not be practical for rapidly testing SFG rickettsiae in field-collected ticks, especially in developing countries which lack the basic isolated environment and trained personnel necessary to complete the requirements.

qPCR

Real-time PCR, or qPCR, is an improved version of PCR that allows a quantified measurement of gene expression (Garibyan and Avashia 2013) (Table 1). It is now considered the 'gold standard' for detecting pathogens in arthropods (Richards 2012). qPCR is able to quantify the target PCR product by analyzing the product during the reaction by using fluorescent dyes (Solanki 2012). The fluorescent dyes and amplified product react together to measure the amount of DNA (Solanki 2012). Benefits of qPCR include its high sensitivity and the capability to provide results in real time (Richards 2012). When compared to traditional PCR, qPCR has less contamination issues, is faster, and less expensive (Renvoise et al 2012; Richards 2012). Major downsides to qPCR involve the need for highly technical skills, the high cost of the necessary equipment, and the molecular facilities required to run the tests (Renvoise et al 2012; Sokhna et al 2013). This technique has been developed and used in a fairly sophisticated field lab in West Africa with electricity (Sokhna et al 2013), however, it is not the best choice for a practical epidemiological field study of SFG rickettsiae in ticks.

LAMP

Loop-mediated isothermal amplification (LAMP) is a relatively new technique for molecular testing (Table 1). The molecular test utilizes one enzyme, Bst DNA polymerase, to replace the old strand with a complementary new strand simultaneously in isothermal conditions (Aonuma et al 2008; Njiru 2012). LAMP is described as rapid, robust, simple, sensitive, and highly specific (Mori et al 2013; Yang et al 2012). The test is rapid because it is less dependent on traditional forms of DNA extraction and purification compared with traditional PCR (Kaneko et al 2007).

This step is bypassed because LAMP is able to endure more exposure to contamination such as biological substances (Aonuma et al 2008; Kaneko et al 2007) and it amplifies DNA from partially processed samples (Njiru 2012). LAMP has been shown to be robust in terms of temperature, elongation time, and pH value (Francois et al 2011).

The test's simplicity stems from the quantity of precipitate formed from the stem-loop structure provided by the two principle primers (Aonuma et al 2008). The test is also simple because of the easy to visualize nature of the precipitate (Njiru 2012). The precipitate forms a white color because of the use of magnesium pyrophosphate (Aonuma et al 2008). Many studies have demonstrated LAMP to be far more sensitive than PCR (Pan et al 2012; Yang et al 2012). In a study testing for a pathogen in mosquitoes, LAMP was shown to be very sensitive for identifying the pathogen in the vector despite contamination (Aonuma et al 2008). The high specificity of LAMP derives from the test's use of 4-6 primers to identify 6-8 regions in the DNA (Njiru 2012). One downside to LAMP is lower ability to multiplex while running the test (Francois et al 2011). In fact, one study showed qPCR to have twenty times the multiplexing capacity compared to LAMP (Pan et al 2012). In conclusion, based on LAMP's fast, robust, easy, sensitive, and specific characteristics, it would be an excellent molecular tool to utilize in the field to test ticks for spotted fever group rickettsiae.

HDA

Helicase Dependent Amplification, HDA, is a molecular tool used to amplify DNA. HDA uses a DNA helicase to unwind the target DNA from the sample (Vincent et al 2004) (Table 1). By making use of a helicase, the initial heating steps required for PCR can be bypassed (Vincent et al 2004). Other features of HDA include a high amplification rate, an ability to obtain results from crude samples, and a relative simplicity (Vincent et al 2004). A downside of this technique is its inability to amplify long target sequences (Vincent et al 2004). In conclusion, HDA represents a

potential tool for an epidemiological study of spotted fever group rickettsiae in field-collected ticks.

Molecular Test Chart

Table 1: PCR, qPCR, LAMP, and HDA comparative chart.

	PCR	**Real Time PCR-Quantitative PCR (qPCR)**	**Loop Mediated Isothermal Amplification (LAMP)**	**Helicase Dependent Amplification (HDA)**
Principle Method	Amplifies DNA from template DNA using primers, nucleotides, and DNA polymerase (Garibyan and Avashia 2013). Yes/No qualitative assay.	PCR that amplifies and quantifies DNA. Results seen in real-time-different from PCR because DNA is quantified (Garibyan and Avashia 2013)	A simple isothermal diagnostic tool utilizing four to six primers used to amplify DNA-different from PCR because assay ran at one temperature-distinct from HDA because primers form loops to amplify DNA (Njiru 2012)	An isothermal technique utilizing a DNA helicase to unwind the DNA and amplify the targeted gene at one temperature-uses only two primers (Vincent et al 2004)
Primers used	gltA and ompA genes (La Scola and Raoult 1997).	gltA, ompA, and ompB genes (Renvoise et al 2012)	ompB gene-GenBank Accession: AY331393 (Pan et al 2012)	
Specificity	ompB gene used to detect *Rickettsia* spp, other genes, ompA used to specify among species (Ngwamidiba et al 2005).	ompB gene used to detect *Rickettsia* spp, other genes, ompA used to specify among species (Ngwamidiba et al 2005).	Highly specific-uses 4-6 primers to identify 6-8 regions of DNA (Njiru 2012)	

Sensitivity	Sensitive to femtograms and below (Francoise et al 2011)	Considered more sensitive than PCR, quantifies DNA amount (Richards 2012)	Highly Sensitive (Pan et al 2012)	
Advantages	Used often, sensitive (Francois et al 2011; Garibyan and Avashia 2013).	Sensitive, provides results in real time (Richards 2012).	Practical for field use, rapid, simple, robust, sensitive, specific, cost effective (Francois et al 2011; Yang et al 2012)	No thermocycler needed, has over 10 million-fold amplification, can use crude samples, simple (Vincent et al 2004)
Disadvantages	Expensive, cannot use in the field easily, labor intensive, time consuming, sensitive to contaminants (Francois et al 2011; Sokhna et al 2013).	Expensive, high technical skills required, need molecular facilities (Renvoise' et al 2012; Sokhna et al 2013)	Less multiplexing (Pan et al 2012)	Not able to amplify long sequences (Vincent et al 2004)

A Brief History of LAMP use with Arthropod-Borne Pathogens

Mosquitoes and other Dipterans

LAMP has been utilized to test for pathogens associated with several arthropods including mosquitoes. In most instances, LAMP has been used to test blood and fecal samples, and generally not arthropod samples. Examples of use in arthropod-borne pathogens include distinguishing between Chikungunya and Dengue viruses in serum samples (Lu et al 2012), characterizing four Dengue Virus serotypes from patients (Teoh et al 2013), and testing blood samples for filarial worms such as Loa Loa (Drame et al 2014) and lymphatic filariasis (Takagi et al 2011; Poole et al 2012), as well as testing blood samples for *Trypanosoma brucei* (Mitashi et al

2013; Mugasa et al 2014). LAMP has also been utilized for all of the five species of malaria parasites as well (Mohon et al 2014). Few studies, however, have used LAMP to detect pathogens in field-collected dipterans.

Tick-borne pathogens

LAMP tests have been created to detect tick-borne pathogens. Examples include *Babesia gibsoni* and all three sub-species of *B. canis* in dog blood samples (Ikadi et al 2004; Muller et al 2010), *Babesia bovis*, *B. bigemina* , *Theileria sergenti* and *T. sinensis* in cattle (Wang et al 2010; Liu et al 2012; Liu et al 2013), and *Theileria lestoguardi* from sheep blood samples (Salih et al 2011). Regarding tick-borne viruses, LAMP assays were developed for a newly discovered Bunyavirus with a suspected tick vector (Huang et al 2014), for tick-borne encephalitis virus in ticks, human blood, and mouse tissue samples (Hayasaka et al 2013), and for Crimean-Congo Hemorrhagic Fever in human serum (Osman et al 2013). Although these LAMP tests detect arthropod-borne pathogens, very few actually tested pathogens in the arthropod vector.

LAMP assays for Rickettsial pathogens

Several studies have created LAMP assays to detect Rickettsial pathogens. Testing field-collected ticks as well as bovine, goat, and lamb blood, Nakao et al (2010) designed two sets of LAMP primers for *Ehrlichia ruminantium* and they did not cross-react with other *Ehrlichia* spp. such as *E. canis*, *E. chaffeensis*, or *E. ewingii*. Other studies have used LAMP to detect *Anaplasma ovis* from field samples of sheep blood (Ma et al 2011) and *Anaplasma phagocytophilum* (Pan et al 2011) and a virulent strain of *Orientia tsutsugamushi* (Paris, 2008; Huber et al 2012) from human blood. A LAMP assay detecting SFG rickettsiae was created using the ompB gene of Rickettsia, which is assumed to be spotted fever group specific (Pan et al 2012). This LAMP assay only tested human blood from patients with confirmed SFG cases.

LAMP for testing arthropods

Few examples exist in which LAMP tests were developed to directly test the arthropod vector. Takagi et al (2011) tested pooled mosquitoes for *Wuchereria bancrofti*. However, this study used laboratory infected mosquitoes instead of field-collected mosquitoes. Nzelu et al (2014) used LAMP to screen field collected sandflies for *Leishmania* parasites and Hayasaka et al (2013) created a LAMP assay to detect tick-borne encephalitis virus in laboratory-infected ticks. It is notable that the LAMP test was not inhibited from detecting the virus by the tick extract which is common problem with using molecular testing of unpurified tick DNA. Focusing on bacterial tick-borne pathogens, Yang et al (2012) tested field collected ticks using a LAMP assay for *Borrelia burgdorferi* and reported a better detection rate than both conventional PCR and nested PCR. Finally, *Ehrlichia ruminantium*, the pathogen responsible for Heartwater, was detected in field collected ticks (Nakao et al 2010). To date, no isothermal assay has been developed to detect *Rickettsia* spp. in field-collected ticks.

In conclusion, LAMP assays have not been frequently utilized to detect pathogens from arthropods. However, assays have been developed and used to test field-collected ticks for *Borrelia burgdorferi* and *Ehrlichia ruminantium* (Nakao et al 2010; Yang et al 2012). Besides the one assay has been created to test for *Rickettsia* spp. (Pan et al 2012), no assay has tested ticks. There is, therefore, a need for a rapid, simple, cost-effective LAMP assay to detect *Rickettsia* spp. in field collected ticks.

CHAPTER III

TICK DISTRIBUTION ALONG THE CHISHOLM TRAIL IN OKLAHOMA

Abstract

Amblyomma americanum is an important tick species involved in the transmission of pathogens of veterinary and public health importance. This species is widely distributed across the United States but particularly in the south-central region. Most data used to develop distribution maps has been either historical or based on passive surveillance. Therefore, there is a need to update distribution maps for *A. americanum* across its distribution area. Throughout the summer of 2014, ticks were collected at state parks and other public use lands using vegetation flagging and carbon dioxide traps. Results from active surveillance activity demonstrated established populations of *A. americanum* 20 out of 21 sampled counties in eastern and central Oklahoma. Ticks were more prevalent in the eastern part of the state compared to the western side. This information augments information provided by previously published studies and shows the importance of updating distribution maps.

Introduction

Lone star ticks, *Amblyomma americanum*, have long been associated with annoyance to humans and other mammals. Not only do they cause painful wounds from their bite, they also are vectors for the pathogens which cause ehrlichiosis and have been linked with Rocky Mountain Spotted Fever (RMSF)-like illnesses (Stromdahl et al 2011; Vaughn et al 2014; CDC 2015). There is a significant knowledge gap throughout the U.S. in the geographic areas where *A. americanum* is known to exist (Springer et al 2014). Distribution studies are important in order to better

understand the ecology of the pathogens transmitted by the vector. By knowing where a vector exists, it is possible to link the vector with disease incidence in humans and animals in a given area. This then can be used by public health officials to inform and warn the public about vector exposure prevention measures to reduce risk of exposure to vector-borne diseases.

Historically, the lone star tick was mainly distributed throughout the eastern part of Oklahoma, especially east the historic Chisholm Trail running through Oklahoma (Fig. 5) (Springer et al 2014). When evaluating the incidence of RMSF in humans between 2000 and 2007, Openshaw et al (2010), reported the incidence of illness in most of the central counties and several western counties of Oklahoma (Fig. 6). Only ten counties did not have any reports of RMSF cases. All counties reporting RMSF cases, except two, had an incidence rate of over or equal to 30/million persons (Openshaw et al 2010). When comparing the two maps (Fig. 5 & 6) (Openshaw et al 2010; Springer et al 2014), there was a considerable lack of data regarding the geographic distribution of potential tick vectors.

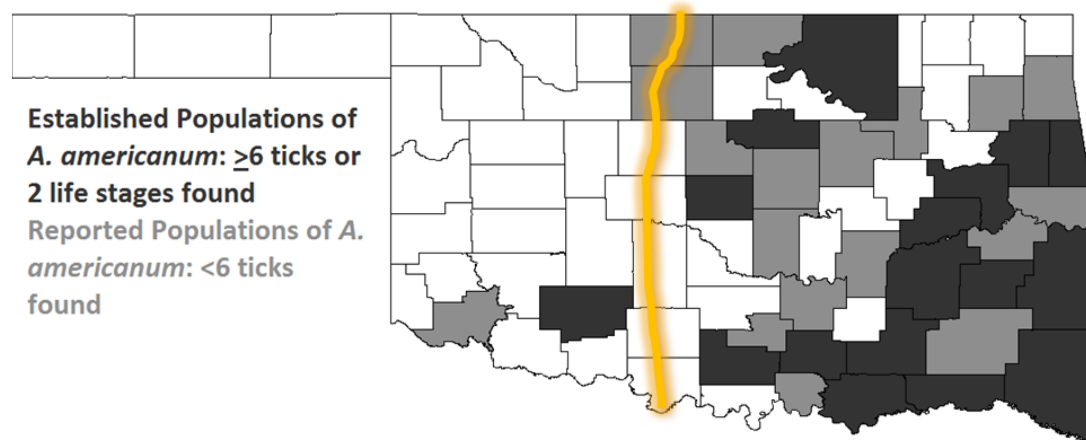

Figure 5. Established and Reported Lone Star Tick Populations prior to summer 2014 as per Springer et al. (2014) with a line representing the historic Chisholm Trail running through Oklahoma.

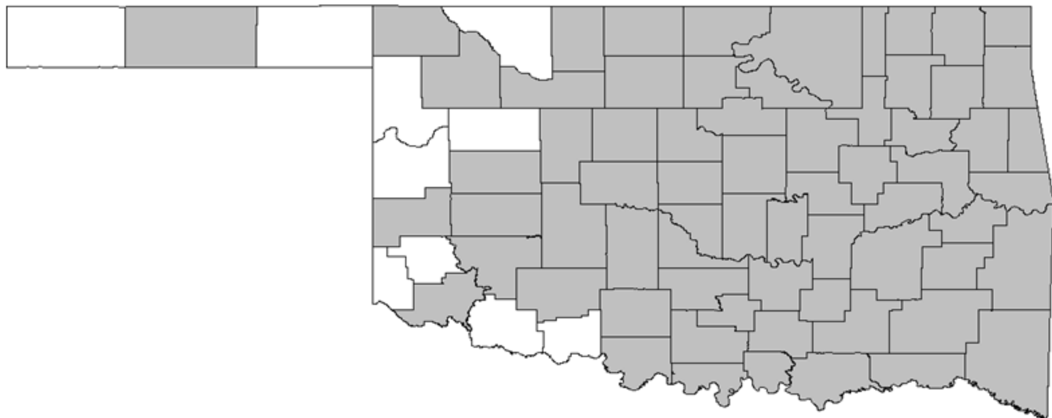

Figure 6. Rocky Mountain spotted fever incidence map 2000-2007 (Openshaw et al 2010).

Previous studies show established *A. americanum* populations mainly in counties east of the Chisholm Trail in Oklahoma, (Fig. 5) (Springer et al 2014). The current study used Highway 81 in Oklahoma as a means to divide the state from north and south, just west of I-35. The historic Chisholm Trail was a cattle drive that ran from San Antonio, TX to Abilene, KS in the late 1800s (Sands 2005). Highway 81 divides the state between two major climatological zones (Fig. 7). The east has a wet, humid climate while the west is dry (Fig. 7) (WebAtlas.com).

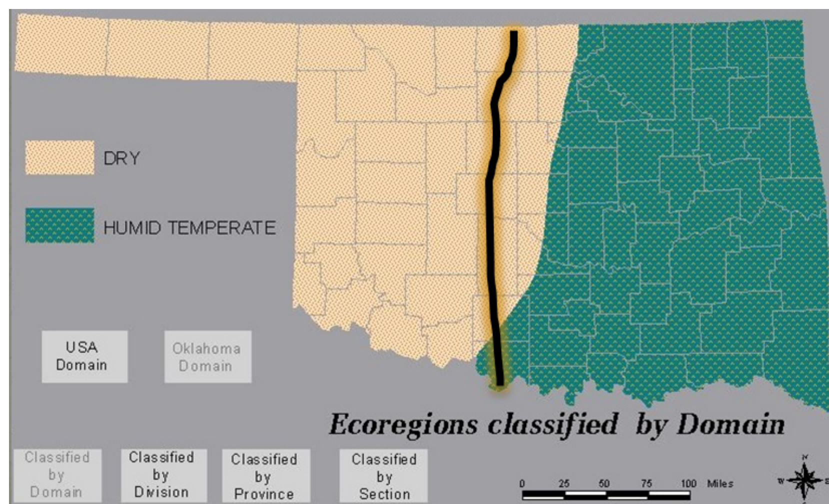

Figure 7. Map of dryer climate versus more humid climate of Oklahoma with Chisholm Trail (black line highlighted with yellow) (WebAtlas.com).

Ticks, especially lone star ticks, generally prefer more humid climates so it is assumed they will be found in the eastern part of Oklahoma. Additionally, Highway 81 divides the state between two major ecological zones (Fig. 8). To the east, there is a mixed oak and cross timbers region while the west has Great Plains (Fig. 8). The eastern part is comprised of woody plants including oak and cedar trees with dense underbrush. This area of Oklahoma has a more humid climate which is associated with Lone Star tick populations and the western part is dryer with less woody plant species.

