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Detection Of Tick-Borne Bacterial Agents In Lone Star Ticks (Amblyomma Americanum) And Various Wildlife In Mississippi

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DETECTION OF TICK-BORNE BACTERIAL AGENTS IN LONE STAR TICKS (*AMBLYOMMA AMERICANUM*) AND VARIOUS WILDLIFE IN MISSISSIPPI

By

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Two studies were conducted to evaluate the presence of tick-borne bacterial agents in *Amblyomma americanum*, lone star tick (LST), and various wildlife in Mississippi. Adult LSTs had DNA evidence of *Ehrlichia chaffeensis* (3.7%), *E. ewingii* (6.3%), and *Borrelia lonestari* (2.6%), while both larval (24.3%) and adult (43.5%) LSTs were positive for a *Rickettsia* spp. by polymerase chain reaction (PCR). White-tailed deer (WTD) were the only wildlife PCR positive for *E. chaffeensis* (18.8%), *B. lonestari* (3.1%), and *Anaplasma phagocytophilum* (3.1%). In addition, WTD had the highest seroprevalence to *B. lonestari* (19.3%) and *E. chaffeensis* (43.9%) antigens while raccoons had the highest seroprevalence to spotted fever group rickettsiae (*R. parkeri* antigen) (73.7%). These studies demonstrate evidence of potentially zoonotic tick-borne agents in LSTs and wildlife in Mississippi underscoring the importance of monitoring these agents for human and animal health.

DEDICATION

I would like to dedicate this research and thesis to my loving parents, Lane and Ronnie, who have loved, supported and believed in me when it was hard to believe in myself. Special dedication goes to my husband, Matthew, who has kept me sane and stood by my side throughout this journey and my friend Banu, for showing me how exciting and rewarding research can be; she is the sole reason I considered Graduate School.

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CHAPTER I

INTRODUCTION

Background

Second only to mosquitoes in public health importance, ticks are important vectors which are capable of transmitting pathogens that impact human and animal health. *Amblyomma americanum* (Acari: Ixodidae), the lone star tick (LST), is an ixodid, three-host tick, known for its aggressive feeding behavior on vertebrate hosts such as white-tailed deer (WTD; *Odocoileus virginianus*), raccoons (*Procyon lotor*), opossums (*Didelphis virginianus*), and even humans (Felz et al. 1996, Kollars et al. 2000, Merten and Durden 2000). Originally considered to be harmless pests, LSTs are now known to harbor and/or transmit several agents such as *Ehrlichia chaffeensis,* the cause of human monocytic ehrlichiosis (HME), *E. ewingii,* causative agent of human and canine granulocytic ehrlichiosis, *Borrelia lonestari,* putative agent of "southern tick-associated rash illness" (STARI), *Rickettsia* spp. (*Rickettsia amblyommii* and *R. parkeri*) which have been associated with human disease, and *Francisella tularensis,* the agent of tularemia (Childs and Paddock 2003, Mixson et al. 2006, Petersen et al. 2008). Although LSTs are the most common tick species in Mississippi, other ticks such as *A. maculatum* and *Ixodes scapularis* are also

found in the state. *Amblyomma maculatum,* the Gulf Coast tick, is the primary vector for *R. parkeri,* the most recently recognized human disease agent that causes “American Boutonneuse Fever” (Sumner et al. 2007). *Ixodes scapularis,* the black-legged tick, is the vector for *Anaplasma phagocytophilum,* which causes human granulocytic anaplasmosis (HGA) (Hodzic et al. 1998) as well as Lyme disease, which will not be discussed further here.

Currently, little is known about tick-borne disease agents in Mississippi. A recent study by Goddard et al. (2003) tested several species of ticks, collected throughout Mississippi, for spotted fever group (SFG) rickettsiae, *E. chaffeensis* and *Borrelia* species. Of these ticks tested by immunofluorescent antibody (FA) staining, 20% were found to be infected with SFG rickettsiae and 22% were positive for *E. chaffeensis*; 15% of the ticks that were positive by FA staining had molecular evidence of SFG rickettsiae. Studies of wildlife in the South have found exposure to and/or infection with some tick-borne agents specifically in Mississippi. One WTD was identified by molecular testing of DNA with *E. chaffeensis* while 53%-100% of deer tested from this state had antibodies to this agent (Dawson et al. 1994, Yabsley et al. 2003). In addition, raccoons in Mississippi have been detected with antibodies to *E. chaffeensis* (Lockhart et al. 1997). Finally, 25% of WTD tested, by indirect immunofluorescent antibody assays, in Mississippi were seropositive to *A. phagocytophilum* (Dugan et al. 2006). To the author"s knowledge, evidence of tick-borne agents in ticks and wildlife from Mississippi are limited to these studies.

Objective

The overall goal of this research was to obtain additional data regarding tick-borne diseases in order to benefit the health care profession and provide information on potential risk of exposure to humans in Mississippi. To meet this objective two studies were conducted to evaluate the prevalence of tick-borne bacterial agents in *A. americanum* and various wildlife species in Mississippi.

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CHAPTER II

LITERATURE REVIEW

Amblyomma americanum

Amblyomma americanum (commonly referred to as the lone star tick (LST) due to the adult female"s characteristic white spot on its scutum) is the most abundant species of tick in the southeastern U.S. The range of LSTs extends from west-central Texas, northward to Iowa, east towards the Atlantic coast and as far north as Maine (Cooley and Kohls 1944, Bishopp and Trembley 1945, Keirans and Lacombe 1998). The distribution of these ticks has expanded over the years due to their introduction into previously un-infested areas on people or animals (Bishopp and Trembley 1945). For example, approximately five thousand ticks were collected between 1946 and 1948 in Long Island, New York, but none were *A. americanum* (Anastos 1947, Collins et al. 1949a, b). In a similar study over twenty years later, in 1971, nearly four thousand ticks were collected but only 19 of them were LSTs (Good 1972). However, in 1986 and 1990, 344 out of 399 (86.2%) ticks collected in Long Island, New York were LSTs (Ginsberg et al. 1991).