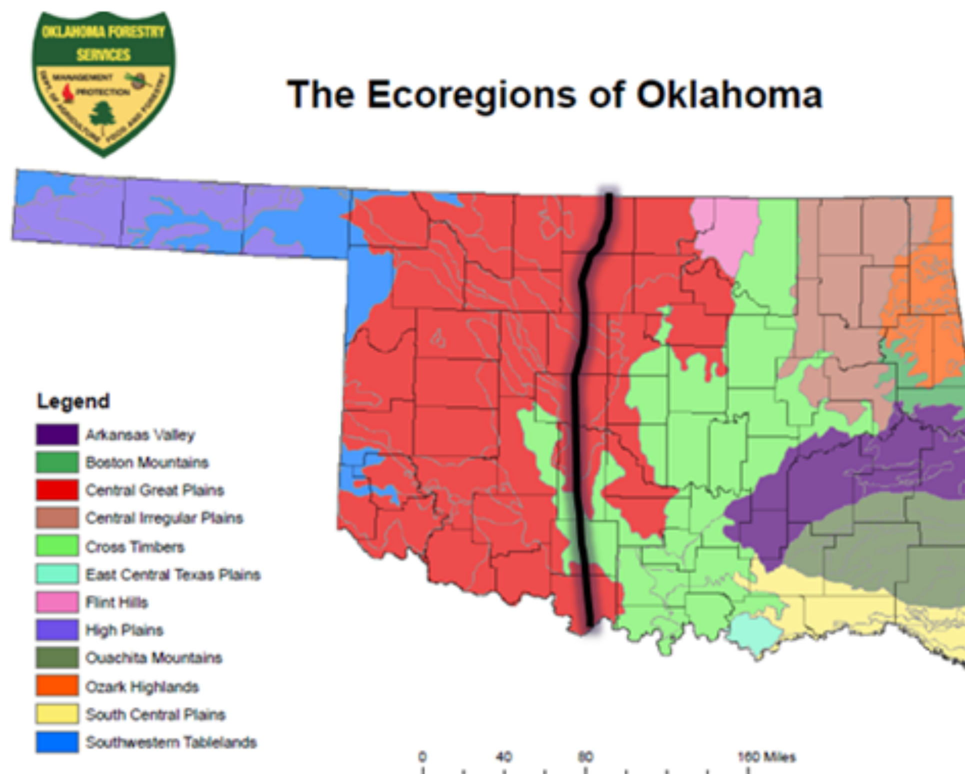

Figure 8. Eco-Regions of Oklahoma with Chisholm Trail (OK Dept Forestry)

The focus of this study, then, was aimed at determining the western boundary for established populations of *A. americanum*. The historical context of the Chisholm Trail provides an excellent landmark in which to explore the distribution of tick species, particularly *A. americanum*, in Oklahoma. The main objective of the study was to identify which counties on either side of and along the Chisholm Trail had established populations of ticks. The hypothesis for this project

was that established populations of *A. americanum* would not be found on the western side of Highway 81 but only found in the counties to the east of the trail. This was because it was felt that the ecological and environmental conditions in counties west of the Chisholm Trail would reduce the likelihood of established Lone Star tick populations. The results of this work are included in Barrett et al. (2015).

Materials and Methods

Tick Population Establishment

Using criteria used by Dennis et al (1998) and Springer et al (2014), established tick population in a county required finding six ticks or more of any life stage or finding two different life stages in one collecting trip. A reported population denoted finding less than six ticks or only one life stage during one sampling event.

Collection Sites

Collection sites were chosen based on areas used by the public, ease of access, and suitable habitat for *A. americanum*. These include, but are not limited to, wildlife management areas (WMA), state parks, national recreation areas, and public parks. The sites were located using Google Maps and locations are reported in Table 2.

Table 2. Collection dates and locations by county along the Chisholm Trail

County	Collection Date	Collection Location
Alfalfa	05/14/14	Salt Plains National Wildlife Refuge
Blaine	05/16/14	Canton WMA
Canadian	05/09/14	Stinchcomb Wildlife Refuge
Caddo	05/15/14	Ft. Cobb WMA
Cleveland	05/09/14	Lake Thunderbird State Park
Cotton	06/27/14	Waurika Lake WMA
Dewey	05/16/14	Canton WMA
Garfield	05/16/14 & 06/02/14	VanBuren Avenue by Hellums Lake & Drummond Flats WMA
Garvin	06/01/14	R. C. Longmire Lake

Grady	06/11/14	Lake Louise Burtschi State Game Reservation
Grant	06/02/14	Rock Island Park near Pond Creek
Jefferson	06/24/14	Waurika Lake WMA
Kingfisher	05/06/14	Gould Bridge
Logan	04/11/14 & 04/23/14	Langston Lake & Liberty Lake
Love	06/24/14	Love Valley WMA
Major	05/16/14	Canton WMA
McClain	06/01/14	Purcell Lake
Murray	06/01/14	Chickasaw National Recreation Area
Pottawatomie	05/09/14	Lake Tecumseh
Stephens	06/24/14 & 06/27/14	Waurika Lake WMA

Collection Methods

Two commonly utilized tick collection methods were employed in this study: vegetation flagging and carbon dioxide traps. The flag for vegetation flagging consisted of a Summer Infant Waterproof Multi Use Pad, White, 27” x 36” (Summer Infant, Amazon.com) stapled on the end of a long wooden pole (Fig. 9) (Ginsberg and Ewing 1989). Vegetation flagging involved performing figure-eight motions with the flag along vegetation (Dantas-Torres 2013). Preferable vegetation for sampling for lone star ticks included woody areas, usually associated with oak, with dense underbrush (Bishopp and Trembley 1945). Vegetation flagging was generally performed over the course of an hour while the carbon dioxide traps attracted ticks.

Carbon dioxide traps utilized dry ice as a source of carbon dioxide to attract ticks (Fig. 10) (Barrett et al 2015). The tarp would be placed among vegetation preferred by *A. americanum*, particularly trees in woody areas. A small-sized, lid-less piece of Tupperware would lie in the middle of the tarp and dry ice would be placed inside the Tupperware. The Tupperware would have holes cut in the sides of it to allow the dry ice sublimate and attract ticks. Ticks were attracted to the carbon dioxide and moved in from the surrounding vegetation and crawled onto the top of the tarp, towards the dry ice.

Carbon dioxide traps were checked every thirty minutes. Underneath the tarp was also checked in case any ticks crawled under the tarp.

Figure 9. Vegetation Flag

Figure 10. Carbon Dioxide Trap

All ticks, from both carbon dioxide traps and flags, were collected with forceps and placed into 70% ethanol. The ticks were taken back to the laboratory for morphological identification.

Laboratory Identification

Ticks were identified using a dissecting microscope. The ticks were identified by species, sex, and life stage using an established key (Kierans and Litwak 1989). After identification, the ticks were grouped into 1.5ml tubes by species and life stage and then placed into -80° C freezer for long term storage.

Results

During the summer of 2014, lone star tick collections were carried out in 21 counties with lone star ticks collected in 20 of those 21 counties along the Chisholm Trail by the end of Summer 2014 (Fig. 11). Established populations of Lone star tick were found in seven counties to the east of the trail (Logan, Cleveland, McClain, Pottawatomie, Garvin, Murray, and Love), seven counties along the trail (Grant, Garfield, Kingfisher, Canadian, Grady, Stephens, and Jefferson), and six counties to the west of the trail (Alfalfa, Major, Dewey, Blaine, Caddo, Cotton, and Woodward) (Fig. 11 & Table 3). No lone star ticks were collected in Woodward County. Collections took place between April and June which corresponded to the peak activity season for *A. americanum* adults and nymphs. Other species collected included: *A. maculatum*, *Dermacentor variabilis*, and *Ixodes scapularis*. *D. variabilis* was collected along with *A. americanum* at most collection sites (Table 2). *I. scapularis* was collected early in the season in April and May (Table 3).

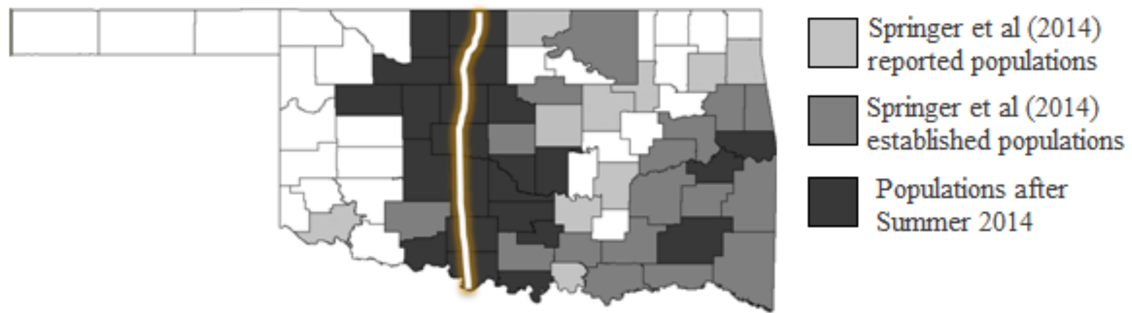

Figure 11. Established and reported Lone Star Tick populations before and after Summer 2014 along the Chisholm Trail.

Table 3: Ticks collected along the Chisholm Trail in Summer 2014

				A. americanum			***D. variabilis***		***A. maculatum***		***I. scapularis***	
Counties	**Collection date**	**# dry ice**	**# flag**	♀	♂	Nymphs	♀	♂	♀	♂	♀	♂
East of Chisholm Trail												
Logan	4/11 & 4/23	0	18	6	6	1					2	3
Cleveland	5/9	12	207	58	90	71						
McClain	6/1	3	9	5	4		1	2				
Pottawatomie	5/9	0	17	5	7		5					
Garvin	6/1	10	4	6	5	1	1	1				
Murray	6/1	15	7	8	6	5	2	1				
Love	6/24	0	8	4	1	1	1	1				
Along the Chisholm Trail												
Grant	6/2	23	7	10	5	13	1	1				
Garfield	5/16 & 6/2	4	19	6	5	3	4	2 (2n)	1			
Kingfisher	5/6	12	0	4	1	3	1	3				
Canadian	5/9	63	6 (Aa)	25	8	36						
Grady	6/11	18	3	6	2	7	3	3				
Stephens	6/24 & 27	5	1	3	2	1		2				
Jefferson	6/24	11	12	13	9		1	1				
West of Chisholm Trail												
Alfalfa	5/14	31	7	17	9	12						
Major	5/16	0	9	2	1	4	1					1
Dewey	5/16	8	0	3	2	1	1	1				
Blaine	5/16	27	0	10	10	7						
Caddo	5/15	24	29	12	14	21	3	3				
Cotton	6/27	11	2	5	1	2	2	1	3			
Woodward	5/21		0									

Discussion

This study identified newly established populations of *A. americanum* populations in 20 out of 21 counties along and around the Chisholm Trail in Oklahoma. This was unknown previous to this study and it provided new evidence of the spread of tick-borne diseases in Oklahoma. A similar study on *A. americanum* distribution in Nebraska showed a change in the distribution of *A. americanum* that had expanded to the north in the state since the late 1980s (Cortinas and Spomer 2013). Much like the report from Cortinas and Spomer (2013), *A. americanum* population distribution has widened in Oklahoma since the last published distribution based on historical studies (Springer et al 2014). Even though the current study only sampled 21 counties, it demonstrated the importance of frequent surveillance of *A. americanum* distribution within a given state. Given the importance of this tick to public and veterinary health, tracking both where the tick is currently established and disease incidence associated with this tick across the state will provide valuable information. This information can be used by public health officials to inform the public concerning the risks of tick infested areas and the potential public health risks associated with this tick.

Another important discovery in this study includes lone star tick populations further to the west than previously recorded (Springer et al 2014). Theories of why lone star tick populations appear in the western counties of Oklahoma include: 1) a migration of tick hosts such as white-tailed deer towards the western part of the state, 2) the tick adaptation to dryer conditions, 3) climate change, or 4) a lack of historical sampling in this region. Previous studies indicate a combination of climate change, landscape modifications, and human activity leads to changes in tick density and distribution (Lėger et al 2013). In terms of climate change, a long-term study on different *Amblyomma* spp. in Zimbabwe concluded higher temperatures and an increase in wet conditions led to an increase in tick populations in areas not previously found (Estrada-Peña 2008). More

research must be conducted to determine how climate change plays a role in Oklahoma's tick population distribution.

This study primarily focused on the active field collection of *A. americanum* in habitats normally associated with lone star ticks (Bishopp and Trembley 1945). Because of this bias, collections of *A. maculatum* and *I. scapularis* were not as numerous as lone star ticks because of the targeted habitat and the season of collection. However, it was interesting to find *D. variabilis* in many of the same locations as *A. americanum*. Even though not found in the same quantities as *A. americanum*, this study demonstrated *D. variabilis* populations can be collected and may overlap with *A. americanum* populations. A possible reason for lower numbers of *D. variabilis* involved a possible increase in resource competition by *A. americanum*, termed 'The Lone Star Effect' (Nadolny et al 2014). Although few studies have researched competition among ectoparasites, one in particular suggests *D. albipictus* out-competes *I. scapularis* for host resources when feeding on white-tailed deer (Baer-Lehman et al 2012). This study did not look at tick hosts, but perhaps *A. americanum* has an edge over *D. variabilis* when locating a host in habitats preferred by both species. Other potential ecological dynamics interacting with tick species distribution could include ecotonal change, predation, mating interactions, and indirect interactions (Bull & Burzacott 2001). In terms of *A. americanum* and *D. variabilis*, this could suggest that climate change could affect how populations are distributed, or the potential impact to a predator of these tick species (Bull & Burzacott 2011). Another factor could be that *A. americanum* have mating pheromones that negate pheromones of *D. variabilis*, or there is a parasite in hosts that affects *D. variabilis* more negatively than *A. americanum* (Bull & Burzacott 2001). Another suggestion of why more *A. americanum* are collected more frequently could also be host preference and availability; *D. variabilis* prefer dogs, raccoons, opossums, and small rodents while *A. americanum* are attracted to multitude of hosts, especially humans (Bishopp & Trembley 1945). This would substantially increase the chances of *A. americanum* being able to establish in areas

when specific host species are limited. More research must be done in order to determine why *A. americanum* seems more abundant than *D. variabilis* especially in the western areas of Oklahoma where it is much more arid and dry.

Several of the limitations in this study involved the relatively short time spent at each location and the targeting of specific habitat to ensure maximum likelihood of encountering *A. americanum* ticks. At each location sampled, carbon dioxide and flagging was done as long as needed for collection of an established lone star tick population. Once a population of lone star ticks was deemed established in the county (six adults or 2 different life stages), everything was packed up and moved to another county site to continue sampling efforts. To know the extent and concentration of lone star ticks as well as other species in each county, more sites in each need to be sampled at different times of the year in order to assess the seasonality of each tick species and life stage. Additionally, by targeting habitat preferred by *A. americanum* (Bishopp and Trembley 1945), we may have missed habitats where other ticks species are more specialized or adapted. As such, this study did not fully evaluate *D. variabilis*, *A. maculatum*, or *I. scapularis* populations. Further research must be conducted in order to have more in depth descriptions of these tick species distributions.

In conclusion, the results from this study identified established *A. americanum* populations in counties to the west of the Chisholm Trail. The results of this study also create a coincidental resemblance to the RMSF incidence map from Openshaw et al (2010). Counties with established lone star tick populations matched up with RMSF disease reports. Even though *D. variablis*, the main historical vector of RMSF, was also found in western counties, *A. americanum* has been implicated as a vector of the spotted fever *R. amblyommii*. The pathogenicity of *R. amblyommii* is unknown, but it is a known symbiont in *A. americanum* and may be involved with SFG rickettsiosis infections in humans (Vaughn et al 2014). This data shows the importance of keeping up to date information on tick populations. Educating the public on how to prevent tick

exposure and reduce the risk of tick-borne disease for themselves and their pets is a necessary aspect to public and veterinary health.

CHAPTER IV

DEVELOPMENT OF A NOVEL MOLECULAR ASSAY TO DETECT *RICKETTSIA* SPP. IN FIELD-COLLECTED TICKS

Abstract

Cases of tick-borne rickettsial diseases are increasing in the Central United States, augmenting the need to develop new surveillance tools to detect changing disease patterns. Loop-mediated isothermal amplification (LAMP) is a novel molecular tool which utilizes four to six primers and runs at one temperature in a simple water-bath. Since the development of this assay, relatively few studies have utilized this novel approach to detect arthropod-borne pathogens in field-collected arthropods with most using LAMP to test blood samples from mammalian hosts. The aim of this project was to design and validate a novel LAMP assay to detect *Rickettsia* spp. in field-collected ticks. All results were compared with a pan-specific PCR assay which targeted the 17 kDa gene of *Rickettsia* spp. The 802 ticks tested using the two assays were collected during the summer of 2014 from various Oklahoma state parks. Preliminary results indicated the two tests correlate, signifying the LAMP assay can be further developed to differentiate rickettsial species. These results show LAMP as a promising molecular surveillance tool which can be used effectively to detect pathogens in field-collected ticks. This assay can then be further developed for use in resource-limited countries to assist with surveillance of tick-borne pathogens.

Introduction

Molecular testing has come a long way since its invention in the mid-20th century (Solanki 2012). Several assays have been designed to detect arthropod-borne pathogens in hosts and the arthropod vector (Richards 2012). PCR is standard molecular detection method, however, new and alternative methods are being established (Richards 2012). Of relevance are Helicase Dependent

Amplification (HDA) and Loop-mediated isothermal AMPlification (LAMP) which are becoming important molecular tools. In most parts of the world, it is difficult to obtain expensive equipment such as a thermocycler and the necessary reagents and primers because of lack of funding or simply access. Equipment requires expensive repairs and the protocols need skilled technicians to run them in a sterile laboratory environment with constant levels of electricity. These monetary and technical constraints make PCR and its derivative assays difficult to perform in developing nations and highlight the opportunity to develop novel assays which can be performed in minimal conditions by operators with limited training.

While developing countries have difficulties accessing the tools and training needed to run assays such as PCR, many also have a high prevalence of disease. Several tick-borne pathogens plague the developing world including numerous *Rickettsia* spp (CDC 2015). In order to determine the prevalence of these pathogens, it is important to test the arthropod vector for the pathogen in order to find the most at risk areas for disease transmission. Combining the high disease prevalence with low budget for molecular equipment acquisition, the importance of low-cost and highly effective molecular assays become obvious. The implementation of LAMP provides an excellent solution to this conundrum.

The aim of this study was to develop a pan-specific assay to detect rickettsia DNA from field-collected ticks. In order to accomplish this aim, we developed a PCR assay to detect the 17 kDa gene of arthropod-borne *Rickettsia* spp. and compared the PCR results to a similarly designed LAMP assay. Ticks collected from state parks in Oklahoma were pooled into groups of up to 10 adult ticks and were tested using both methods. The ticks were also tested with a previously published PCR assay designed to detect *Ehrlichia chaffeensis*. This research project hypothesized that the LAMP assay would have similar if not better results than the PCR assay. It is anticipated that the LAMP assay would be flexible to allow for modification for future field testing of ticks not only in the United States but also in developing countries where it is critically needed.

Materials and Methods

Samples for Testing

Sources for DNA samples of bacterial strains used: Stocks of cell cultured bacteria or DNA samples were generously provided by the following laboratories:

1. Dr. William Nicholson (CDC Atlanta) supplied *R. rickettsii*, *E. chaffeensis*, and *A. phagocytophilum*.
2. Dr. Katherine Kocan and Dr. Edmour Blouin (Oklahoma State University) supplied *A. phagocytophilum*.
3. Dr. Ed Shaw (Oklahoma State University) supplied *R. amblyommii*, *R. montanensis*, *R. parkeri*, and *Coxiella burnetii*.
4. Dr. Jiang and Dr. Allen Richards (Rickettsial Diseases Research Program, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD) supplied *R. conorii* and *R. africae*.
5. Dr. Azad and Dr. Magda Beier (University of Maryland at Baltimore) supplied *R. typhi* and *R. felis*.
6. Dr. Li Ma (Oklahoma State University) supplied *Salmonella typhi* and *Escherichia coli*.

End Point Polymerase Chain Reaction

Rickettsia Species

Primer Design: The web interface application, Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi), was used to design end-point PCR primers located over the 17 kda gene of *Rickettsia* spp. This gene was chosen because it was reported to be a broad spectrum gene unique to *Rickettsia* spp (Pan et al 2012). Considered Accession numbers from NCBI GenBank included KC464548, KC107823, GU723477, GU723476, DQ176856, AY189818, J03371, AY281069,

KC713872, U11013, DQ517291, EU828788, EF689730, JN378400, EF102237, U17008, EF689732, GU395295, and DQ402377. An alignment was performed using ClustalW2 (.http://www.ebi.ac.uk/Tools/msa/clustalw2/). The last date of accession of NCBI for sequence retrieval was April 7, 2014 at 9:00 AM CST. Fig. 12 shows the location of forward and reverse PCR primers binding (red). Concerning alignment of primers with other published sources, the forward primer sequence was already published by Jiang et al (2012). To the authors' knowledge, the reverse primer has not yet been published. Customized 5'-AT rich nucleotide tails were added to both the forward and reverse primers based on previous published work (Arif et al 2014). These 5'-AT rich nucleotide tails increase the melting temperature and allow the primers to be more sensitive (Arif et al 2014).

ATGACAAATCTTTCCAATTTATTTATTTGTAGTTTAACAAAGAAAATATAAAATTCA
ATTATAATGAGGTTCTGTGAACAAAATTAAAAATAATATTTTAAAATACAAATAATT
TAAATTTTGTTCACAGAATCTAGTTTTAGCTTTACAAAATTCTAAAAACCATATACTT
ATTAATTATATATTAATTTAGAGAGAATTATATGAAACTATTATCTAAAATTATGATT
ATAGCTCTTGCAACTTCTATGTTACAAGCCTGTAACGGTCCGGGCGGTATGAATAAA
CAAGGTACAGGAACACTTCTTGGCGGTGCTGGCGGCGCATTACTTGGTTCTCAATTC
GGTAAGGGCAAAGGACAGCTTGTTGGAGTAGGTGTAGGTGCATTACTTGGAGCAGT
TCTTGGTGGACAAATCGGTGCAGGTATGGATGAACAGGATAGAAGACTTGCAGAGC
TTACCTCACAGAGAGCTTTAGAAACAGCTCCTAGTGGTAGTAACGTAGAATGGCGTA
ATCCGGATAACGGCAATTACGGTTACGTAACACCTAATAAAACTTATAGAAATAGC
ACTGGTCAATATTGCCGTGAGTACACTCAAACAGTTGTAATAGGCGGAAAACAACA
AAAAGCATACGGTAATGCATGCCGCCAACCTGACGGACAATGGCAAGTTGTGAATT
GATAGACAAAACGTCATTGCGAGGAAAATTACGAAGTAATT

RED = our 17kd 80bp product for general rickettsia primers

Blue, yellow, purple and green = Aligned sequences for four LAMP primers

Figure 12: Location of PCR and LAMP primers on Rickettsial 17Kda gene

PCR Protocol: PCR reactions were run using GoTaq® Green Master Mix (Promega) in a C1000 Touch™ Thermal Cycler (Bio-Rad). Each reaction consisted of 12.5 µl GoTaq® Green Master

Mix, 9.5 µl nuclease-free water, 1 µl 5µM forward primer, 1 µl 5µM reverse primer, and 1 µl template for a total of a 25 µl reaction.

The PCR cycling protocol consists of an initiation step of four minutes at 95° C then, 40 cycles of denaturation at 95° C, annealing at 61° C, and extension at 72° C. A final extension occurred for four minutes at 72° C. Samples were stored at 4° C until visualization. Optimization of the primers occurred using two temperature gradients: One used a 24° C temperature gradient starting at 43° C to 67° C while the second further optimized the reaction using a 5° C gradient between 58.5° C and 63.5° C.

Primer Verification: The primers were verified using sensitivity and specificity testing. A sensitivity test was carried out using concentrations beginning at 4.7 ng/µl down to 1 fg/µl. Specificity testing included a broad range of closely related *Rickettsia* spp. with DNA from cultured strains of *R. rickettsii*, *R. parkeri*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. montanensis*, *R. felis*, and *R. typhi* was performed. The bands from the specificity *Rickettsia* spp. gel were extracted with a PureLink™ Quick Gel Extraction Kit (Invitrogen) and then sequenced at the Oklahoma State University Core Facility. All resulting sequences were compared using BLAST analysis to verify the reaction. An additional specificity panel was run using three *Rickettsia* spp. (*R. rickettsii*, *R. parkeri*, and *R. typhi*), three closely related bacteria species (*E. chaffeensis*, *Anaplasma phagocytophilum*, and *Coxiella burnetii*) and more distantly related bacteria species (*Escherichia coli*, and *Salmonella typhi*).

Ehrlichia chaffeensis

***Ehrlichia chaffeensis* PCR primers and assay verification**: Nested PCR primers specific for *Ehrlichia chaffeensis* were obtained from the scientific literature (Dawson et al 1996). A temperature gradient for the outside primers ECC and ECB as well as a specificity for the inside primers HE1 and HE3 using a strain of *E. chaffeensis* was performed. Modifications include

changing the annealing temperature of the outside primer PCR protocol from 73° C to 45° C. PCR reactions were run using GoTaq® Green Master Mix (Promega USA) in a C1000 Touch™ Thermal Cycler (Bio-Rad). Each reaction totaled 25 µl which consisted of 12.5 µl GoTaq® Green Master Mix (Promega), 9.5 µl nuclease free water, 1 µl 5µM forward primer, 1 µl 5µM reverse primer, and 1 µl template. The protocol for ECC and ECB, the outside primers, began with an initiation step for three minutes at 95° C followed by two cycles of denaturation at 94° C for one minute, annealing at 45° C for two minutes, and extension at 72° C for 90 seconds, followed by 38 cycles of denaturation at 94° C for one minute, annealing at 45° C for two minutes, and extension at 72° C for 91 seconds. A final elongation step at 72° C occurred for five minutes. The samples were stored at 4° C until run with the nested primers HE1 and HE3. The nested primer protocol for HE1 and HE3 began with two cycles of denaturation at 94° C for one minute, annealing at 55° C for one minute, and extension at 72° C for 15 seconds, followed by 38 cycles of denaturation at 94° C for one minute, annealing at 55° C for one minute, and extension at 72° C for 16 seconds. A final elongation step at 72° C occurred for five minutes. The samples were stored at 4° C until visualization.

Loop-Mediated Isothermal Amplification

Rickettsia Species

LAMP Primer Design: LAMP primers were designed using web interface software, Primer Explorer version 4 software (http://primerexplorer.jp/elamp4.0.0/index.html). The NCBI GenBank accession numbers used were the same as for the end-point PCR primer design. Four primers (F3, B3, FIP, and BIP) were designed. Specific locations of the primers on the 17kDa gene are shown in Fig. 12. Two of the LAMP primers overlapped with sequences previously published by Jiang et al (2012) which include the forward primer (R17K128f2) as well as the

reversed Taq Probe (R17K202). To the authors' knowledge, the rest of the LAMP primers do not match with other published primers targeting the *R. rickettsii* 17kDa gene.

LAMP Protocol: All LAMP reactions were performed using the OmniAmp® RNA & DNA LAMP kit (Lucigen, Middleton, WI). Each reaction totaled 25 µl consisting of 10.7 µl nuclease free water, 2.5 µl 10x DNA Polymerase Buffer C, 0.8 µl dnTPs (25 mM each), 1.5 µl 100 mM $MgSO_4$, 4 µl Betaine, 1 µl 5mM F3 primer, 1 µl 5mM B3 primer, 1 µl 20mM FIP, 1 µl 20mM BIP, and 0.5 µl OmniAmp DNA Polymerase, 50x, of reaction with 25 µl of mineral oil on top of each reaction for a total of 50 µl per reaction. The mineral oil was used as an aid to avoid contamination and to keep contaminants out of the reaction. In addition to LAMP kit (Lucigen) components, 25 mM dnTPs were added to the mixture (Thermo Scientific, United States).

The LAMP reactions were run at 65° C for an hour in GeneMate (BioExpress VWR) followed by five minutes at 95° C to denature the enzyme, thus stopping further DNA amplification. All samples were stored at 4° C until visualization. LAMP reaction temperature was optimized using gradient temperature assays. The first temperature gradient used the F3 and B3 primers between 50° C and 60° C. Subsequent LAMP temperature gradients used all four primers with the first temperature gradient between 65° C and 70° C and the second between 62° C and 67° C.

A magnesium sulfate and betaine gradient assays were also used to determine the optimum concentrations of $MgSO_4$ and betaine. $MgSO_4$ concentrations tested were 4 mM, 6 mM, 8 mM, and 10 mM while the betaine concentrations tested were 0.1 M, 0.3 M, 0.5 M, and 0.7 M. The MgSO4 and betaine concentrations were tested using one of each the $MgSO_4$ and betaine concentrations respectively for each reaction.

LAMP Primer Validation: Validation of the primers was done using sensitivity and specificity assays. A sensitivity assay was carried out using a dilution gradient from 4.7 ng/µl down to 1 femtogram/µl of rickettsial DNA from cultured *R. rickettsii*. Specificity testing included DNA

from cultured strains of a broad range of closely related *Rickettsia* spp. including *R. rickettsii*, *R. parkeri*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. montanensis*, *R. felis*, and *R. typhi*. An additional specificity assay was tested using three *Rickettsia* spp. (*R. rickettsii*, *R. parkeri*, and *R. typhi*), three closely related bacteria species (*E. chaffeensis*, *Anaplasma phagocytophilum*, and *Coxiella burnetii*) and two more distantly related bacteria species (*Escherichia coli*, and *Salmonella typhi*).

PCR and LAMP Visualization

Result Visualization: Gel electrophoresis was used to visualize results of PCR and LAMP reactions. 1X TBE buffer was used in RunOne™ Electrophoresis Cell (Embi Tec, San Diego, CA) at 100 V. Products were visualized on a 2% agarose gel stained with ethidium bromide (0.5µl/ml). Migration time varied between 24-32 minutes for PCR reactions and between 30-37 minutes for LAMP reactions. A 100 base pair ladder was used as a reference for product size (Life Technologies, USA). Gels were visualized using a White Light/UV transilluminator (VWR® USA) and results recorded with a digital CP3800 Canon digital camera (Canon USA) and printed with a Selphi printer (Canon USA).

Samples for Testing

Field-collected tick samples: Tick samples were collected from state parks across Oklahoma between May and August 2014. State parks included those in the east, central, and west regions of the state. Eastern state parks include Sequoyah, Greenleaf, Robbers Cave, Lake Wister, and Lake Tenkiller state parks. Lake Thunderbird accounted for the central state park. Boiling Springs and Roman Nose state parks were the parks in the western portion of the state. Tick species collected include *Dermacentor variabilis*, *Ixodes scapularis* and *Amblyomma americanum*. Ticks were collected by methodically flagging popular hiking trails. Ticks were stored in 70% ethanol

for preservation until DNA extraction. A total of 802 adult ticks were used for this study. Collection methods are described in Chapter 3.

DNA extraction from tick samples: DNA extraction methods were modified from a previously described extraction method (Salazar 2015). Ticks were washed in two sets of de-ionized water followed by a final wash in 70% ethanol. After washing, ticks were bisected using razor blades after which the blade was rinsed with 70% ethanol in between individual ticks. One half of the tick was put in an empty and clean 0.5 ml tube and stored at -80 °C for long term storage and as reference. The other half of the tick was stored in a vial (VWR USA) and DNAzol® Direct (MRC, Cincinnati, OH). 100 µl of DNAzol Direct sample processing reagent for direct PCR was used for each (100 µl/10mg) of tick tissue mass. No ticks required more than 100 µl of DNAzol. Each tick and DNAzol sample was heated at 80-90 °C for fifteen minutes which allowed tick components to loosen and break up easier (Salazar 2015). After heating, 20 1.0 mm diameter zirconia/silica beads (BioSpec, Bartlesville, OK) and two 2.3 mm diameter zirconia/silica beads (BioSpec, Bartlesville, OK) were added to the tick half and the DNAzol. Tick halves, DNAzol, and zirconia/silica beads were placed in a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) for two consecutive three minute cycles at medium speed. Bead-beating allowed tick components to break apart extract the DNA. After bead-beating, the tubes were centrifuged for one minute at 12,000 RPG in a Legend Micro 21R centrifuge (Thermo Scientific USA). The liquid was pipetted into a fresh 0.5 ml tube (VWR USA) and stored at -20° C until DNA testing.

DNA from all collected ticks was pooled into groups up to 10 ticks for pathogen testing. Pools were separated by the month they were collected in, the state park in which they were collected, and individual tick species. Prior to testing, pools of DNA were created from the extracted samples as described above. One µl of DNA from each tick, up to 10 individual ticks, consisted of one pool. The DNA concentration of each pool was measured using a NanoDrop 2000

Spectrophotometer (Thermo Scientific USA) and the measured concentration was diluted to 240 ng/µl, if necessary, to prevent false negative results (Overzier et al 2013).

Verification of Tick DNA: Eight random tick pools were chosen to verify tick DNA. The primers used were TQ16S+1F and TQ16S-2R (Halos et al 2004; Crowder et al 2010). The protocol involved an initial denaturation at 95° C for five minutes followed by 30 cycles of denaturation at 94° C for one minute, annealing at 55° C for one minute, extension at 72° C for one minute with a final extension at 72° C for five minutes (Salazar 2015). The reaction was stored at 4° C until visualization.

Assay Protocols using pooled tick samples: No changes in the protocol were made for any of the tick pools. Since the DNA concentration for all tick pools was measured and diluted, 1 µl of the tick pool DNA was used as a template for reactions.

Positive Sample Verification: Ten pooled tick samples were randomly chosen and tested for verification using the *Rickettsia* and *Ehrlichia* PCR protocols. Random tick pools for the *Rickettsia* PCR protocol were samples 4, 11, 20, 28, 42, 43, 45, 53, 69, and 84. Random tick pools for the *Ehrlichia* PCR protocol were samples 16, 17, 26, 27, 28, 31, 42, 48, 49, and 76. The positive bands were gel extracted using a PureLink™ Quick Gel Extraction Kit (Invitrogen) and then sequenced at the Oklahoma State University Core Facility. These sequences were then searched in the nucleotide BLAST database to verify that the primers amplified the target.

Analysis of tick data: Because tick DNA was tested by the three assays in pools instead of individually, the maximum likelihood estimation (MLE) was used in Microsoft Excel 2010 (Microsoft Corp, Redmond, WA) to approximate true infection rate of *Rickettsia* spp. and *Ehrlichia chaffeensis* in the field-collected tick samples. This analysis has been used by others focused on tick-borne pathogens in field-collected ticks (Nadolny et al 2014; Russart et al 2014).

The software used to perform MLE is available from the Centers for Disease Control (CDC, 2013). When all samples are 100% positive, the MLE result is N/A as observed in Table 6.

Results

PCR and LAMP primer set

The thermodynamic characteristics for end-point PCR and LAMP primers are described in Table 4. The sequences for the primers from 5'-3' are: ATA CTA CTA TAC TAT ATC ATA TGG TAA GCT CTG CAA GTC TTC TAT CCT for the forward primer and ATA CTA CTA TAC TAT ATC ATA TGT GCA TTA CTT GGA GCA GTT CTT G for the reverse primer. The PCR primers amplified a 124 base pair sequence. The PCR Rickettsia forward primer is 48 base pairs long and the PCR Rickettsia reverse primer is 45 base pairs long. The GC%, how many G and C nucleotides in each primer, content for the forward and reverse primers is 32.6% and 33.3%, respectively. The ΔG (standard-state free energy of reaction) for both the forward and reverse primers were 0.9kcal/mol and 1.9kcal/mole, respectively.