Amblyomma americanum is an ixodid, three-host tick which obtains its blood meals from various vertebrate hosts. Immature stages are found on small

to large mammals including a variety of ground-feeding birds, raccoons (*Procyon lotor*), opossums (*Didelphis virginianus*), skunks (*Mephitis nigra*), cottontail rabbits (*Sylvilagus floridanus*), squirrels (*Sciurus* spp.), red fox (*Vulpes vulpes*), and white-tailed deer (WTD; *Odocoileus virginianus*). Adult LSTs may parasitize medium to large mammals including raccoons, WTD, coyotes (*Canis latrans*), red fox, and opossums, as well as wild turkeys (*Meleagris gallopavo*) (Cooley and Kohls 1944, Zimmerman et al. 1988, Kollars et al. 1999). Interestingly, the most common host species for *A. americanum* immature stages differs among geographic locations. For example, the immature stages in central Tennessee prefer eastern cottontail rabbits (Zimmerman et al. 1988) however, in the state of Virginia, rabbits were not a common host (Sonenshine et al. 1965). All stages of these aggressive feeders are commonly found attached to humans (Felz et al. 1996, Merten and Durden 2000). One report showed that over one hundred ticks each were found on a group of ten soldiers within a two-week period on Little Rock Air Force Base, Arkansas, which was historically infested with lone star ticks (Goddard and McHugh 1990). In fact, LSTs are typically the most common tick found on people. In a five-year study of ticks parasitizing humans in Georgia and South Carolina, 758 out of 913 (83%) of those ticks were *A. americanum* (Felz et al. 1996). In addition, a ten-year study in Mississippi concluded that 53% of all tick bites reported during that time were from LSTs (Goddard 2002).

Though it is less likely to be as active in midwinter, *A. americanum* can be active throughout the year in the southern states (Bishopp and Trembley 1945). The activity of nymphal and adult stages is highest in the spring, during which

there is a peak in their abundance between the months of April and June. The numbers of adult and nymphal LSTs decline towards the end of summer while the abundance of larvae increases (Paddock and Childs 2003). In a two-year study in Mississippi, questing adult LSTs were most active from April to early July. Nymphal populations followed a pattern similar to adult *A. americanum* in Mississippi, but reached a bi-modal peak in April/May and in August/September. In contrast, larvae were at their peak from late June to October, however, they were also occasionally found in March and April (Goddard 2007).

Amblyomma americanum as a Vector for Bacteria

In general, ticks are excellent vectors for bacteria, having few natural enemies, being essentially long-lived, and using a wide host range with the tendency to feed on several different hosts throughout their life cycle. Until the early 1990s, *A. americanum* was considered to be mainly a nuisance (Childs and Paddock 2003). However, today, it is a known or suspected vector for numerous zoonotic bacteria. Bacterial agents transmitted by lone star ticks include *Borrelia lonestari*, putative agent of “southern tick-associated rash illness” (STARI), *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis (HME), *E. ewingii*, a causative agent of human and canine granulocytic ehrlichiosis, *Francisella tularensis*, the agent of tularemia, *Rickettsia amblyommii,* a suspected pathogen, and possibly *R. parkeri*, the cause of “American Boutonneuse Fever”.

Ehrlichia chaffeensis

Ehrlichia chaffeensis, in the family *Anaplasmataceae,* is an obligate intracellular bacterium which invades mononuclear cells and replicates in clusters known as morulae, which are modified early endosomes (Barnewall et al. 1997, Popov et al. 1998). First isolated in 1991 from a patient in Ft. Chaffee, Arkansas, this gram-negative bacterium is the etiologic agent of human monocytotropic (or monocytic) ehrlichiosis (HME) (Anderson et al. 1991, Dawson et al. 1991, Rikihisa 1999). In the late 1980s, several observations eventually led to the suspicion that *A. americanum* was a vector for *E. chaffeensis.* For example, the peak activity of adult and nymphal LSTs correlated with the highest occurrence of HME from May through July and the distribution of LSTs overlapped with cases of HME (Eng et al. 1990, Lockhart et al. 1995). Molecular evidence for the role of *A. americanum* was first shown with the detection of DNA from *E. chaffeensis* in LSTs from five states using PCR (Anderson et al. 1993). Later, another study showed WTD from a single location had antibodies against *E. chaffeensis* following the introduction of *A. americanum* to that area (Lockhart et al. 1995). *Amblyomma americanum* was finally implicated as a vector when antibodies to *E. chaffeensis* were identified in WTD in areas where LSTs were present, as compared to areas where LSTs were scarce and most deer were seronegative (Lockhart et al. 1996). Transmission of *E. chaffeensis* by the LST was first shown experimentally in 1995 and has since been demonstrated several more times (Ewing et al. 1995, Varela-Stokes 2007a, b). Prevalence of *E. chaffeensis* infection in LSTs ranges from 0%-29% (Anderson et al. 1992b, Anderson et al.