The LAMP primers F3, B3, FIP, and BIP, have sequences 5'-3' of: TGT TAC AAG CCT GTA ACG G, TCC TGT TCA TCC ATA CCT G, GAG AAC CAA GTA ATG CGC CGG GCG GTA TGA ATA AAC AAG G, and AAT TCG GTA AGG GCA AAG GAC CAC CGA TTT GTC CAC CAA, respectively. The melting temperatures of each of the LAMP primers are 52.4° C for F3, 51.2° C for B3, 66.8° C for FIP, and 67.9° C for BIP. The GC% for each F3, B3, FIP, and BIP are: 47.4%, 47.4%, 50.0%, and 48.7%, respectively.

Table 4: Sequences and Thermodynamics of PCR and LAMP primers

Target Species	Primer Code	Primer Sequence (5'-3')	Amplicon (bp)	Length (bp)	Tm	GC%	ΔG
Rickettsia spp.	PCR Rickettsia Forward	5'- ATA CTA CTA	124	48	60.4° C	32.6%	0.9kcal/mol

		TAC TAT ATC ATA TGG TAA GCT CTG CAA GTC TTC TAT CCT -3’					
	PCR Rickettsia Reverse	5’- ATA CTA CTA TAC TAT ATC ATA TGT GCA TTA CTT GGA GCA GTT CTT G - 3’	124	45	61.1° C	33.3%	1.9kcal/mol
Rickettsia spp.	LAMP F3	5’-TGT TAC AAG CCT GTA ACG G - 3’	N/A	19	52.4° C	47.4%	
	LAMP B3	5’- TCC TGT TCA TCC ATA CCT G - 3’	N/A	19	51.2° C	47.4%	
	LAMP FIP	5’- GAG AAC CAA GTA ATG CGC CGG	N/A	40	66.8° C	50.0%	

		GCG GTA TGA ATA AAC AAG G - 3’					
	LAMP BIP	5’- AAT TCG GTA AGG GCA AAG GAC CAC CGA TTT GTC CAC CAA -3’	N/A	39	67.9° C	48.7%	

Tm: melting temperature

GC%: Percentage of Guanine and Cytosine

ΔG: free energy of reaction

PCR 17kDa primers for end-point PCR

Assay Optimization

Rickettsia PCR temperature gradients: The 24 °C span temperature gradient (Fig. 13) for the end point Rickettsia PCR primers demonstrated a wide range of potential annealing temperatures. Based on these results, a second assay involving 5 °C span temperature gradient was performed, identifying 61.0 °C as the best temperature from the 24 °C temperature gradient.

The 5 °C end point *Rickettsia* temperature gradient (Fig. 14) showed a wide range of potential annealing temperatures for this primer set. From these results, the temperature 61 °C was chosen as the best temperature and was used in the PCR protocol.

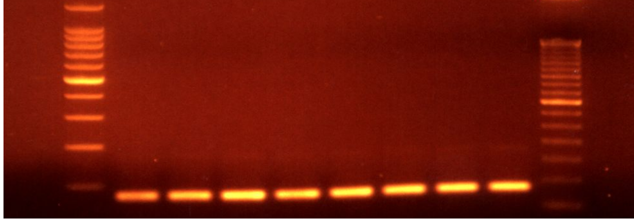

Figure 13: Rickettsia 17kDa 24° C Span Temperature Gradient, 2% agarose gel run for 28 minutes. Lanes 1) Blank, 2) 100 bp ladder, 3) 65.2° C, 4) 63.7° C, 5) 61.0° C, 6) 56.2° C, 7) 50.4° C, 8) 45.8° C, 9) 42.7° C, 10) 41.2° C, 11) 100 bp ladder, 12) Negative Control.

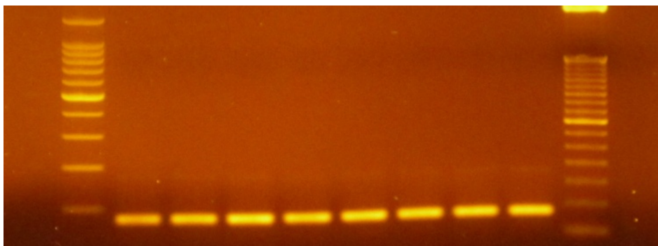

Figure 14: Rickettsia 17kDa 5° C Span Temperature Gradient, 2% agarose gel run for 30 minutes at 100V. Lanes 1) Blank, 2) 100 bp ladder, 3) 63.5° C, 4) 63.2° C, 5) 62.7° C, 6) 61.7, 7) 60.5° C, 8) 59.5° C, 9) 58.8° C, 10) 58.5° C, 11) 100 bp ladder, 12) Negative control

Rickettsia Sensitivity and Specificity Testing

Rickettsia Sensitivity Panels: Figures 15 and 16 indicate the Rickettsia 17kDa primer set for end-point PCR amplify Rickettsia spp. including: *R. rickettsii*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. felis*, *R. montanensis*, *R. parkeri*, and *R. typhi*. The end-point PCR primers do not amplify other closely related species such as *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *Coxiella burnetii* nor other bacterial species like *Escherichia coli* or *Salmonella typhi*.

Table 5 shows selected BLAST results after gel excision, DNA purification, and sequencing of eight *Rickettsia* spp. amplified with the 17kDa PCR primer set. These eight species include: *R. rickettsii*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. felis*, *R. montanensis*, *R. parkeri*, and *R. typhi*.

The BLAST results show that the primers amplify a conserved region on the 17kDa Rickettsia gene.

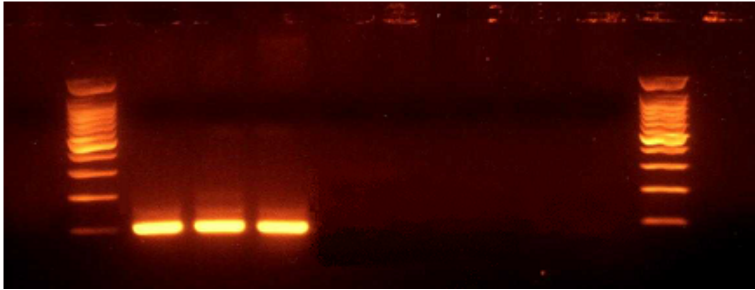

Figure 15: Rickettsia 17kDa Specificity Panel, 2% agarose gel run for 30 minutes at 100V. Lanes 1) Negative control, 2) 100 bp ladder, 3) *R. rickettsii*, 4) *R. parkeri*, 5) *R. typhi*, 6) *Ehrlichia chaffeensis*, 7) *Anaplasma phagocytophilum*, 8) *Coxiella burnetii*, 9) *Escherichia coli*, 10) *Salmonella typhi*, 11) 100 bp ladder, 12) Blank

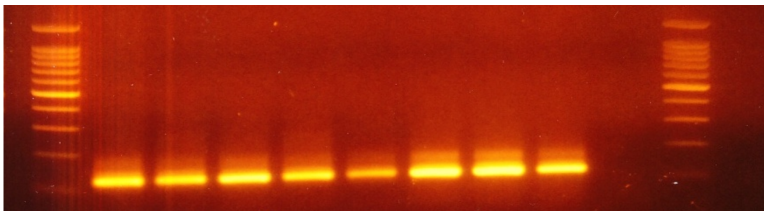

Figure 16: Rickettsia 17 kDa Rickettsia Species Specificity Panel, 2% agarose gel run for 30 minutes at 100V. Lanes 1) 100 bp ladder, 2) *R. rickettsii*, 3) *R. africae*, 4) *R. amblyommii*, 5) *R. conorii*, 6) *R. felis*, 7) *R. montanensis*, 8) *R. parkeri*, 9) *R. typhi*, 10) Negative control, 11) 100 bp ladder, 12) Blank

Table 5: Selected BLASTn results of sequences from 17kDA *Rickettsia* spp. after gel extractions

Scientific Name	Origin	% ID	e^-	NCBI BLAST Accession Number
R. rickettsii				
Rickettsia rickettsii	USA	98%	9e-17	CP006010.1
Rickettsia rickettsii	Brazil	98%	9e-17	KC845924.1
Rickettsia sibirica	China	98%	9e-17	JX945527.1
Rickettsia africae	Greece	98%	9e-17	KF646137.1

Rickettsia rickettsii	USA	98%	9e-17	CP003318.1
R. africae				
Rickettsia africae	Greece	98%	9e-17	KF646137.1
Rickettsia rickettsii	Brazil	98%	9e-17	KC845924.1
Rickettsia conorii	Sicily, Italy	98%	9e-17	JN182795.1
Rickettsia africae	Ethiopia	98%	9e-17	CP001612.1
Rickettsia africae	Antigua and Barbuda	98%	9e-17	EU072682.1
R. amblyommii				
Rickettsia amblyommii	Tallulah, LA	100%	3e-17	KC003473.1
Rickettsia amblyommii	USA	100%	3e-17	CP003334.1
Rickettsia amblyommii	Mexico	100%	3e-17	EU828788.1
Rickettsia amblyommii	Brazil	100%	3e-17	DQ517291.1
Rickettsia amblyommii	Brazil	100%	3e-17	AY375162.1
R. conorii				
Rickettsia conorii	Sicily, Italy	98%	1e-16	JN182795.1
Rickettsia conorii	France	98%	1e-16	AE006914.1
Rickettsia conorii	Sicily, Italy	96%	5e-15	JE182790
Rickettsia rickettsii	USA	98%	1e-16	CP006010.1
Rickettsia africae	Mediterranean Area	98%	1e-16	KF646137.1
R. felis				
Rickettsia felis	Bangladesh	100%	8e-18	KP308188.1
Rickettsia felis	India	100%	8e-18	KM365086.1
Rickettsia felis	Sicily, Italy	100%	8e-18	KM0060859.1
Rickettsia felis	Yucatan, Mexico	100%	8e-18	KF241853.1
Rickettsia felis	Sweden	100%	8e-18	GU182891.1
R. montanensis				
Rickettsia raoultii	China	100%	3e-18	KT261761.1
Candidatus *Rickettsia vini*	Czech Republic	100%	3e-18	KP713672.1
Rickettsia montanensis	Los Alamos, NM	100%	3e-18	CP003340.1
Rickettsia montanensis	USA	100%	3e-18	DQ402377.1
Rickettsia rhipicephali	Los Alamos, NM	100%	3e-18	CP003342.1
R. parkeri				
Rickettsia parkeri	USA	100%	2e-17	CP003341.1
Rickettsia parkeri	Brazil	100%	2e-17	EF102237.1
Rickettsia peacockii	Saskatchewan, Canada	100%	2e-17	HF935073.1
Rickettsia rickettsii	Arizona, USA	100%	2e-17	CP003307.1
Rickettsia rickettsii	Colombia	100%	2e-17	CP003306.1
R. typhi				
Rickettsia typhi	Galveston, TX	94%	3e-12	KJ648944.1
Rickettsia typhi	Mexico	94%	3e-12	KC469609.1
Rickettsia typhi	Yucatan, Mexico	94%	3e-12	JX189507.1
Rickettsia typhi	USA	94%	3e-12	CP003398.1
Rickettsia typhi	USA	94%	3e-12	AE017197.1

Rickettsia Sensitivity Panel

The 17kDa PCR primer set is sensitive down to 0.0001 ng/µl (Fig. 17).

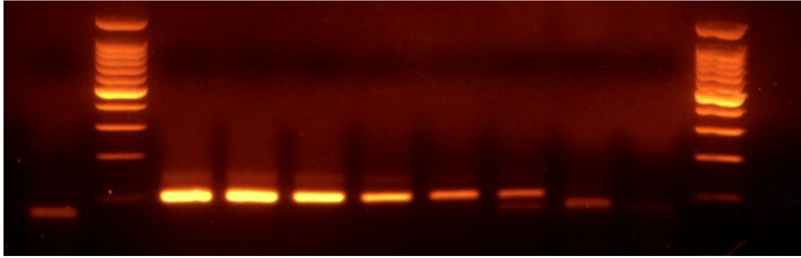

Figure 17: 17 kDa *Rickettsia* spp. PCR sensitivity of primer set, 2% agarose gel run for 30 minutes at 100V. Lanes 1) Negative control, 2) 100 bp ladder, 3) 4.7 ng/µl, 4) 1 ng/µl, 5) 0.1 ng/µl, 6) 0.01 ng/µl, 7) 0.001 ng/µl (1 picogram/µl), 8) 0.0001 ng/µl, 9) 0.00001 ng/µl, 10) 0.000001 ng/µl (1 femtogram/µl), 11) 100 bp ladder, 12) Blank

Testing of Field Collected Ticks

The presence of tick DNA was confirmed in this study (Fig. 18). Eight randomly chosen state park tick pools were used to test for tick DNA using published primers. The use of the 17kDa primer set with pools of field-collected ticks from state parks is demonstrated in Fig. 19. The negative control has no band except a slight primer dimer at ~90 bp. The primer dimer at ~90 bp matches with the primers, with sequences of 45 bp and 48 bp for the forward and reverse primers, respectively, binding together to form a product of 93 bp when nothing else is there to be amplified. The positive control was positive in at 124 bp. A non-specific band appeared in tick samples at ~200 base pairs. The phylogenetic tree of the *Rickettsia* spp. (Fig. 20) sequences from gel extraction indicates a conserved region used for amplification.

Ten randomly chosen positive tick pool samples were chosen to be sequenced. Once sequenced, the BLAST results from the 124 base pair band showed high % of sequence identity with *R. amblyommii* (Table 6). Eight randomly chosen positive tick pool samples were chosen to

sequence the interesting 200 bp band from the tick samples. The BLAST results showed a high % of sequence identity with *R. amblyommii* (Table 7).

All tick pool samples from Fig. 18 were positive. Out of 90 tick pools, 86 were positive with the *Rickettsia* spp. end point PCR primers. The negative tick pool results from the 17kDa PCR primer set include those from Robbers Cave with ticks collected in July, Boiling Springs with ticks collected in May, Roman Nose with ticks collected in May, and the *I. scapularis* from Greenleaf. Table 6 shows details.

Verification of Tick DNA

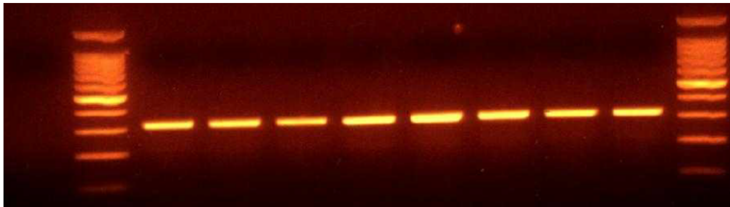

Figure 18: Verification end-point PCR for Tick DNA, 2% agarose gel run for 30 minutes at 100V. Lanes 1) Blank, 2) 100 bp ladder, 3)-10) randomly chosen state park tick pools: 1, 14, 15, 40, 53, 55, 62, and 76, 11) 100 bp ladder

Representative sample of 17kDa Rickettsia PCR with tick pools

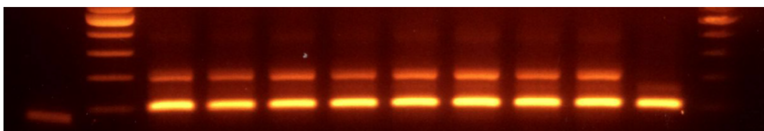

Figure 19. 17 kDa *Rickettsia* spp. PCR primers with Tick Pools, 2% agarose gel, ran at 100V. Lanes 1) Negative control, 2) 100 bp ladder, 3)-10) State Park tick pools 1-8, 11) Positive control, *R. rickettsii*, 12) 100 bp ladder.

Table 6. BLAST results from randomly chosen positive samples of Rickettsia 17 kDa primer set with State Park pools.

Scientific Name	Origin	% ID	e-	NCBI BLAST Accession Number
Rickettsia amblyommii	Tallulah, LA	98%	4e-18	KC003473.1
Uncultured *Rickettsia* sp.	Stillwater, OK	100%	4e-18	GQ302894.1
Rickettsia raoultii	China	96%	2e-18	KT261761.1
Rickettsia amblyommii	Mexico	98%	4e-18	EU828788.1
Rickettsia amblyommii	Argentina	98%	4e-18	DQ517291.1

Table 7. BLAST results from randomly chosen positive results from 17 kDa Rickettsia PCR primers, sequences from 200 bp excised band.

Scientific Name	Origin	%ID	e-	NCBI BLAST Accession Number
Rickettsia amblyommii	Tallulah, LA	99%	2e-61	KC003473.1
Rickettsia amblyommii	Texas, USA	99%	2e-61	EF689730.1
Rickettsia amblyommii	Brazil	99%	2e-61	DQ517291.1
Rickettsia raoultii	Mudanjiang City, China	98%	1e-59	JX885457.1
Rickettsia amblyommii	Brazil	98%	1e-59	KJ534311.1

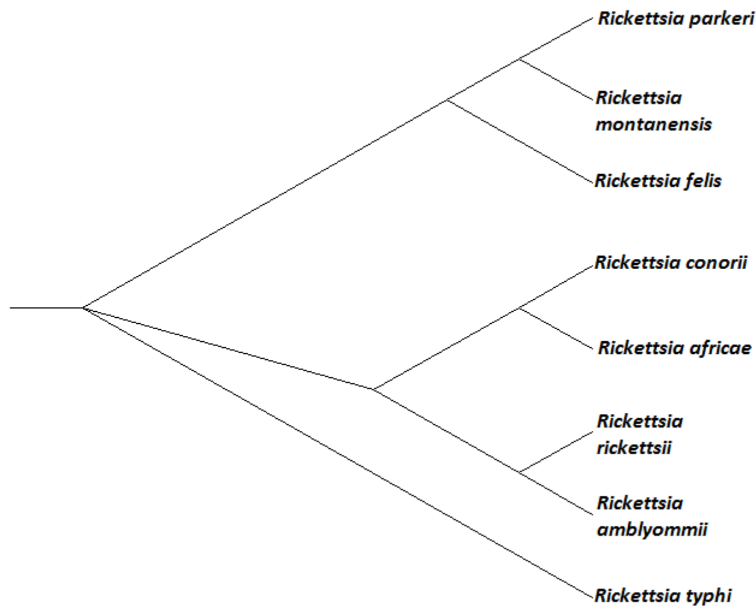

Figure 20. Phylogenetic relationship of PCR diagnostic sequences obtained by PCR from the 17 kDa gene from tested *Rickettsia* spp.