1993, Lockhart et al. 1997a, Lockhart et al. 1997b, Yu et al.1997, Burket et al. 1998, Roland et al. 1998, Steiner et al. 1999, Ijdo et al. 2000, Irving et al. 2000, Stromdahl et al. 2000, Whitlock et al. 2000, Wolf et al. 2000, Stromdahl et al. 2001, Steiert and Gilfoy 2002, Goddard et al. 2003, DeShields et al. 2004, Long et al. 2004, Mixson et al. 2004, Varela et al. 2004a, Schulze et al. 2005, Mixson et al. 2006, Varela-Stokes 2007b, Apperson et al. 2008, Stromdahl et al. 2008, Yabsley et al. 2008, Yabsley et al. 2009). Apart from one study that detected *E. chaffeensis* in larval LSTs, there is no convincing evidence suggesting transovarial transmission of this agent; it has also been shown that acquisition of this organism primarily occurs when the nymphal stage feeds on WTD (Rikihisa 1999, Stromdahl et al. 2001, Long et al. 2003).

Ehrlichia ewingii

The agent of both human and canine granulocytotropic (granulocytic) ehrlichiosis, *E. ewingii,* is an intracellular bacterium that invades granulocytes in clusters of morulae (Anziani et al. 1990, Popov et al. 1998). Closely related to *E. chaffeensis* and *E. canis* based on the 16S rRNA sequence, this agent was first considered to be a strain of *E. canis* and was not named *E. ewingii* until 1992 (Ewing et al. 1971, Anderson et al. 1992a). Transmission of this agent by *A. americanum* was first demonstrated experimentally in 1990, when infected LSTs were placed on susceptible dogs (Anziani et al. 1990). Since then, *E. ewingii* DNA has been detected in LSTs from Florida, Georgia, Missouri, New Jersey, New York, North Carolina, and Oklahoma with the rate of infection ranging from

0% to19% (Murphy et al. 1998, Wolf et al. 2000, Steiert and Gilfoy 2002, DeShields et al. 2004, Varela et al. 2004a, Schulze et al. 2005, Mixson et al. 2006, Yabsley et al. 2009).

Ehrlichia ewingii, initially known only as a cause of canine granulocytic ehrlichiosis, was recognized as an agent of human ehrlichiosis in 1999 (Buller et al. 1999). A few *E. ewingii* associated human ehrlichiosis cases have been documented since that time, with the majority of patients being immunocompromised (Ratnasamy et al. 1996, Buller et al. 1999, Paddock et al. 2001, Liddell et al. 2003, Hamilton et al. 2004, Paddock et al. 2005). Clinically and serologically, the human ehrlichioses caused by *E. chaffeensis* and *E. ewingii* are almost identical, leading many to question if cases of *E. ewingii* have mistakenly been reported as *E. chaffeensis* (Buller et al. 1999, Paddock et al. 2005). As of today, there has been no successful culture isolation of *E. ewingii* (Anderson et al. 1992a).

Borrelia lonestari

The Borreliae are spirochetes, which are slender, helically coiled motile bacteria in the family *Spirochaetaceae*. Borreliae are commonly referred to as gram-negative bacteria, however, due to the unique outer membrane of these bacteria compared to other bacteria, they are technically neither gram-negative nor gram-positive (Barbour and Hayes 1986, Shapiro and Gerber 2000).

Erythema migrans (EM), the bull"s eye rash that may occur in Lyme disease, has been associated with the bite of *A. americanum* ticks, especially in

the southeastern United States. However, antibodies to *Borrelia burgdorferi,* the causative agent of Lyme disease, have not been detected in these patients" sera nor has *B. burgdorferi* been cultured from skin biopsies (Campbell et al. 1995, Masters and Donnell 1995, Kirkland et al. 1997, Masters et al. 1998, Felz et al. 1999, Armstrong et al. 2001, Wormser et al. 2005b). In addition, LSTs are not known to have a high prevalence of *B. burgdorferi* infection, there is no evidence showing they are able to transmit this agent, and their saliva has been shown to be borreliacidal towards *B. burgdorferi* (Piesman and Sinsky 1988, Mukolwe et al. 1992, Ryder et al. 1992, Sanders and Oliver 1995, Piesman and Happ 1997, Stromdahl et al. 2001, Stromdahl et al. 2003, Ledin et al. 2005, Taft et al. 2005, Murphree et al. 2009). These observations led physicians to suspect a condition distinct from Lyme disease called “southern tick-associated rash illness” (STARI), “Southern Lyme”, or “Master"s disease” (Masters et al. 1998, James et al. 2001).

In 1996, using sequence analysis of the 16S rRNA and flagellin (*flaB*) genes, a novel *Borrelia* sp. was identified in LSTs, which some called, tentatively, *B. lonestari* (Barbour et al. 1996). *Borrelia lonestari* was thought to be responsible for STARI cases, but its role has become controversial and, while some consider it the putative agent of this condition, others suspect other pathogens (Armstrong et al. 2001, Burkot et al. 2001, James et al. 2001, Wormser et al. 2005b, Billeter et al. 2007b). For example, a study conducted using skin biopsies from thirty patients with EM showed they had neither evidence of *B. lonestari* nor *B. burgdorferi* infection (Wormser et al. 2005b). In contrast, *B. lonestari* DNA has been detected in LSTs removed from people in