***Ehrlichia chaffeensis* PCR**

The temperature gradient shows a wide range of temperatures amplified by the ECC and ECB primers (Fig. 21). The nested PCR HE1 and HE3 primers for *E. chaffeensis* amplified the target *E. chaffeensis* (Fig. 22).

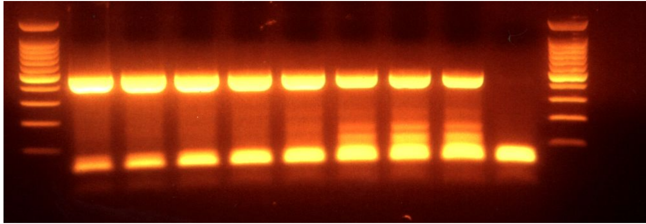

Figure 21. *Ehrlichia chaffeensis* ECC and ECB primer set Temperature Gradient, 2% agarose gel, ran at 100V. Lanes 1) 100 bp ladder, 2) 73.6° C, 3) 71.7° C, 4) 68.8° C, 5) 64.5° C, 6) 59.0° C, 7) 55.0° C, 8) 51.7° C, 9) 49.6° C, 10) Negative control, 11) 100 bp ladder, 12) Blank

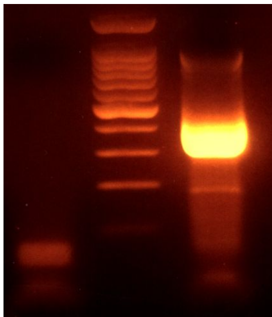

Figure 22. *Ehrlichia chaffeensis* HE1 and HE3 nested primer verification, 2% agarose gel, ran at 100V. Lanes 1) Negative control, 2) 100 bp ladder, 3) Positive control *E. chaffeensis*

<u>Testing Field-Collected Ticks</u>

Figures 23 and 24 show results from state park tick pool samples using the Ehrlichia nested PCR primers HE1 and HE3.

Ten randomly chosen positive state park tick pools were chosen to be sequenced. The BLAST results of these sequences were aligned with *E. chaffeensis*. Selected BLAST results are presented in Table 8.

From all the state park tick pools, 25 out of 90 were positive for *E. chaffeensis* using the nested primers. Detailed results of all state park tick pools are presented in Table 6.

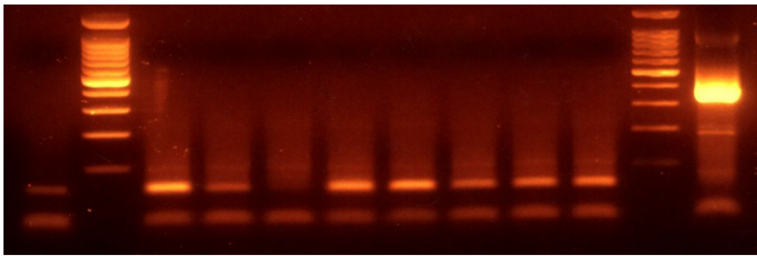

Figure 23. Ehrlichia PCR State Park Tick Pool samples with nested primers HE1 and HE3, 2% agarose, ran at 100V for 30 minutes. Lanes: 1) Negative control, 2) 100 bp ladder, 3)-10) State Park tick pools 1-8, 11) 100 bp ladder, 12) Positive control, *E. chaffeensis*

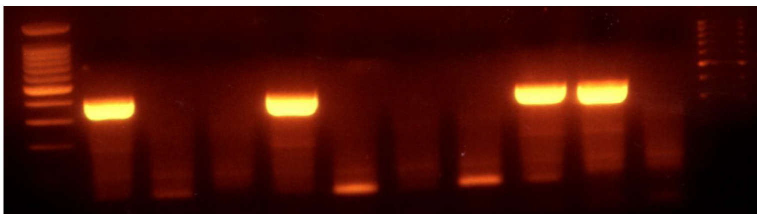

Figure 24. Ehrlichia PCR State Park Tick Pool samples, 2% agarose, ran at 100V for 30 minutes. Lanes: 1) 100 bp ladder, 2)-11) State Park Tick Pools 9-18, 12) 100 bp ladder

Table 8. BLAST results from randomly chosen positive samples of *Ehrlichia chaffeensis* HE1 and HE3 nested primer set with State Park tick pools.

Scientific Name	**Origin**	**%ID**	**e^-**	**NCBI BLAST Accession Number**
Ehrlichia chaffeensis	USA	99%	2e-176	CP007480.1
Ehrlichia chaffeensis	USA	99%	2e-176	CP007479.1
Ehrlichia chaffeensis	Arkansas, USA	99%	2e-176	CP000236.1

Ehrlichia chaffeensis	South Korea	99%	2e-171	EU564810.1
Ehrlichia chaffeensis	Jeju Island, South Korea	99%	3e-175	EU181141

Pan-Specific *Rickettsia* spp. LAMP Assay

Assay Optimization

Temperature Gradients

The thermocycling temperature gradient for the LAMP F3 and B3 primers shows a wide range of potential annealing temperatures for these primers (Fig. 25).

The LAMP temperature gradient using all four LAMP primers (F3, B3, FIP, and BIP) shows smears at all temperatures between 65-70 °C (Fig. 26). The best showing temperature is 65 °C.

A second temperature gradient was performed using all four LAMP primers (F3, B3, FIP, and BIP). This one used temperatures ranging from 62-67 °C (Fig. 27). All temperatures have a faint smear. The best temperature is 65.1 °C.

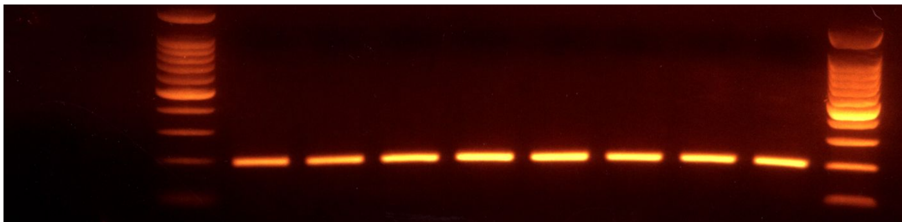

Figure 25. Rickettsia LAMP F3 and B3 primer set at 5 µM concentration Temperature Gradient PCR with positive control *R. rickettsii*, 2% agarose gel, ran at 100V for 30 minutes. Lanes 1) Blank, 2) Negative control, 3) 100 bp ladder, 4) 60.0° C, 5) 59.3° C, 6) 58.1° C, 7) 56.3° C, 8) 54.0° C, 9) 52.3° C, 10) 50.9° C, 11) 50.0° C, 12) 100 bp ladder.

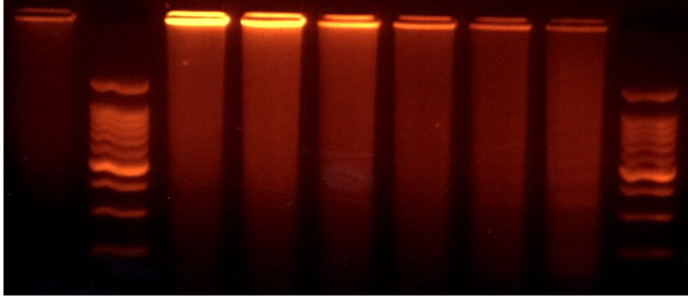

Figure 26. Rickettsia LAMP Temperature Gradient 65-70° C using *R. rickettsii* as template, 2% agarose gel, ran at 100V for 35 minutes. Lanes: 1) Negative control, 2) 100 bp ladder, 3) 70° C, 4) 69.1° C, 5) 68.2° C, 6) 67° C, 7) 66.2° C, 8) 65° C, 9) 100 bp ladder

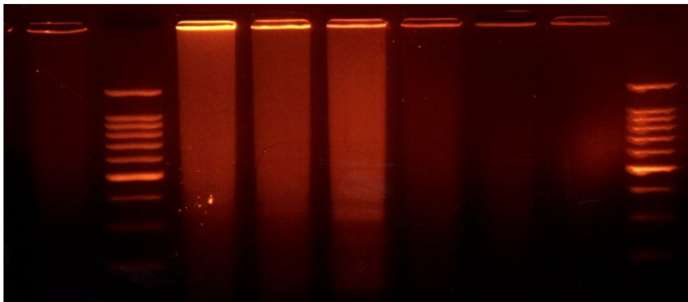

Figure 27. Rickettsia LAMP Temperature Gradient 62-67° C using *R. rickettsii* as template, 2% agarose gel, ran at 100V for 35 minutes. Lanes: 1) Negative Control, 2) 100 bp ladder, 3) 67° C, 4) 66.1° C, 5) 65.1° C, 6) 64.1° C, 7) 63.2° C, 8) 62° C, 9) 100 bp ladder

Specificity Panels

Figure 28 indicates the pan-specific *Rickettsia* spp. LAMP primers amplified eight Rickettsia spp. including: *R. rickettsii*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. felis*, *R. montanensis*, *R. parkeri*, and *R. typhi*. The smears varied in brightness because the templates used had different concentrations of DNA. The LAMP primers did not amplify closely related species such as *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *Coxiella burnetii* or other distantly related bacteria like *Escherichia coli* or *Salmonella typhi* (Fig. 29).

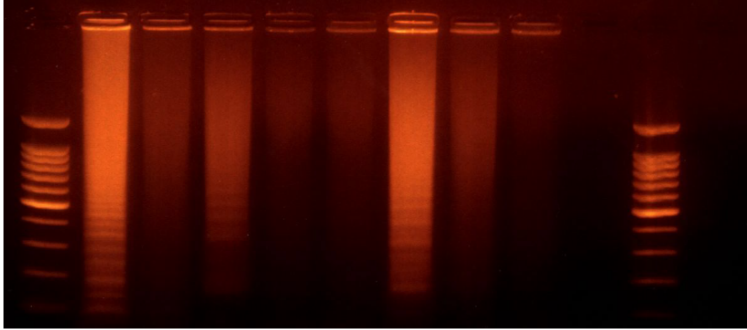

Figure 28. LAMP *Rickettsia* spp. specificity panel, 2% agarose gel, ran at 100V for 35 minutes. Lanes 1) 100 bp ladder, 2) *R. africae*, 3) *R. amblyommii*, 4) *R. conorii*, 5) *R. felis*, 6) *R. montanensis*, 7) *R. parkeri*, 8) *R. rickettsii*, 9) *R. typhi*, 10) Negative control, 11) 100 bp ladder, 12) Blank.

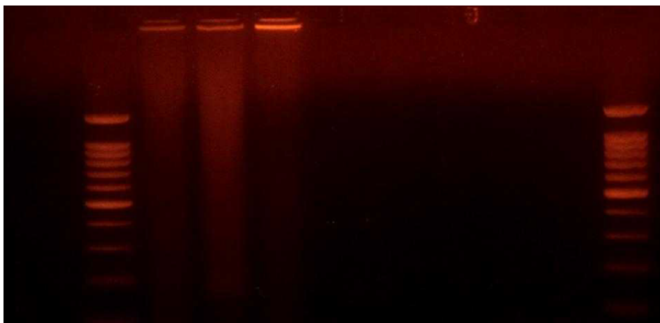

Figure 29. LAMP Rickettsia spp. specificity, 2% agarose , ran at 100V for 35 minutes. Lanes 1) Negative control, 2) 100 bp ladder, 3) *R. rickettsii*, 4) *R. parkeri*, 5) *R. typhi*, 6) *Ehrlichia chaffeensis*, 7) *Anaplasma phagocytophilum*, 8) *Coxiella burnetii*, 9) *Escherichia coli*, 10) *Salmonella typhi*, 11) 100 bp ladder

Sensitivity Panel

The LAMP Rickettsia primers show smears for all concentrations ranging from 4.7 ng/µl down to 1 femtogram/µl (Fig. 30).

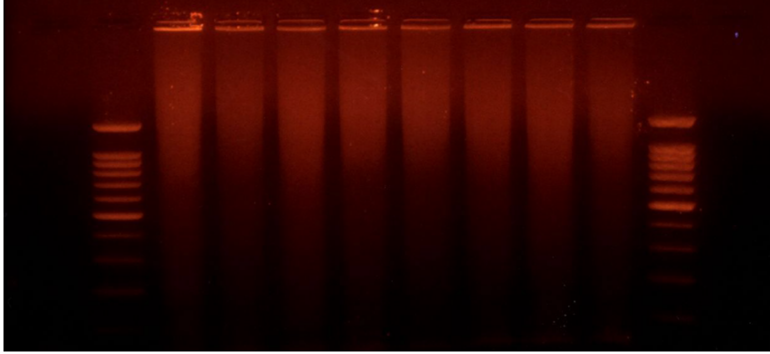

Figure 30. LAMP *Rickettsia* spp. sensitivity panel using *R. rickettsii* as template, 2% agarose, ran at 100V for 35 minutes. Lanes 1) Negative control, 2) 100 bp ladder, 3) 4.7 ng/µl, 4) 1 ng/µl 5) 0.1 ng/µl, 6) 0.01 ng/µl, 7) 0.001 ng/µl (1 picogram/µl), 8) 0.0001 ng/µl, 9) 0.00001 ng/µl, 10) 0.000001 ng/µl (1 femtogram/µl), 11) 100 bp ladder, 12) Blank.

MgSO4 and Betaine Concentration Gradient

Figures 31 and 32 demonstrates the effect of different combinations of MgSO4 and Betaine concentrations in the amplification of LAMP product. Concentrations chosen for the assay were 0.7 Betaine and 8 mM MgSO4.

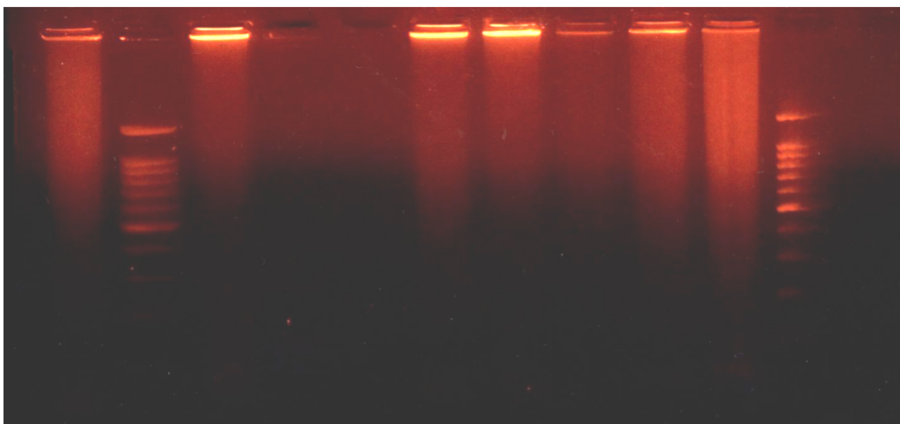

Figure 31. LAMP *Rickettsia* MgSO4/Betaine Concentration Gradient, *R. rickettsii* as template, 2% agarose gel, ran at 100V for 35 minutes. Lanes 1) Positive control, 2) 100 bp ladder, 3) 0.1 M Betaine & 4 mM MgSO4, 4) 0.1 M Betaine & 6mM MgSO4, 5) 0.1 M Betaine & 8 mM MgSO4,

6) 0.1 M Betaine & 10 mM MgSO4, 7) 0.3 M Betaine & 4 mM MgSO4, 8) 0.3 M Betaine & 6 mM MgSO4, 9) 0.3 M Betaine & 8 mM MgSO4, 10) 0.3 M Betaine & 10 mM MgSO4, 11) 100 bp ladder, 12) Negative control.

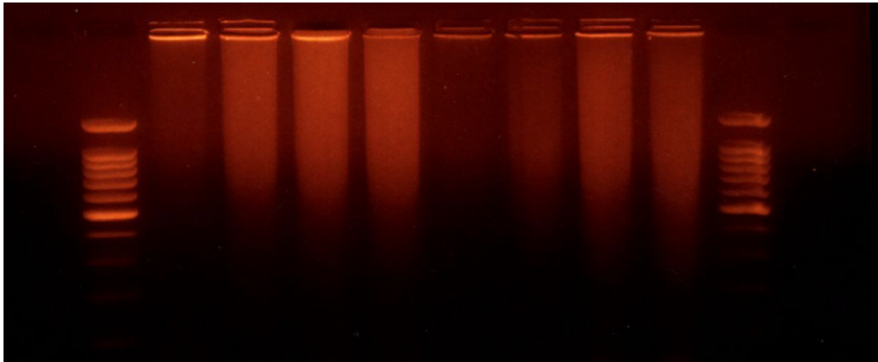

Figure 32. LAMP *Rickettsia* MgSO4/Betaine Concentration Gradient, *R. rickettsii* as template, 2% agarose gel, ran at 100V for 35 minutes. Lanes 1) Blank, 2) 100 bp ladder, 3) 0.5 M Betaine & 4 mM MgSO4, 4) 0.5 M Betaine & 6 mM MgSO4, 5) 0.5 M Betaine & 8 mM MgSO4, 6) 0.5 M Betaine & 10 mM MgSO4, 7) 0.7 M Betaine & 4 mM MgSO4, 8) 0.7 M Betaine & 6 mM MgSO4, 9) 0.7 M Betaine & 8 mM MgSO4, 10) 0.7 M Betaine & 10 mM MgSO4, 11) 100 bp ladder, 12) Blank

Testing Field-Collected Ticks

Figure 33 shows selected state park tick pool results, all of these tick pools were positive. Detailed results are found in Table 6.

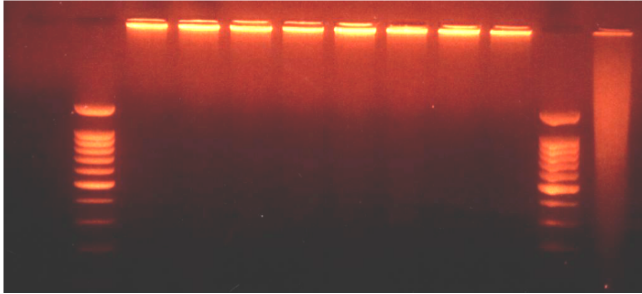

Figure 33. LAMP *Rickettsia* State Park Tick pools with *R. rickettsii* as positive control, 2% agarose gel, ran at 100V for 35 minutes. Lanes 1) Negative control, 2) 100 bp ladder, 3)-10) State Park Tick Pools-1-8, 11) 100 bp ladder, 12) Positive control.

State Park Tick Pool Results

The end-point PCR SFGR assay detected Rickettsial DNA in 86/87 samples and the LAMP assay detected Rickettsial DNA in all 87 samples (Table 9). The LAMP assay detected Rickettsial DNA in pools of single *D. variabilis* and *I. scapularis* as opposed to the PCR assay.

Prevalence of Rickettsia in field collected ticks from Oklahoma state parks is 100% for all species tested: *A. americanum*, *D. variabilis*, and *I. scapularis* (Table 9). Prevalence of *Ehrlichia chaffeensis* in field collected ticks in Oklahoma state parks varied by species. *D. variabilis* and *I. scapularis* both had 0% prevalence and *A. americanum* had a prevalence of 27.6% (Table 9).

Table 9. Prevalence of *Rickettsia* spp. and *Ehrlichia chaffeensis* in ticks collected from state parks in Oklahoma, summer 2014.

State Park	Month	Assay	Tick species	# positive pools	# pools tested	# of ticks	Positive pools (%)	MLE infection rate (%)
Greenleaf SP	May	17kDa[a]	*A. americanum*	5	5	50	100	N/A
		LAMP[b]	*A. americanum*	5	5	50	100	N/A
		Ehrlichia[c]	*A. americanum*	0	5	50	0	0.0
		17kDa	*I. scapularis*	0	1	1	0	0.0
		LAMP	*I. scapularis*	1	1	1	100	N/A
		Ehrlichia	*I. scapularis*	0	1	1	0	0.0
	June	17kDa	*A. americanum*	15	15	195	100	N/A
		LAMP	*A. americanum*	15	15	195	100	N/A
		Ehrlichia	*A. americanum*	4	15	145	26.6	30.64
	July	17kDa	*A. americanum*	1	1	10	100	N/A
		LAMP	*A. americanum*	1	1	10	100	N/A
		Ehrlichia	*A. americanum*	0	1	10	0	0.0
Sequoyah SP	May	17kDa	*A. americanum*	36	36	352	100	N/A
		LAMP	*A. americanum*	36	36	352	100	N/A
		Ehrlichia	*A. americanum*	18	36	352	50	66.80
	June	17kDa	*A. americanum*	8	8	74	100	N/A
		LAMP	*A. americanum*	8	8	74	100	N/A
		Ehrlichia	*A. americanum*	0	8	74	0	0.0
	July	17kDa	*A. americanum*	6	6	53	100	N/A
		LAMP	*A. americanum*	6	6	53	100	N/A
		Ehrlichia	*A. americanum*	1	6	53	16.7	18.86
	August	17kDa	*A. americanum*	1	1	5	100	N/A
		LAMP	*A. americanum*	1	1	5	100	N/A
		Ehrlichia	*A. americanum*	0	1	5	0	0.0
Lake Thunderbird SP	May	17kDa	*A. americanum*	8	8	73	100	N/A
		LAMP	*A. americanum*	8	8	73	100	N/A
		Ehrlichia	*A. americanum*	1	8	73	12.5	13.69
	June	17kDa	*A.*	1	1	3	100	N/A

			americanum					
		LAMP	*A. americanum*	1	1	3	100	N/A
		Ehrlichia	*A. americanum*	0	1	3	0	0.0
Lake Tenkiller SP	June	17kDa	*A. americanum*	2	2	13	100	N/A
		LAMP	*A. americanum*	2	2	13	100	N/A
		Ehrlichia	*A. americanum*	0	2	13	0	0.0
	July	17kDa	*A. americanum*	1	1	1	100	N/A
		LAMP	*A. americanum*	1	1	1	100	N/A
		Ehrlichia	*A. americanum*	0	1	1	0	0.0
Lake Wister SP	May	17kDa	*A. americanum*	1	1	4	100	N/A
		LAMP	*A. americanum*	1	1	4	100	N/A
		Ehrlichia	*A. americanum*	0	1	4	0	0.0
Robbers Cave SP	June	17kDa	*A. americanum*	0	1	2	0	0.0
		LAMP	*A. americanum*	1	1	2	100	N/A
		Ehrlichia	*A. americanum*	0	1	2	0	0.0
	July	17kDa	*A. americanum*	1	1	1	100	N/A
		LAMP	*A. americanum*	1	1	1	100	N/A
		Ehrlichia	*A. americanum*	0	1	0	0	0.0
Boiling Springs SP	May	17kDa	*D. variabilis*	0	1	1	0	0.0
		LAMP	*D. variabilis*	1	1	1	100	N/A
		Ehrlichia	*D. variabilis*	0	1	1	0	0.0
Roman Nose SP	May	17kDa	*D. variabilis*	0	1	1	0	0.0
		LAMP	*D. variabilis*	1	1	1	100	N/A
		Ehrlichia	*D. variabilis*	0	1	1	0	0.0

[a] 17kDa = Pan-specific Rickettsia 17kDa PCR assay

[b] LAMP = Pan-specific Rickettsia LAMP assay

[c] Ehrlichia = Nested Ehrlichia PCR specific for *E. chaffeensis*

Discussion

This is the first study to develop a functional LAMP assay that can be used to test Rickettsial pathogens in field-collected ticks. While field-collected ticks were used as the template in a

LAMP assay to detect *Borrelia burgdorferi* (Yang et al 2012), other LAMP studies involving Rickettsial species did not test DNA from field-collected ticks (Nakao et al, 2010; Ma et al, 2011; Pan et al 2012). The 17kDa Rickettsial LAMP assay provides a novel molecular tool which amplifies the targeted product in field-collected ticks and it compares effectively with the 17kDa Rickettsial end-point PCR assay when detecting *Rickettsia* spp. in wild ticks. Using both assays, it was possible to compare prevalence rates of pathogens in state parks in Oklahoma.

Most studies focused on detecting Rickettsial pathogens in ticks begin with a screening phase to assess whether any rickettsial pathogens are present before moving to more specific assays to determine which species are present (Nadolny et al. 2014, Russart et al. 2014). This screening assay normally is based on the 17kDa gene which is pan-specific for both SFG and Typhus group rickettsiae. This is the reason that both the end-point PCR assay and LAMP assay are based on the 17kDa gene because both will be used as the screening phase in future diagnostic strategies. Future studies will begin developing other LAMP assays based on other genes to focus on specific species of rickettsia.

The 17 kDa Rickettsia end-point PCR primers are quality primers for detecting Rickettsial pathogens as they amplify DNA at a wide range of temperatures, detect several *Rickettsia* spp., and have an easy to visualize band on gel electrophoresis. The PCR bands that were sequenced amplified a conserved region on the 17kDa gene based on a phylogenetic tree analysis. BLAST results from these sequences show potential amplification of several *Rickettsia* spp. Although the primers have several benefits, some limiting factors also exist. These primers each have a 22 base pair long nucleotide extension added to aid in sensitivity (Arif et al 2014). The extension allows the detection and sequencing of a ~125 base pair sequence which is relatively short for direct sequencing and BLAST analysis. Because of the short length, the sequence may become mistaken with the ~90 base pair band which results from the primers binding together when there is nothing to detect (a primer dimer). The primer dimer becomes apparent especially in the larger

specificity testing. The bands detected using DNA from other bacteria are hard to detect if it is a positive band or a primer dimer. The short sequence also interferes with sequencing of the gel extracted band as the results vary between readable and unreadable sequences.

One surprising result when using the modified 17kDa primer set with DNA from field collected ticks was the presence of an additional ~200 base pair non-specific band found only in DNA extracted from field-collected ticks. This result was not anticipated as the band was not present in any of the samples from cultured DNA used in specificity testing. This non-specific band, when sequenced, matched *R. amblyommii*, a symbiotic rickettsial species associated with Lone star ticks. It is possible that *R. amblyommii* in ticks has a different phase or presentation than when in culture which may account for the extra band. Further research is needed to understand the nature of this second product.

Based on the phylogenetic tree (Fig. 20), the DNA extraction from the PCR primers categorizes *Rickettsia* spp. into three groups based on the 17 kDa ompB Rickettsial gene. One group contains *R. parkeri*, *R. montanensis*, and *R. felis*, another group contains *R. conorii*, *R. africae*, *R. rickettsii*, and *R. amblyommii*, and the third group contains *R. typhi*. The findings of this study agree with the results from previous studies which has divided the ompB rickettsial gene family into four groups. Group one contains *R. rickettsii* grouped with *R. conorii*. Group two contains *R. montanensis* with other *Rickettsia* spp. Group three contains *R. felis* with other *Rickettsia* spp. and group four contains *R. typhi* in its own group (Roux and Raoult 2000). Another genetic study places *R. africae*, *R. conorii*, and *R. parkeri* in a group closely related with *R. rickettsii*, another group containing *R. montanensis* closely related to *R. felis*, and *R. typhi* alone in its own group (Ngwamidiba et al 2005). Both of these studies show the ompB region of the gene is a highly conserved region ideal for detecting all *Rickettsia* spp. (Roux and Raoult 2000; Ngwamidiba et al 2005).

The results from this study demonstrate that the rickettsial LAMP assay has potential in the future for detection of rickettsial pathogens from field-collected ticks. This LAMP assay has a specific temperature range which indicates practical usage in field stations where electricity currents are hard to maintain. Results indicate the LAMP assay is sensitive to 1 femtogram/µl with all smears having similar brightness. This indicates that the LAMP assay is sensitive for detecting even low concentrations of *Rickettsia* spp. The LAMP primers also detect a wide range of *Rickettsia* spp. but did not detect other closely-related bacteria. This high specificity correlates to other similar LAMP primer sets such as one detecting *Anaplasma ovis* (Ma et al 2011) or other studies focused on other members of the order Rickettsiales (Pan et al 2011; Pan et al 2012). The major limiting factor for the assay at the moment is the visualization step which, up to now, has only occurred using gel electrophoresis. Although all laboratory samples used DNA from cultured rickettsia stocks had bright smears, results from field-collected ticks were faint and difficult to distinguish between positive and negative samples. Other studies have used colorimetric dyes like naphthalene blue or casein or even SYBR Green (Pan et al 2012). A sensitive visualization step to the assay would increase the usefulness the assay for use in field settings.

The comparison of the PCR and LAMP Rickettsial assays developed in this study has pros and cons. In regards to temperature, the PCR primers detect a wide range of temperatures, all with bright, observable bands. The LAMP assay detects with a narrow temperature range, producing a distinguishable smear. Both assays were tested with sensitivity and specificity panels. The PCR assay only detected down to .0001 ng/µl while the LAMP primers were sensitive down to 1 femtogram/µl. Both PCR and LAMP assays were specific to eight different *Rickettsia* spp. including *R. rickettsii*, *R. africae*, *R. amblyommii*, *R. conorii*, *R. felis*, *R. montanensis*, *R. parkeri*, and *R. typhi*. Both assays also were specific to *Rickettsia* spp. and did not amplify other closely or distantly related bacterial species. In regards to of sensitivity, the LAMP primers are more sensitive; but, both assays showed similar specificity to *Rickettsia* spp. It was easier to visualize

the results from the end-point PCR assay compared with the LAMP assay. When testing the assays with DNA from field-collected ticks, the PCR primers showed a bright band for the amplified target of ~125 base pairs but the LAMP assay produced smears which were not easily distinguished. This is definitely where a colorimetric component could dramatically improve the visualization of the LAMP target. In summary, the end-point PCR primers were better in terms of amplification temperatures and visualization while the LAMP primers provided better sensitivity. This corresponds with previous studies which also demonstrated that LAMP assays are more specific than end-point PCR (Ma et al 2011; Pan et al. 2012). Both assays had the same specificity results which differed from other studies which indicate that LAMP primers may be more specific than end-point PCR (Ma et al 2011). Further work needs to be done with the LAMP system to refine and improve the visualization to ensure that true positives are being detected.

Applying the developed assays to field-collected ticks has provided insights into tick-borne pathogen prevalence in Oklahoma State Parks. All but four of the state park tick pools (Robbers Cave *A. americanum* collected in June, Boiling Springs *D. variabilis* collected in May, Roman Nose *D. variabilis* collected in May, and Greenleaf *I. scapularis* collected in May) were positive using the end-point PCR Rickettsial 17 kDa primer set. The *Ehrlichia chaffeensis* HE1 and HE3 nested primers amplified *E. chaffeensis* according to sequence and BLAST data. Using the maximum likelihood estimation (MLE) to approximate true infection rate, our results (27.6%) were similar to those found in the region. Although difficult to visualize the results, the LAMP assay detected positive results in all tick pools, including the *D. variabilis* and *I. scapularis* ticks. This was different than the results from the end-point PCR assay and is probably due to the increased sensitivity of the LAMP assay in detecting *Rickettsia* spp. in field-collected tick samples. In total, while it remains to be established how pathogen infection rates in field-collected ticks impacts risk for humans or animals, it is apparent from the data that there is a high prevalence of *Rickettsia* spp. infection in ticks collected from Oklahoma state parks. Future

studies are needed to determine how these infection rates might change over time and between state parks.

In conclusion, this is the first LAMP assay to detect Rickettsial pathogens in field-collected ticks. *Rickettsia* spp. specific end-point PCR primers were created as a means to confirm LAMP assay results. Both sets of primers amplified regions on the 17kDa gene. The end-point PCR assay amplified products in a wide range of temperatures, was sensitive to 0.0001ng/µl, had high specificity for *Rickettsia* spp., and had a bright band on visualization at ~125 base pairs. Limitations include the non-specific banding at ~200 base pairs which matched sequences for *R. amblyommii*. The LAMP assay had a narrow temperature range in which products were amplified, was sensitive to 1 fg/µl, had high specificity for *Rickettsia* spp., and did not have an easy to distinguish positive smear from field-collected ticks upon visualization. Comparisons between the two assays indicate the PCR assay to be better in terms of amplification temperature range and visualization while the LAMP assay was more sensitive. While both assays had the same specificity, the LAMP assay detected *Rickettsia* spp. in 100% of field-collected tick pools and the end-point PCR assay detected *Rickettsia* spp. in 98.9% of field-collected tick pool samples. This indicated the possibility that the LAMP assay performed better than the end-point PCR assay in regards to field-collected tick samples, but there is a need to develop a visualization to confirm positive reactions. The maximum likelihood estimation (MLE) of *Rickettsia* spp. and *Ehrlichia chaffeensis* were also determined. While the MLE of Rickettsial pathogens in field-collected ticks was not applicable because they were all positive, the MLE for *E. chaffeensis* was between 13.69 and 66.80. Although preliminary, these prevalence rates give a preview of tick-borne pathogen risk in state parks in Oklahoma. It appears from the data that Rickettsial pathogens are present in *A. americanum* during all summer months and in all state parks in which ticks were collected.

CHAPTER V

CONCLUSION

Tick-borne diseases are the most important vector-borne diseases currently impacting the United States and Europe. While studies continue to augment our understanding of the specific ecological and environmental factors contributing to the unique nidi of infection involving vector, host, and pathogen, there is still much to be learned in order to understand how local conditions and factors contribute to the spread of a particular tick-borne disease in a given area. This is especially the case in the south-central region of the United States where long term endemicity of SFG rickettsiosis and Ehrlichiosis is intersecting with emerging pathogens to produce dramatic increases in incidence of tick-borne diseases among humans and animals across a highly diverse ecosystem. The objectives of this thesis research focused on two important aspects will assist in any effective surveillance program: 1) updating the current distribution maps for important tick vectors and 2) developing a low-cost, sensitive molecular assay for which to test field-collected ticks for pathogens.

Objective one focused on the distribution of the most important tick vector in the Southern United States, *Amblyomma americanum*. By focusing on the counties surrounding the historic Chisholm Trail, this study used active surveillance to determine that Lone star ticks have established populations throughout the region and into the western counties. This was a surprising result because of the arid, dry ecosystems in the region. This information augments earlier published studies and shows the importance of updating distribution maps as well as providing new information from which to interpret current distributions of tick-borne diseases throughout the state.

Objective two focused on the development and field-testing of an innovative assay for screening field collected ticks for Rickettsial pathogens. Validated by a pan-specific end-point PCR assay which targeted the 17 kDa gene of *Rickettsia* spp. and 802 ticks collected during the summer of 2014 from various Oklahoma state parks, preliminary results indicated the two tests correlated well, signifying the LAMP assay as a promising molecular surveillance tool which can be used effectively to detect pathogens in field-collected ticks and can be further developed for use in resource-limited countries. The results from these two objectives have and will continue to be valuable in the quest for a better understanding of the ecology of tick-borne diseases in the Great Plains region.

REFERENCES

Adjemian, J., Krebs, J., Mandel, E., & McQuiston, J. (2009). Spatial clustering by disease severity among reported Rocky Mountain Spotted Fever cases in the United States, 2001-2005. *American Journal of Tropical Medicine and Hygiene , 80* (1), 72-77.

Amsden, J., Warmack, S., & Gubbins, P. (2005). Tick-borne bacterial, rickettsial, spirochetal, and protozoal infectious diseases in the United States; a comprehensive review. *Pharmacotherapy , 25* (2), 191-210.

Anderson, B., Dawson, J., Jones, D., & Wilson, K. (1991). *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *Journal of Clinical Microbiology , 29* (12), 2838-2842.

Anderson, B., Sims, K., Olson, J., Childs, J., Piesman, J., Happ, C., et al. (1993). *Amblyomma americanum*: a potential vector of human ehrlichiosis. *American Journal of Tropical Medicine and Hygiene , 49* (2), 239-244.