nine states (Stromdahl et al. 2003). Despite this, it has only been associated with one case of erythema migrans (James et al. 2001). The prevalence of *B. lonestari* DNA in LSTs ranges from 0%-12% and has been documented in several states including Alabama, Arkansas, Delaware, Florida, Georgia, Kansas, Kentucky, Maryland, Missouri, New Jersey, New York, North Carolina, South Carolina, Tennessee, Texas, and Virginia (Armstrong et al. 1996, Barbour et al. 1996, Armstrong et al. 2001, Burkot et al. 2001, James et al. 2001, Rich et al. 2001, Bacon et al. 2003, Goddard et al. 2003, Stegall-Faulk et al. 2003, Stromdahl et al. 2003, Clark 2004, Varela et al. 2004a, b, Bacon et al. 2005, Schulze et al. 2005, Taft et al. 2005, Wormser et al. 2005b, Mixson et al. 2006, Schulze et al. 2006, Varela-Stokes 2007b, Apperson et al. 2008, Clay et al. 2008, Stromdahl et al. 2008, Jordan et al. 2009, Murphree et al. 2009, Yabsley et al. 2009). For example, of ticks tested in northeastern Georgia, 4/398 (1%) were positive for *B. lonestari,* and in New Jersey there was a 5.8% prevalence of infection (Varela et al. 2004a, Schulze et al. 2006). Isolation of this bacterium was successfully accomplished in 2004 (Varela et al. 2004b).

Rickettsia amblyommii

Rickettsiae in the genus *Rickettsia* are obligate intracellular bacteria which are classified into the scrub typhus group, the typhus group, and the spotted fever group (SFG). There are numerous SFG rickettsiae, some of which are non-pathogenic and others that are known to cause human disease (Raoult and Olson 1999). *Rickettsia rickettsii,* the agent of Rocky Mountain spotted fever, is a

pathogenic SFG rickettsia that was initially suspected to be transmitted by *A. americanum.* However, it was later determined that *R. amblyommii* was common in LSTs and not *R. rickettsii* (Goddard and Norment 1986). *Rickettsia amblyommii* was originally isolated from LSTs in 1974 (Burgdorfer et al. 1981). The prevalence of LSTs infected with *R. amblyommii* has been reported to range from 0%-84% (Mixson et al. 2006, Apperson et al. 2008, Stromdahl et al. 2008, Yabsley et al. 2009). The pathogenicity of this bacterium is unknown; however it may cause mild illness (Sanchez et al. 1992, Apperson et al. 2008). Recent evidence suggests that *R. amblyommii* could be the agent of STARI, since a tick removed from a STARI patient had molecular evidence of *R. amblyommii* but not of *B. lonestari* (Billeter et al. 2007a).

Rickettsia parkeri

Rickettsia parkeri, a member of the spotted fever group rickettsiae, was isolated from *A. maculatum,* commonly referred to as the Gulf Coast tick (GCT), in 1937 (Parker et al. 1939). Since then, several studies have recognized *A. maculatum* as the primary vector of *R. parkeri* due to the vast abundance of naturally infected *A. maculatum* ticks in the southeast (Philip and White 1955, Loving et al. 1978, Philip et al. 1978, Sumner et al. 2007). In addition, LSTs are capable of successfully maintaining and transmitting *R. parkeri* experimentally; and some have been detected with a natural infection (Goddard and Norment 1986, Goddard 2003, Cohen et al. 2009, Yabsley et al. 2009). Studies have confirmed that this agent is transstadially and transovarially transmitted by both

A. americanum and *A. maculatum* (Goddard 2003). In a study on seasonal activity of *Amblyomma* spp. in Mississippi, only adult *A. maculatum* were easily collected by a dragging technique while all stages of *A. americanum* were commonly collected by this method. In addition, unlike *A. americanum, A. maculatum* typically do not feed on humans (Goddard 2007). For example, throughout a ten-year study in Mississippi only two nymphal *A. maculatum* were reported biting humans (Goddard 2002). Thus, since little is known about the ecology and natural history of *R. parkeri,* either *A. americanum* or *A. maculatum* are likely a vector for this spotted fever group rickettsia, though *A. maculatum* is most likely the primary vector.

Rickettsia parkeri was considered non-pathogenic until 2002, when it was isolated from a patient"s eschar, or area of necrosis at the site the tick bite (Paddock et al. 2004). Generally, individuals infected with this agent experience mild illness characterized by fever, myalgia, headache, and an eschar (Paddock et al. 2008). The proposed name for this illness is “American Boutonneuse Fever” (ABF) due to the clinical similarities with “Boutonneuse Fever”, a disease that occurs in Europe and Africa (Goddard 2004). To date, ABF has been confirmed and/or suspected in twelve patients from Alabama, Florida, Kentucky, Maryland, Mississippi, South Carolina, and Virginia (Whitman et al. 2007, Paddock et al. 2008).

Anaplasma phagocytophilum

Anaplasma phagocytophilum (order Rickettsiales) is an obligate intracellular, gram-negative, coccoid bacterium that infects granulocytes, usually neutrophils. Originally, referred to as the Human Granulocytic Ehrlichiosis agent (HGE agent), *Ehrlichia phagocytophila*, and *Ehrlichia equi,* the bacterium was designated *A. phagocytophilum* when these agents were reclassified to represent one species of bacteria which infects several vertebrates, such as mice, dogs, horses, ruminants and humans (Dumler et al. 2001, Dumlet et al. 2005). This agent differs from the bacteria described in the sections above because it is transmitted, not by *A. americanum,* but by two *Ixodes* species in the U.S., *Ixodes scapularis* and *Ixodes pacificus*. *Ixodes scapularis,* the deer/black-legged tick, is the tick vector for *A. phagocytophilum* in the eastern U.S. while *I. pacificus,* the western black-legged tick, is the vector of this bacterium in the western states (Keirans et al. 1996).

Human granulocytic anaplasmosis (HGA), formerly known as HGE (human granulocytic ehrlichiosis), was identified in 1994 as an illness associated with *A. phagocytophilum.* HGA is most commonly reported from the upper midwestern and northeastern states, where *I. scapularis* are most prevalent and likely to bite humans (Chen et al. 1994). An average of 1.6 cases per million people is reported every year. In 1999 and 2007, the Centers for Disease Control and Prevention reported 203 and 834 cases of HGA respectively (CDC 2001, 2009). HME and HGA have similar clinical manifestations. Depending on severity, individuals who become infected with HGA may experience fever, body

aches, malaise, headache, chills, shock, hemorrhage, and possibly death (Bakken et al. 1994, Hardalo et al. 1995, Jahangir et al. 1998, Dumler et al. 2005).

Francisella tularensis

Francisella tularensis causes tularemia, a disease commonly referred to as “rabbit fever” and “deer fly fever”. This gram-negative coccobacillus can be transmitted through numerous routes such as contact, inhalation, ingestion, and by insect or tick bites (Hopla 1974, Taylor et al. 1991, Ellis et al. 2002, Petersen and Schriefer 2005). *Amblyomma americanum* is an important tick vector for this agent. LSTs are able to transmit *F. tularensis* while feeding and can maintain the bacterium transstadially (Hopla 1953, Petrov 1966, Hopla 1974). Unfed larval LSTs have tested positive for *F. tularensis* suggesting that transovarial transmission occurs, however, this has not been reproduced experimentally (Calhoun and Alford 1955, Hopla 1955, 1974). Prevalence of *F. tularensis* in *A. americanum* is generally low; in fact, an early study showed that less than 0.05% of ticks tested were infected with this agent (Calhoun and Alford 1955).

Human disease is caused primarily by two subspecies of *F. tularensis*, subspecies *tularensis* (type A) and *holarctica* (type B). Unlike type B infection, which occurs throughout the Northern Hemisphere and only occasionally uses vectors, type A is more commonly vector-associated and presumably confined to North America; it is further broken down into clades A1, in the east, and A2 in the west (Johansson et al. 2004, Farlow et al. 2005, Staples et al. 2006). There are

six distinct clinical forms of tularemia depending on the route of entry, the most common form being ulceroglandular tularemia. This form of disease is characterized by a skin ulcer at the site of infection subsequent to the bite of an infected vector, or other mode of entry into the skin (Eliasson et al. 2006). General symptoms that may be associated with tularemia include fever, malaise, headache, chills, and fatigue. In addition, some forms of tularemia are associated with high morbidity and mortality.

Reservoir Hosts for Tick-Borne Bacteria

White-Tailed Deer

White-tailed deer are commonly found infested with ticks. All mobile stages of LSTs prefer to feed on WTD (Bloemer et al. 1986, 1988). For example, in Kentucky and Tennessee, some deer were found infested with as many as 205 adult, 479 nymphal, and 1,150 larval LSTs (Bloemer et al. 1988). As a result, WTD are known or suspected hosts of many tick-transmitted bacteria including: *E. chaffeensis, E. ewingii, B. lonestari* and *A. phagocytophilum.* Based on serology, PCR, and cell culture evidence of infection, WTD are considered the principle reservoir host for *E. chaffeensis* (Dawson et al. 1994a, Lockhart et al. 1997a, b). Several populations of WTD in the Southeast have been found with antibodies to or infection with this tick-borne disease agent (Dawson et al. 1994a, Lockhart et al. 1996, Lockhart et al. 1997b, Muller-Anneling et al. 2000, Yabsley et al. 2003). To date, the seroprevalence of WTD to *E.chaffeensis* (or a closely

related *Ehrlichia* sp.) has been reported to be as high as 100% in the Southeast, specifically in Mississippi (Dawson et al. 1994a, Lockhart et al. 1995, Lockhart et al. 1996, Lockhart et al. 1997a, b, Irving et al. 2000, Yabsley et al. 2003, Paddock and Yabsley 2007). The number of WTD that are PCR positive with *E. chaffeensis* has only been reported to be 5% in Mississippi while in other states it has been reported as high as 40% (Yabsley 2003). Within the range of the LST, deer can also be found with natural infection to another *Ehrlichia* species, *E. ewingii*. For instance, 0%-20.3% of deer tested from Arkansas, Kentucky, North Carolina, and Missouri were positive for *E. ewingii* (Yabsley et al. 2002, Arens et al. 2003). WTD are only a suspected reservoir of this tick-borne agent because little is known about the natural history of *E. ewingii*. Thus far, all attempts to isolate *E. ewingii* in cell culture have failed, however experimental transmission of this agent from naturally infected WTD to uninfected fawns via blood inoculation has been successful (Yabsley et al. 2002).

As of today, WTD are the only vertebrate known as a potential reservoir for *B. lonestari.* Attempts to experimentally infect animals other than deer, such as rodents, calves, dogs, and lizards, have been unsuccessful (Grigery et al. 2005, Moyer et al. 2006). Furthermore, infection with or exposure to *B. lonestari* has been detected in naturally infected WTD from Arkansas, Georgia, North Carolina, and South Carolina (Belongia et al. 1997, Moore et al. 2003). Lastly, because adult *I. scapularis* parasitize WTD, these animals are believed to be consistently exposed to *A. phagocytophilum*, and hence are considered potential reservoirs for this agent (Lane et al. 1991, Mount et al. 1997). Exposure to and

molecular evidence of *A. phagocytophilum* has been documented in wild WTD in several states, including Mississippi (25% IFA positive; 0% PCR positive) (Belongia et al. 1997, Mount et al. 1997, Little et al. 1998, Walls et al. 1998, Magnarelli et al. 1999a, Bakken and Dumler 2000, McQuiston et al. 2003, Magnarelli et al. 2004, Dugan et al. 2006). In general, *A. phagocytophilum* is more endemic in the northeastern U.S. and Pacific coast, and although it is detected in the south-central United States, it is less common; the density of deer in these two regions is similar (Dumler et al. 2005).