Anderson, J., Andreadlis, T., Vossbrinck, C., Tirrell, S., Wakem, E., French, R., et al. (1999). Isolation of West Nile virus from mosquitoes, crows, and a cooper's hawk in Connecticut. *Science , 286* (5448), 2331-2333.

Aonuma, H., Suzuki, M., Iseki, H., Perera, N., Nelson, B., Igarashi, I., et al. (2008). Rapid identification of plasmodium-carrying mosquitoes using loop-mediated isothermal amplification. *Biochemical and Biophysical Research Communications , 376*, 671-676.

Arif, M., Aguilar-Moreno, G., Wayadande, A., Fletcher, J., & Ochoa-Corona, F. (2014). Primer modification improves rapid and sensitive in vitro and field-deployable assays for detection of high plains virus variants. *Applied and environmental microbiology , 80* (1), 320-327.

Armstrong, P.M., Rich, S.M., Smith, R.D., Hartl, D.L., Spielman, A., & Telford, S.R. 3rd. (1996). A new Borrelia infecting Lone Star ticks. *Lancet*, 347(8993), 67-68.

Baer-Lehman, M., Light, T., Fuller, N., Barry-Landis, K., Kindlin, C., & Stewart, R. (2012). Evidence for competition between *Ixodes scapularis* and *Dermacentor albipictus* feeding concurrently on white-tailed deer. *Experimental and Applied Acarology , 58* (3), 301-314.

Barbour, A.G. Maupin, G.O., Teltow, G.J., Carter, C.J., & Piesman, J. (1996). Identification of an uncultivable Borrelia species in the hard tick *Amblyomma americanum*: possible agent of a lyme disease-like illness. *The Journal of Infectious Diseases , 173* (2), 403-409.

Barrett, A., Little, S., & Shaw, E. (2014). *Rickettsia amblyommii* and *R. montanenesis* infection in dogs following natural exposure to ticks. *Vector-Borne and Zoonotic Diseases , 14* (1), 20-25.

Barrett, A., Noden, B., Gruntmeir, J., Holland, T., Mitcham, J., Martin, J., et al. (2015). County scale distribution of *Amblyomma americanum* (Ixodida: Ixodidae) in Oklahoma: addressing local deficits in tick maps based on passive reporting. *Journal of Medical Entomology*, 52(2), 269-273.

Beall, M., Alleman, A., Breitschwerdt, E., Cohn, L., Couto, C., Dryden, M., et al. (2012). Seroprevalence of *Ehrilichia canis*, *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in dogs in North America. *Parasites & Vectors , 5*, e29.

Bishopp, F., & Trembley, H. (1945). Distribution and hosts of certain North American ticks. *The Journal of Parasitology , 31* (1), 1-54.

Bowman, D., Little, S., Lorentzen, L., Shields, J., Sullivan, M., & Carlin, E. (2009). Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: results of a national clinic-based serologic survey. *Veterinary Parasitology , 160* (1), 138-148.

Bradley, K., Bergman, D., Woods, J., Crutcher, J., & Kirchhoff, L. (2000). Prevalence of American trypanosomiasis (Chagas disease) among dogs in Oklahoma. *Journal of the American Veterinary Medical Association , 217* (12), 1853-1857.

Bull, C., & Burzacott, D. (2001). Temporal and spatial dynamics of a parapatric boundary between two Australian reptile ticks. *Molecular Ecology , 10* (3), 639-648.

Buller, R., Arens, M., Hmiel, S., Paddock, C., Sumner, J., Rikihisa, Y., et al. (1999). *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *The New England Journal of Medicine , 341* (3), 148-155.

Carmichael, J., & Fuerst, P. (2010). Molecular detection of *Rickettsia bellii*, *Rickettsia montanensis*, and *Rickettsia rickettsii* in a *Dermacentor variabilis* tick from nature. *Vector-Borne and Zoonotic Diseases , 10* (2), 111-115.

Castellaw, A., Showers, J., Goddard, J., Chenney, E., & Varela-Stokes, A. (2010). Detection of vector-borne agents in lone star ticks, *Amblyomma americanum* (Acari: Ixodidae), from Mississippi. *Journal of Medical Entomology , 47* (3), 473-476.

CBS News. (2014, May 28). Rare Heartland virus kills man in Oklahoma. Online: http://www.cbsnews.com/news/rare-heartland-virus-kills-man-in-oklahoma/ Accessed March 31, 2016).

Centers for Disease Control and Prevention (CDC). (2012). Mosquito species in which West Nile virus has been detected, United States, 1999-2012. Online: http://www.cdc.gov/westnile/resources/pdfs/mosquitospecies1999-2012.pdf. (Accessed March 31, 2016).

CDC. (2013). Tularemia - United States, 2001-2010. MMWR Morb Mortal Wkly Rep, 62, 963-966.

CDC. (2015). Tickborne Diseases of the U.S. Online: http://www.cdc.gov/ticks/diseases/ (Accessed March 8, 2016).

CDC. (2016). Zika Virus. Online: http://www.cdc.gov/zika/ (Accessed March 31, 2016).

Chen, S., Dumler, J., Bakken, J., & Walker, D. (1994). Identification of a granulocytotropic ehrlichia species as the etiologic agent of human disease. *Journal of Clinical Microbiology , 32* (3), 589-595.

Childs, J., & Paddock, C. (2003). The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. *Annual Review Entomology , 48*, 307-337.

Clarke, C., Bradley, K., Wright, J., & Glowicz, J. (2013). Case report: emergence of autochthonous cutaneous leishmaniasis. *American Journal of Tropical Medicine and Hygiene , 88* (1), 157-161.

Cortinas, R., & Spomer, S. (2013). Lone star tick (Acari: Ixodidae) occurrence in Nebraska: historical and current perspectives. *Journal of Medical Entomology , 50* (2), 244-251.

Crowder, C., Rounds, M., Phillipson, C., Picuri, J., Matthews, H., Halverson, J., et al. (2010). Extraction of total nucleic acids from ticks for the detection of bacterial and viral pathogens. *Journal of Medical Entomology , 47* (1), 89-94.

Dahlgren, F., Holma, R., Paddock, C., Callinan, L., & McQuiston, J. (2012). Fatal Rocky Mountain Spotted Fever in the United States, 1999-2007. *American Journal of Tropical Medicine and Hygiene , 86* (4), 713-719.

Dantas-Torres, F. (2007). Rocky Mountain Spotted Fever. *The Lancet Infectious Diseases , 7* (11), 724-732.

Dantas-Torres, F., Lia, R., Capelli, G., & Otranto, D. (2013). Efficiency of flagging and dragging for tick collection. *Experimental and Applied Acarology , 61* (1), 119-127.

Davidson, W., Lockhart, J., Stallknecht, D., & Howerth, E. (1991). Susceptibility of red and fray foxes to infection by *Ehrlichia chaffeensis*. *Journal of Wildlife Diseases , 35* (4), 696-702.

Dawson, J. E., Biggie, K. L., Warner, C. K., Cookson, K., Jenkins, S., Levine, J. F., & Olson J. G. 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. AJVR 57(8): 1175-1179**.**

Demma, L., Traeger, M., Nicholson, W., Paddock, C., Blau, D., Eremeeva, M., et al. (2005). Rocky Mountain Spotted Fever from an unexpected tick vector in Arizona. *The New England Journal of Medicine , 353* (6), 587-594.

Dennis, D., Nekomoto, T., Victor, J., Paul, W., & Piesman, J. (1998). Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* (Acari: Ixodidae) in the United States. *Journal of Medical Entomology , 35* (5), 629-638.

Dobson, A., & Foufopoulos, J. (2001). Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London , 356*, 1001-1012.

Drame, P., Fink, D., Kamgno, J., Herrick, J., & Nutman, T. (2014). Loop-mediated isothermal amplification (LAMP) for the rapid and semi-quantitative detection of loa loa infection. *Journal of Clinical Microbiology* , 1-23.

Drexler, N.A., Dahlgren, F.S., Heitman, K.N., Massung, R.F., Paddock, C.D., & Behravesh, C.B. (2016). National Surveillance of Spotted Fever Group Rickettsioses in the United States, 2008-2012. *American Journal of Tropical Medicine and Hygiene*, 94, 26-34.

Dumler, J., & Walker, D. (2005). *Rickettsia typhi* (murine typhus). *Principles and practices of infectious diseases , 6*, 2306-2309.

Durden, L., Oliver, J., Banks, C., & Vogel, G. (2002). Parasitism of lizards by immature stages of the blacklegged tick, *Ixodes scapularis* (Acari, Ixodidae). *Experimental and Applied Acarology , 26*, 257-266.

Edwards, K., Goddard, J., Jones, T., Paddock, C., & Varela-Stokes, A. (2011). Cattle and the natural history of *Rickettsia parkeri* in Mississippi. *Vector-borne and Zoonotic Diseases , 11* (5), 485-491.

Eisen, L. (2007). A call for renewed research on tick-borne *Francisella tularensis* in the Arkansas-Missouri primary national focus of Tularemia in humans. *Journal of Medical Entomology , 44* (3), 389-397.

Eisen, R., Mead, P., Meyer, A., Pfaff, L., Bradley, K., & Eisen, L. (2008). Ecoepidemiology of tularemia in the southcentral United States. *American Journal of Tropical Medicine and Hygiene, 78* (4), 586-594.

Estrada-Pena. (2008). Climate, niche, ticks, and models: what they are and how we should interpret them. *Parasitology Research , 103* (1), 87-95.

Ewing, S., Panciera, R., Kocan, K., Ge, N., Welsh, R., Olson, R., et al. (1997). A winter outbreak of anaplasmosis in a nonendemic area of Oklahoma: a possible role for *Dermacentor albipictus*. *Journal of Veterinary Diagnostic Investigation , 9*, 206-208.

Folkema, A., Holma, R., & McQuiston, J. C. (2012). Trends in clinical diagnoses of rocky mountain spotted fever among American Indians, 2001-2008. *American Journal of Tropical Medicine and Hygiene , 86* (1), 152-158.

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E., Boehme, C., Notomi, T., et al. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *Immunology and Medical Microbiology , 62*, 42-48.

Fritzen, C., Huang, J., Westby, K., Freye, J., Dunlap, B., Yabsley, M., et al. (2011). Infection prevalences of common tick-borne pathogens in adult lone star ticks (*Amblyomma americanum*)

and American dog ticks (*Dermacentor variabilis*) in Kentucky. *The American Journal of Tropical Medicine and Hygiene , 85* (4), 718-723.

Gage, K., Ostfeld, R., & Olson, J. (1995). Nonviral vector-borne zoonoses associated with mammals in the United States. *Journal of Mammalogy , 76* (3), 695-715.

Garibyan, L., & Avashia, N. (2013). Polymerase chain reaction. *Journal of Investigative Dermatology , 133*, e6.

Garvin, S., Noden, B., Dilwith, J., Fox, S., Payton, M., & Barker, R. (2015). Sylvatic infestation of Oklahoma reptiles with immature (Acari: Ixodidae). *Journal of Medical Entomology , 52* (5), 873-878.

Ginsberg, H., & Ewing, C. (1989). Comparison of flagging, walking, trapping, and collecting from hosts as sampling methods for northern deer ticks, *Ixodes dammini*, and lone-star ticks, *Amblyomma americanum* (Acari: Ixodidae). *Experimental & Applied Acarology , 7* (4), 313-322.

Gray, M. (2012). Mitochondrial evolution. *Cold Spring Harbor Perspectivies in Biology* , 1-16.

Halos, L., Jamal, T., Vial, L., Maillard, R., Suau, A., Le Menach, A., et al. (2004). Determination of an efficient and reliable method for DNA extraction from ticks. *Veterinary Research , 35* (6), 709-713.

Harkess, J., Ewing, S., Brumit, T., & Mettry, C. (1991). Ehrilichiosis in children. *Pediatrics , 87* (2), 199-203.

Hayasaka, D., Aoki, K., & Morita, K. (2013). Development of simple and rapid assay to detect viral RNA of tick-borne encephalitis virus by reverse transciption-loop-mediated isothermal amplification. *Virology Journal , 68*, 1-10.

Hopla, C. (1960). The transmission of tularemia organisms by ticks in the southern states. *Southern Medical Journal , 53*, 92-97.

Huang, X., Hu, X., Ma, H., Du, Y., Ma, H., Kang, K., et al. (2014). Detection of new bunyavirus RNA by reverse transcription-loop-mediated isothermal amplification. *Journal of Clinical Microbiology , 52* (2), 531-535.

Huber, E., Ji, D., Howell, L., Zhang, Z., Chen, H., Ching, W., et al. (2012). Loop-mediated isothermal amplification assay targetting the 47-Kda gene of *Orientia tsutsugamushi*: A rapid and sensitive alternative to real-time PCR. *Medical Microbiology and Diagnosis , 1* (4), 1-5.

Hulstine, R. (2015, May 27). *New Tick-Borne Virus Confirmed In Oklahoma.* Retrieved November 19, 2015, from News9 Oklahoma's Own: http://www.news9.com/story/29168577/new-tick-borne-virus-confirmed-in-oklahoma

Ikadi, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., et al. (2004). Molecular evidence of infections with *Babesia gibsoni* parasites in Japan and evaluation of the diagnostic

potential of a loop-mediated isothermal amplification method. *Journal of Clinical Microbiology , 42* (6), 2465-2469.

James, A., D, L., Wormser, G., I, S., MA, M., & BJB, J. (2001). *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. *The Journal of Infectious Diseases , 183* (12), 1810-1814.

Jiang, J., Stromdahi, E., & Richards, A. (2012). Detection of *Rickettsia parkeri* and Candidatus *Rickettsia andeanae* in *Amblyomma maculatum* gulf coast ticks collected from humans in the United States. *Vector-Borne and Zoonotic Diseases , 12* (3), 175-182.

Jiang, J., Yarina, R., Miller, M., Stromdahl, E., & Richards, A. (2010). Molecular detection of *Rickettsia amblyommii* in *Amblyomma americanum* parasitizing humans. *Vector-Borne and Zoonotic Diseases , 10* (4), 329-340.

Johnson, M., Adams, J., McDonald-Hamm, C., Wendelboe, A., & Bradley, K. (2015, April 8). Seasonality and survival associated with three outbreak seasons of West Nile virus disease in Oklahoma- 2003, 2007, and 2012. *Journal of Medical Virology* , 1-8.

Johnson, R., Schmid, G., Hyde, F., Steigerwalt, A., & Brenner, D. (1984). *Borrelia burgdorferi* sp. nov.: etiological agent of Lyme disease. *International Journal of Systematic Bacteriology , 34* (4), 496-497.

Kaneko, H., Kawana, T., Fukushima, E., & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods , 70*, 499-501.

Keirans, J., & Litwak, T. (1989). Pictorial key to the adults of hard ticks, family Ixodidae (Ixodida: Ixodoidea), east of the Mississippi River. *Journal of Medical Entomology , 26* (5), 435-448.

Kjos, S., Snowden, K., & Olson, J. (2009). Biogeography and *Trypanosoma cruzi* infection prevalence of Chagas disease vectors in Texas, USA. *Vector Borne and Zoonotic Diseases , 9* (1), 41-50.

Kocan, A., Levesque, G., Whitworth, L., Murphy, G., Ewing, S., & Barker, R. (2000). Naturally occuring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. *Emerging Infectious Disease , 6* (5), 477-480.

Kocan, A., Mukolwe, S., Murphy, G., Barker, R., & Kocan, K. (1992). Isolation of *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) from *Ixodes scapularis* and *Dermacentor albipictus* ticks (Acari: Ixodidae) in Oklahoma. *Journal of Medical Entomology , 29* (4), 630-633.

Kosoy, O., Lambert, A., Hawkinson, D., Pastula, D., Goldsmith, C., Hunt, D., et al. (2015). Novel thogotovirus associated with febrile illness and death, United States, 2014. *Emerging Infectious Diseases , 21* (5), 760-764.

La Scola, B., & Raoult, D. (1997). Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. *Journal of Clinical Microbiology , 35* (11), 2715-2727.

Lanciotti, R., Roehrig, J., Deubel, V., Smith, J., Parker, M., Steel, K., et al. (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science , 286* (5448), 2333-2337.

Leger, E., Vourc'h, G., Vial, L., Chevillon, C., & McCoy, K. (2013). Changing distributions of ticks: causes and consequences. *Experimental and Applied Acarology , 59* (1), 219-244.

Little, S., Beall, M., Bowman, D., Chandrashekar, R., & Stamaris, J. (2014). Canine infection with *Dirofiliaria immitis*, *Borrelia burgdorferi*, *Anaplasma* spp., and *Ehrlichia* spp. in the United States, 2010-2012. *Parasites and Vectors , 7* (257), 1-9.

Little, S., O'Connor, T., Hempstead, J., Saucier, J., Reichard, M., Meinkoth, K., et al. (2010). *Ehrlichia ewingii* infection and exposure rates in dogs from the southcentral United States. *Veterinary Parasitology , 172*, 355-360.

Liu, A., Guan, G., Du, P., Gou, H., Liu, Z., Liu, J., et al. (2012). Loop-mediated isothermal amplification (LAMP) method based on two species-specific primer sets for the rapid identification of Chinese *Babesia bovis* and *B. bigemina*. *Parasitology International , 61*, 658-663.

Liu, A., Guan, G., Du, P., Gou, H., Zhang, J., Liu, Z., et al. (2013). Rapid identification and differntiation of *Theileria sergenti* and *Theileria sinensis* using a loop-mediated isothermal amplification (LAMP) assay. *Veterinary Parasitology , 191*, 15-22.

Lockhart, J., Davidson, W., Stallknecht, D., Dawson, J., & Howerth, E. (1997). Isolation of *Ehrlichia chaffeensis* from wild white-tailed deer (*Odocoileus virginianus*) confirms their role as natural reservoir hosts. *Journal of Clinical Microbiology , 35* (7), 1681-1686.

Logan, T., Fox, J., & Hair, J. (1985). The prevalence and distribution of Anaplasmosis in Oklahoma. *Journal of Oklahoma Veterinary Medical Association , 37*, 96-99.