Raccoons

Immature and adult stages of LSTs are often found parasitizing raccoons (Cooney and Burgdorfer 1974, Zimmerman et al. 1988). Because these animals are commonly found in a close proximity to humans they are considered a potentially important reservoir for *E. chaffeensis* (Riley et al. 1998). Antibodies reactive to *E. chaffeensis* antigen have been detected in raccoons from Florida, Georgia, Mississippi, North Carolina, South Carolina, Texas, Virginia, and West Virginia, with the highest prevalence‟s ranging from 80%-90% (Lockhart et al. 1997b, Comer et al. 2000).

Opossums

Studies conducted on opossums have produced some evidence that they may be involved in the natural history of *E. chaffeensis* and *A. phagocytophilum* (Anderson et al. 1992b). Antibodies to *E. chaffeensis* have been detected in

opossums (Lockhart et al. 1997b). However, one study did not find any opossums positive for *E. chaffeensis* by IFA or PCR (Dugan et al. 2005). Additionally, opossums have been reported to have serologic or molecular evidence of *A. phagocytophilum* (Levin et al. 2002).

Feral Swine

Species of ticks that have been found parasitizing feral swine include: *A. cajennense, A. maculatum, A. americanum, Dermacentor variabilis, D. nitens, I. scapularis, Ornithodoros turicata,* and *Otobius megnini* (Shender et al. 2002, Straw and Taylor 2006). To the author"s knowledge, there are no studies on the tick-borne agents, transmitted by these ticks, of feral swine in the U.S. However, in Spain, feral swine have been reported to have serological evidence of exposure to a *Rickettsia* spp. (*R. slovaca*) (Ortuno et al. 2007). Similarly, feral swine in the U.S. may potentially be exposed to and or infected with a *Rickettsia* species or other tick-borne agent.

Other Hosts

Canids, goats, lemurs, foxes, and rabbits have been shown to have natural infection, experimental susceptibility, or exposure to *E. chaffeensis* (Lockhart et al. 1997b, Davidson et al. 1999, Dugan et al. 2000, Kocan et al. 2000, Liddell et al. 2003, Yabsley et al. 2004). Dogs have been implicated as important vertebrate hosts of *E. ewingii* (Anderson et al. 1992a, Dawson et al. 1996, Greig et al. 1996, Murphy et al. 1998, Buller et al. 1999, Kordick et al.

1999, Yabsley et al. 2002, Childs and Paddock 2003, Liddell et al. 2003, Loftis et al. 2008). They have been reported to have a high prevalence of *E. ewingii* infection, for example in a recent study, 91% of domestic dogs tested PCR positive for this agent (Liddell et al. 2003). Smalls mammals are considered reservoirs for *A. phagocytophilum* and *F. tularensis.* The major host for *A. phagocytophilum* is the white-footed mouse (*Peromyscus leucopus)* (Des Vignes and Fish 1997, Magnarelli et al. 1999b). Cottontail rabbits are common hosts of type A *F. tularensis* (Morner 1992).

Animal and Public Health Significance

Considering the LSTs‟ seemingly expanding wide geographic range, high population densities, and aggressive feeding habits on humans and animals, information pertaining to the disease agents associated with these ticks are of significant medical and veterinary importance.

Human Monocytic Ehrlichiosis

Human Monocytic Ehrlichiosis has become of significant public health interest since the first human case was identified in 1986 (Maeda et al. 1987). To date, cases have been reported in 47 states, with the majority originating where LSTs and WTD are most abundant (Eng et al. 1990, McQuiston et al. 1999). The wide geographic distribution of the disease is most likely associated with patients who traveled to endemic areas prior to being diagnosed. A three-year prevalence study in Cape Girardeau, Missouri showed an average annual

occurrence of 3.2 cases per 100,000 people (Olano et al. 2003b). In 2006, a total of 578 cases of HME were reported in the U.S. (McNabb et al. 2008). However, the number of cases is most likely an underestimate due to underreporting as a result of disease unfamiliarity. HME can result in morbidity, cause severe illness if not treated, and can possibly lead to death. Two to three weeks after a tick bite, the disease initially presents as a flu-like illness with a fever, headache, and myalgia. In the minority of cases, nausea, anorexia, dizziness, cough, rash, abdominal tenderness, confusion, and stiff neck occur (Eng et al. 1990, Marty et al. 1995, Rikihisa 1999, Olano et al. 2003a, Walker et al. 2004). On average, individuals most commonly diagnosed with HME are over forty years of age, male, work or venture into areas where the tick vector is prevalent, and are immunocompromised (Fishbein et al. 1994, Rikihisa 1999, Paddock and Childs 2003). Patient hospitalization occurs in 41-70% of cases (Fishbein et al. 1994, Standaert et al. 1995, Carpenter et al. 1999, Rikihisa 1999, Standaert et al. 2000, Paddock and Childs 2003, Zhang et al. 2007). Case-fatality has reached as high as 5%, but is typically found at less than 5%, and on average, is approximately 2.7% (Fishbein et al. 1994, McQuiston et al. 1999, Childs and Paddock 2003, Zhang et al. 2007). With rapid diagnosis and proper antimicrobial therapy (typically doxycycline) the risk for major complications and death has decreased (Fishbein et al. 1994).

Southern Tick-Associated Rash Illness

In 1984, an illness similar to Lyme disease, associated with an *A. americanum* bite, was first described (Schulze et al. 1984). As of today STARI has been reported in Alabama, Georgia, Kentucky, Maryland, Missouri, North Carolina, New Jersey, and South Carolina (Schulze et al. 1984, Masters et al. 1994, Campbell et al. 1995, Armstrong et al. 1996, Barbour et al. 1996, Kirkland et al. 1997, Masters et al. 1998, Felz et al. 1999, Shapiro and Gerber 2000, Armstrong et al. 2001, James et al. 2001, Moore et al. 2003, Haddad et al. 2005). Patients with STARI report erythema migrans which is a bull"s eye-like rash at the site of the tick-bite. Other symptoms are typically mild and may include a flu-like illness with fatigue, headache, fever, malaise and/or joint and muscle pain (Campbell et al. 1995, Kirkland et al. 1997, Masters et al. 1998, Felz et al. 1999, James et al. 2001, Wormser et al. 2005a, Murphree et al. 2009). Since this illness is similar to Lyme disease, it is treated the same, usually with doxycycline; however, symptoms have been shown to resolve without treatment, whereas Lyme disease may have chronic manifestations (Masters 1993, Wormser et al. 2000, Haddad et al. 2005, Wormser et al. 2005b). Due to STARI"s similarity to Lyme it is often reported as Lyme, leaving the true magnitude of this syndrome unknown (Masters et al. 1994, Armstrong et al. 2001).

Borrelia lonestari has been considered the putative agent for STARI since the mid 1990s; however, it has only been detected from one patient (James et al. 2001). In later studies, *B. lonestari* DNA was not detected in the skin biopsies of four patients who suffered a LST bite, and neither *B. lonestari* nor *Borrelia*

burgdorferi DNA was found in thirty patients thought to have STARI (Bacon et al. 2003, Wormser et al. 2005b). As a result, many believe another agent, or no agent at all, is responsible for this disease. Recently, a LST was tested for multiple bacterial agents, including *Borrelia*, after a woman developed a rash at the site of attachment. The tick was negative for all bacterium except *Rickettsia amblyommii,* leading researchers to consider this rickettsial agent as the cause of STARI (Billeter et al. 2007a).

Other Tick-Borne Diseases of Importance

Although HME and STARI are the most notable human diseases transmitted by LSTs, the agents causing the other diseases mentioned above are still significant, despite not being as frequently reported. For instance, human ehrlichiosis is caused by *E. ewingii*, *A. phagocytophilum* can cause HGA, and *F. tularensis* is the agent of tularemia. Additionally, some human disease has been associated with *R. amblyommii* and *R. parkeri*.

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CHAPTER III

THE LONE STAR TICK, *AMBLYOMMA AMERICANUM* (ACARI: IXODIDAE), AS A VECTOR OF TICK-BORNE AGENTS IN MISSISSIPPI

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Abstract

This study evaluated *Amblyomma americanum* (lone star tick) in Mississippi for the presence of *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis, *E. ewingii*, causative agent of human and canine granulocytic ehrlichiosis, *Borrelia lonestari*, putative agent of "southern tick-associated rash illness" (STARI), *Francisella tularensis*, the agent of tularemia, and *Rickettsia* spp., particularly *R. amblyommii*, a suspected pathogen. We collected adult *A. americanum* from four regions of Mississippi: Northeast, Northwest, Southeast, and East. Of the ticks collected, 192 were dissected and DNA was extracted for nested PCR assays to detect the above bacteria. In all,

2.6% of ticks had evidence of *Borrelia* sp., 3.7% for *E. chaffeensis*, 6.3% for *E. ewingii*, and 43.5% for a *Rickettsia* species. As determined by sequencing, most *Rickettsia* spp. were *R. amblyommii*. In addition, 42 pools (total of 950) of larval *A. americanum* collected in Southwest Mississippi were tested for the presence of *E. chaffeensis* and *Rickettsia* species. Of the pools tested by PCR, 9 out of 42 (21.4%) were positive for a *Rickettsia* sp., most often, *R. amblyommii*; none had evidence of *E. chaffeensis*, supporting the ability of LSTs to transovarially transmit *R. amblyommii* but not *E. chaffeensis*. This study demonstrates *E. chaffeensis*, *E. ewingii*, *B. lonestari*, and *R. amblyommii* in *A. americanum* by PCR for the first time in Mississippi. Understanding the prevalence and epidemiology of these agents in Mississippi should increase awareness of tick-borne disease in the medical community.

Key Words *Amblyomma americanum*, tick-borne agents, Mississippi

Commonly referred to as the lone star tick (LST), *Amblyomma americanum* is the most abundant tick species in the southeastern United States. LSTs are three-host ticks which obtain their blood meals from various vertebrate hosts. All stages of these aggressive feeders are commonly found attached to humans (Felz et al. 1996, Merten and Durden 2000). There is considerable human exposure to LSTs and their associated pathogens. One study showed that a group of ten soldiers reported over one hundred ticks each within a two-week training period on Little Rock Air Force Base, Arkansas, which was historically infested with LSTs (Goddard and McHugh 1990). In addition, a ten-

year study at the Mississippi Department of Health revealed 53% of all tick bites reported during that time were from LSTs (Goddard 2002).