Lu, X., Li, X., Mo, Z., Jin, F., Wang, B., Zhao, H. S., et al. (2012). Rapid identification of Chikungunya and Dengue virus by a real-time reverse transcription-loop-mediated isothermal amplification method. *American Journal of Tropical and Medical Hygiene , 87* (5), 947-953.

Ma, M., Liu, Z., Sun, M., Yang, J., Guan, G., Li, Y., et al. (2011). Development and evaluation of a loop-mediated isothermal amplification method for rapid detection of *Anaplasma ovis*. *Journal of Clinical Microbiology , 49* (6), 2143-2146.

Mani, R., Reichard, M., Morton, R., Kocan, K., & Clinkenbeard, K. (2012). Biology of *Francisella tularensis* subspecies holarctica live vaccine strain in the tick vector *Dermacentor variabilis*. *PLoS ONE , 7* (4), e35441.

McMullan, L., Folk, S., Kelly, A., MacNeil, A., Goldsmith, C., Metcalfe, M., et al. (2012). A new phlebovirus associated with severe febrile illness in Missouri. *The New England Journal of Medicine , 367* (9), 834-841.

McQuiston, J., Holman, R., Groom, A., Kaufman, S., Cheek, J., & Childs, J. (2000). Incidence of Rocky Mountain Spotted Fever among American Indians in Oklahoma. *Public Health Reports (1974-) , 115* (5), 469-475.

McQuiston, J., Zemtsova, G., Perniciaro, J., Hutson, M., Singleton, J., Nicholson, W., et al. (2012). Afebrile spotted fever group rickettsia infection after a bite from a *Dermacentor variabilis* tick infected with *Rickettsia montanensis*. *Vector-Borne and Zoonotic Diseases , 12* (12), 1059-1061.

Mitashi, P., Hasker, E., Ngoyi, D., Pyana, P., Lejon, V., Van der Veken, W., et al. (2013). Diagnostic accuracy of loopamp *Trypanosoma brucei* detection kit for diagnosis of human *African trypanosomiasis* in clinical samples. *PLOS Neglected Tropical Diseases , 7* (10), e2504.

Mixson, T., Campbell, S., Gill, J., Ginsberg, H., Reichard, M., Schulze, T., et al. (2006). Prevalence of Ehrlichia, Borrelia, and Rickttsial agents in *Amblyomma americanum* (Acari: Ixodidae) collected from nine states. *Journal of Medical Entomology , 43* (6), 1261-1268.

Mohon, A., Elahi, R., Khan, W., Haque, R., Sullivan Jr, D., & Alam, M. (2014). A new visually improved and sensitive loop mediated isothermal amplification (LAMP) for diagnosis of symptomatic falciparum malaria. *Acta Tropica , 134*, 52-57.

Moncayo, A., Cohen, S., Fritzen, C., Huang, E., Yabsley, M., Freye, J., et al. (2010). Absence of *Rickettsia rickettsii* and occurrence of other spotted fever group rickettsiae in ticks from Tennessee. *The American Journal of Tropical Medicine and Hygiene , 83* (3), 653-657.

Moraru, G., Goddard, J., Paddock, C., & Varela-Stokes, A. (2013). Experimental infection of cotton rats and bobwhite quail with Rickettsia parkeri. *Parasites and Vectors , 6* (70), 1-5.

Mori, Y., Hidetoshi, K., & Tsugunori, N. (2013, March 29). Loop-mediated isothermal amplification (LAMP): recent progress in research and development. *Japanese Infectious Chemotherapy* .

Mugasa, C., Katiti, D., Boobo, A., Lubega, G., Schalig, H., & Matovu, E. (2014). Comparison of nucleic acid sequence-based amplification and loop-mediated isothermal amplification for diagnosis of human African trypanosomiasis. *Diagnostic Microbiology and Infectious Disease , 78*, 144-148.

Mukolwe, S., Kocan, A., & Wyckoff, J. (1992). Serological survey for Lyme disease in domestic dogs and white-tailed deer from Oklahoma. *Annals New York Academy of Sciences* , 172-177.

Muller, H., Aysul, N., Liu, Z., Salih, D., Karagenc, T., Beyer, D., et al. (2010). Development of a loop-mediated isothermal amplification (LAMP) assay for rapid diagnosis of *Babesia canis* infections. *Transboundary and Emerging Diseases , 57*, 63-65.

Nadolny, R., Wright, C., Sonenshine, D., Hynes, W., & Gaff, H. (2014). Ticks and spotted fever group rickettsiae of southeastern Virginia. *Ticks and Tick-borne Diseases , 5*, 53-57.

Nakao, R., Stromdahl, E., Magona, J., Faburay, B., Namangala, B., Malele, I., et al. (2010). Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Ehrlichia ruminantium*. *BMC Microbiology , 296*, 1-10.

Nichols Heitman, K; Dahlgren, FS; Drexler, NA; Massung, RF; Barton Behravesh, C (2010). Increasing incidence of Ehrlichiosis in the United States: a summary of national surveillance. *American Journal of Tropical Medicine and Hygiene, 94* (1),52-60.

Ngwamidiba, M., Blanc, G., Ogata, H., Raoult, D., & Fournier, P. (2005). Phylogenetic study of *Rickettsia* species using sequences of the autotransporter protein-encoding gene sca2. *Annual New York Academy of Sciences* (1063), 94-99.

Njiru, Z. (2012). Loop-mediated isothermal amplication technology: towards point of care diagnostics. *Neglected Tropical Diseases , 6* (6), e1572.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research , 28* (12), e63-e63.

Nzelu, C., Gomez, E., Caceres, A., Sakurai, T., Martini-Robles, L., Uezato, H., et al. (2014). Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for Leishmania infection. *Acta Tropica , 132*, 1-6.

Oliver, J., Gregory, J., Cummins, G., & Joiner, M. (1993). Immature *Ixodes scapularis* (Acari: Ixodidae) parasitizing lizards from the southeastern USA. *The Journal of Parasitology , 79* (5), 684-689.

Okatlas.com. (2004, July). Ecoregions Classified by Domain. Retrieved July 5, 2015, from Web Atlas of Oklahoma: http://www.okatlas.org/okatlas/biotic/ecoregions.html.

Oklahoma Forestry Services. (2007). The Eco Regions of Oklahoma. Retrieved July 6, 2015, from: http://www.forestry.ok.gov/ecoregions-of-oklahoma.

Openshaw, J., Swerdlow, D., Krebs, J., Holman, R., Mandel, E., Harvey, A., et al. (2010). Rocky Mountain Spotted Fever in the United States, 2000-2007: interpreting contemporary increases in incidence. *The American Journal of Tropical Medicine and Hygiene , 83* (1), 174-182.

Osman, H., Eltom, K., Musa, N., Bilal, N., Elbashir, M., & Aradaib, I. (2013). Development and evaluation of loop-mediated isothermal amplification assay for detection of Crimean Congo hemorrhagic fever virus in Sudan. *Journal of Virological Methods* .

Overzier, E., Pfister, K., Herb, I., Mahling, M., Bock, G., & Silaghi, C. (2013). Detection of tick-borne pathogens in roe deer (*Capreolus capreolus*), in questing ticks (*Ixodes ricinus*), and in ticks infesting roe deer in southern Germany. *Ticks and tick-borne diseases , 4* (4), 230-328.

Pagac, B., Miller, M., Mazzei, M., Nielsen, D., Jiang, J., & Richards, A. (2014). *Rickettsia parkeri* and *Rickettsia montanensis*, Kentucky and Tennessee, USA. *Emerging Infectious Diseases , 20* (10), 1750-1752.

Pan, L., Zhang, L., Wang, G., & Lie, Q. (2012). Rapid, simple, and sensitive detection of the ompB gene of spotted fever group rickettsia by loop-mediated isothermal amplification. *BioMed Central Infectious Diseases , 12*, 254.

Pan, L., Zhang, L., Wang, G., Liu, Q., Yu, Y., Wang, S., et al. (2011). Rapid, simple, and sensitive detection of *Anaplasma phagocytophilum* by loop-mediated isothermal amplification of the msp2 gene. *Journal of Clinical Microbiology , 49* (12), 4117-4120.

Paras, K., Little, S., Reichard, M., & Reiskind, M. (2012). Detection of *Dirofilaria immitis* and Ehrilichia species in coyotes (*Canis latrans*), from rural Oklahoma and Texas. *Vector-Borne and Zoonotic Diseases , 12* (7), 619-621.

Paras, K., O'Brien, V., & Reiskind, M. (2014). Comparison of the vector potential of different mosquito species for the transmission of heartworm, *Dirofilaria immitis*, in rural and urban areas in and surrounding Stillwater, Oklahoma, USA. *Medical and Veterinary Entomology , 28* (1), 60-67.

Paris, D. H. (2008). Simple, rapid and sensitive detection of *Orientia tsutsugamushi* by loop-isothermal DNA amplification. *The Royal Society of Tropical Medicine and Hygiene* , 1239-1246.

Parola, P., Paddock, C., Socolovschi, C., Labruna, M., Mediannikov, O., Kernif, T., et al. (2013). Update on tick-borne Rickettsioses around the world: a geographic approach. *Clinical Microbiology Reviews , 26* (4), 657-702.

Poole, C., Tanner, N., Zhang, Y., Evans Jr, T., & Carlow, C. (2012). Diagnosis of brugian filariasis by loop-mediated isothermal amplification. *PLOS Neglected Tropical Diseases , 6* (12), e1948.

Prioleau, W., Parker, E., Bradham, R., & Gergorie, H. (1976). *Dirofilaria immitis* (dog heartworm) as a pulmonary lesion in humans. *The Annals of Thoracic Surgery , 21* (5), 382-385.

Raoult, D., & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology , 10* (4), 694-719.

Renvoise, A., Rolain, J., Socolovschi, C., & Raoult, D. (2012). Widespread use of real-time PCR for rickettsial diagnosis. *Immunology and Medical Microbiology , 64*, 126-129.

Richards, A. (2012). Worldwide detection and identification of new and old rickettsiae and rickettsial diseases. *Immunology and Medical Microbiology , 64*, 107-110.

Rodgers, S., Welsh, R., & Stebbins, M. (1994). Seroprevalence of bovine anaplasmosis in Oklahoma from 1977 to 1991. *Journal of Veterinary Diagnostic Investigation , 6* (2), 200-206.

Roux, V., & Raoult, D. (2000). Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (ompB). *International Journal of Systemic and Evolutionary Microbiology , 50* (4), 1449-1455.

Russart, N., Dougherty, M., & Vaughan, J. (2014). Survey of ticks (Acari: Ixodidae) and tick-borne pathogens in North Dakota. *Journal of Medical Entomology , 51* (5), 1087-1090.

Salazar, J. (2015). *Detection of tick-borne pathogens in lab reared tick colonies and wild populations.* Master's Thesis, Oklahoma State University, Entomology and Plant Pathology, Stillwater.

Salih, D., Ali, A., Liu, Z., Bakheit, M., Taha, K., El Imam, A., et al. (2011). Development of a loop-mediated isothermal amplification method for detection of *Theileria lestoquardi*. *Parasitology Res* , 1-6.

Sands, K. (2005). Texas history 101. *Texas Monthly , 33* (4), 1. Accessed at March 31, 2016 at http://www.texasmonthly.com/articles/texas-history-101-18/.

Savage, H., Godsey Jr, M., Lamber, A., Pannella, N., Burkhalter, K., Harmon, J., et al. (2013). First detection of heartland virus (Bunyaviridae: Phlebovirus) from field collected arthropods. *The American Journal of Tropical Medicine and Hygiene , 89* (3), 445-452.

Simon, F., Siles-Lucas, M., Morchon, R., Gonzalez-Miguel, J., Mellado, I., Carreton, E., et al. (2012). Human and animal dirofilariasis: the emergence of a zoonotic mosaic. *Clinical microbiology reviews , 25* (3), 507-544.

Sokhna, C., Mediannikov, O., Fenollar, F., Bassene, H., Diatta, G., Tall, A., et al. (2013). Point-of-care laboratory of pathogen diagnosis in rural Senegal. *Neglected Tropical Diseases , 7* (1), e1999.

Solanki, G. (2012). Polymerase chain reaction. *International Journal of Pharmacological Research , 2* (3), 98-102.

Springer, Y., Eisen, L., Beati, L., James, A., & Eisen, R. (2014). Spatial distribution of counties in the continental United States with records of occurrence of *Amblyomma americanum* (Ixodida: Ixodidae). *Journal of Medical Entomology , 51* (2), 342-351.

Starkey, L., Barrett, A., Chandrashekar, R., Stillman, B., Tyrrell, P., Thatcher, B., et al. (2014). Development of antibodies to and PCR detection of Ehrlichia spp. in dogs following natural tick exposure. *Veterinary Microbiology , 173*, 379-384.

Starkey, L., Barrett, A., Chandrashekar, R., Thatcher, B., Tyrrell, P., & Little, S. (2015). Persistent *Ehrlichia ewingii* infection in dogs after natural tick infestation. *Journal of Veterinary Internal Medicine , 29*, 552-555.

Stromdahl, E., Jiang, J., Vince, M., & Richards, A. (2011). Infrequency of *Rickettsia rickettsii* in *Dermacentor variabilis* removed from humans, with comments on the role of other human-biting

ticks associated with spotted fever group Rickettsiae in the United States. *Vector Borne Zoonotic Diseases , 11* (7), 969-977.

Stromdahl, E., Vince, M., Billingsley, P., Dobbs, N., & Williamson, P. (2008). *Rickettsia amblyommii* infecting *Amblyomma americanum* larvae. *Vector-Borne and Zoonotic Diseases , 8* (1), 15-24.

Takagi, H., Itoh, M., Kasai, S., Yahathugoda, T., Weerasooriya, M., & Kimura, E. (2011). Development of loop-mediated isothermal amplification method for detecting *Wuchereria bancrofti* DNA in human blood and vector mosquitoes. *Parasitology International , 60*, 493-497.

Taylor, J., Istre, G., McChesney, T., Satalowich, F., Parker, R., & McFarland, L. (1991). Epidemiological characteristics of human Tularemia in the southwest-central states, 1981-1987. *American Journal of Epidemiology , 133* (10), 1032-1038.

Teel, P.D., Ketchum, H.R., Mock, D.E., Wright, R.E., & Strey, OF. (2010). The Gulf Coast tick: a review of the life history, ecology, distribution, and emergence as an arthropod of medical and veterinary importance. *Journal of Medical Entomology* 47(5), 707-722.

Teoh, B., Sam, S., Tan, K., Johari, J., Danlami, M., Hooi, P., et al. (2013). Detection of dengue viruses using reverse transcription-loop-mediated isothermal amplification. *BMC Infectious Diseases , 13*, 387.

Varela-Stokes, A. S. (2007). Transmission of Ehrilichia chaffeensis from lone star ticks (*Amblyomma americanum*) to white-tailed deer (Odocoileus virginianus). *Journal of Wildlife Diseases , 43* (3), 376-381.

Vaughn, M., Delisle, J., Johnshon, J., Daves, G., Williams, C., Reber, J., et al. (2014). Seroepidemiologic study of human infections with spotted fever group rickettsiae in North Carolina. *Journal of Clinical Microbiology , 52* (11), 3960-3966.

Vincent, M., Xu, Y., & Kong, H. (2004). Helicase-dependent isothermal DNA amplification. *European Molecular Biology Organization Reports , 5* (8), 795-800.

Wang, L., He, L., Fang, R., Song, Q., Tu, P., Jenkins, A., et al. (2010). Loop-mediated isothermal amplification (LAMP) assay for detection of Theileria sergenti infection targeting the p33 gene. *Veterinary Parasitology , 171*, 159-162.

Weaver, S., & Reisen, W. (2010). Present and future arboviral threats. *Antiviral Research , 85*, 328-345.

Yang, J., Guan, G., Nu, Q., Liu, Z., Li, Y., Liu, J., et al. (2012). Development and application of a loop-mediated isothermal amplification assay for rapid detection of *Borrelia burgdorferi* s. I. in ticks. *Transboundary and Emerging Diseases* , 1-7.

VITA

Jaclyn Elizabeth Martin

Candidate for the Degree of

Master of Science

Thesis: DISTRIBUTION OF TICKS OF MEDICAL AND VETERINARY IMPORTANCE ALONG THE CHISHOLM TRAIL AND DEVELOPMENT OF A MOLECULAR ASSAY TO DETECT *RICKETTSIA* SPP. IN FIELD-COLLECTED TICKS IN OKLAHOMA

Major Field: Entomology and Plant Pathology

Education:

- Bachelor of Science (Entomology) Oklahoma State University, Stillwater, OK. 2015.
- Completed the requirements for the Master of Science in Entomology and Plant Pathology at Oklahoma State University, Stillwater, Oklahoma in May, 2016.

Experience: Linnaean Team Member: Southwestern Branch Champions 2014 and 2015

Professional Memberships: Entomological Society of America

Presentations (underline=presenter)**:**

- Martin, J., Noden, BH. Where the wind comes sweepin' down the plain: an update on vector-borne disease research in Oklahoma. Presentation at SW Branch ESA, San Antonio, TX (Feb 2014).
- Martin, JE, Noden, BH. 2014. Along the Chisholm Trail: Establishing the distribution of Lone Star tick (*Amblyomma americanum*) populations in Oklahoma. Presentation at Annual ESA, Portland, OR (Nov 2014).
- Martin, J., Noden, BH.. Risk of exposure to ticks and tick-borne pathogens in state parks in Oklahoma. Presentation at the SW Branch ESA, Tulsa, OK (February 2015). (2nd Place Master's Ten-minute paper)
- Martin, J, Noden, BH. Detection of *Rickettsia* spp in field-collected ticks using loop-mediated isothermal amplification (LAMP). Presentation at Annual ESA, Minneapolis, MN (Nov 2015).

Publications:

- Barrett, AW, Noden, BH, Gruntmeir, JM, Holland, T, Mitcham, JR, Martin, JE, Johnson, EM, Little, SE. 2015. County scale distribution of *Amblyomma americanum* in Oklahoma: addressing local deficits in tick maps based on passive reporting. Journal of Medical Entomology. 52(2): 269–273.