In a recent study, Goddard et al. (2003) tested several tick species in Mississippi for spotted fever group (SFG) rickettsiae, *Ehrlichia chaffeensis* and *Borrelia* species using immunofluorescent antibody staining (FA) and subsequent polymerase chain reaction (PCR). Of these ticks tested by FA, 20% were infected with SFG rickettsiae, 22% with *E. chaffeensis*, and none were positive for *Borrelia* spp; by PCR, 15% were positive for SFG rickettsiae, while no other agent was detected. Aside from this data, there is limited information about the presence of tick-borne diseases in Mississippi. However, similar studies in the southern U.S. have found that the prevalence of infected LSTs has been as high as 29% for *E. chaffeensis*, 19% for *E. ewingii,* and up to 12% for *B. lonestari* (Roland et al. 1998, Mixson et al. 2006).

In this study, we focused on the following tick-borne bacterial agents: *E. chaffeensis,* causative agent of human monocytic ehrlichiosis (HME); *E. ewingii*, causative agent of human and canine granulocytic ehrlichiosis; *Borrelia lonestari*, putative agent of "southern tick-associated rash illness" (STARI); *Francisella tularensis,* the agent of tularemia; *Rickettsia amblyommii,* a suspected pathogen; and *Rickettsia parkeri,* a recently recognized human disease agent. We chose these particular bacteria because they are known or putative causes of recognized tick-borne diseases in the United States, and have been detected in ticks and/or animals in the Southeast.

Materials and Methods

During April-June of 2008, over 700 adult LSTs were collected by tick drag cloth from four regions of Mississippi: East (Noxubee National Wildlife Refuge in Noxubee County and Columbus Air Force Base, Golden Triangle Regional Airport, and Tombigbee State Park in Lowndes County), Northwest (Wall Doxey State Park in Marshall County), Northeast (Tishomingo State Park in Tishomingo County), and South (Natchez State Park in Adams County) (Figure 3.1). Live LSTs were brought back to the lab, transferred to vials, and maintained alive in humidity chambers until processing.

In addition, 4,000 larval LSTs were collected by drag cloth over a 2-year period (during July 2001-June 2003) from the Copiah County Wildlife Management Area in southwest Mississippi (Figure 3.1). Two 300m lanes were systematically sampled weekly, checking the cloth every 10m for attached ticks. Larval broods were sub-sampled and 950 larvae were returned to the lab, separated into 42 pools in vials of ethanol, and analyzed.

We dissected live adult LSTs (n= 192) and placed tissues in 100 µL phosphate buffered saline (PBS; pH 7.4) and froze these at -80º C prior to extraction. In preparing larval ticks for extraction, ethanol was removed from the vials with a pipette, ticks were rinsed with 100µl PBS, and then crushed with a pestle. DNA was extracted from 50 µL tick tissue slurry or crushed larval ticks using the Illustra Tissue and Cells Genomic Prep Mini Spin Kit (GE Healthcare, Piscataway, NJ) following the manufacturer"s protocol for tissues.

We tested all ticks for the tick mitochondrial 16S rRNA gene to confirm that tick DNA was present, and thus, that the DNA extraction was successful (Black and Piesman 1994). All extracted samples were tested for the presence of *B. lonestari*, *E. chaffeensis*, *E. ewingii*, *Rickettsia* spp., and *F. tularensis* by nested PCR. Nested genus-wide PCR assays were used to amplify a segment of the *flaB* gene for *Borrelia* spp., the *fopA* gene of *F. tularensis,* and the 17-kDa or *rompA* genes for *Rickettsia* species (Fulop et al. 1996, Moore et al. 2003, Paddock et al. 2004). For both *Ehrlichia* spp. nested PCR assays using unique primers specific for either species were used to amplify a segment of the 16S rRNA gene (Anderson et al. 1992, Dawson et al. 1994). All products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Representative samples positive by PCR were sent to Eurofins MWG Operon (Huntsville, AL) for sequencing. Sequences were aligned using ClustalX and subjected to the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) to determine nearest species identity.

Results and Discussion

We detected tick mitochondrial 16S rDNA in 99.5% (191/192) adult ticks. The numbers of adult ticks positive with a tick-borne bacterial agent are listed in Table 3.1 and range from 0-43.5%; this is broken down by location in Figure 3.2. Co-infections were detected in eleven adult LSTs. Ten LSTs were co-infected with a *Rickettsia* spp. and either *Borrelia lonestari* (4), *E. chaffeensis* (1), or *E.*

ewingii (5). In addition, another tick was co-infected with both *E. chaffeensis* and *E. ewingii*. Tick DNA was detected in 88.1% (37/42) of larval ticks and of those ticks, 24.3% (9/37) were positive for a *Rickettsia* spp. with a minimum infection rate of 1.4% (1/70)-5.9% (1/17). No larval LSTs had evidence of *E. chaffeensis* DNA. Additionally, all sequences were 100% identical to published sequences for respective organisms in NCBI, including *E. chaffeensis, E. ewingii, B. lonestari,* and *R. amblyommii*, however five adult ticks were 100% identical to multiple *Rickettsia* spp. including *R. amblyommii, R. parkeri, R. conorii, R. Rickettsii*, and *R. peacockii*. In addition, one adult tick that was initially positive by *17kDa* PCR was not successfully sequenced and, after PCR and sequencing of the *gltA,* was closest in identity (95%) to an uncultured *Rickettsia* sp. (GenBank DQ887279). All larvae were 100% identical to *R. amblyommii* with one exception. One larval pool was identical to a *Rickettsia* sp. detected in LST from Delaware by subsequent PCR and sequencing of the *gltA* gene (GenBank AF031496).

Overall, *A. americanum* had the highest prevalence of infection for *Rickettsia* spp., specifically *R. amblyommii.* No adult ticks tested PCR positive for *F. tularensis* which was anticipated since the prevalence of this agent in LSTs has been reported to be less than 0.05% and human cases of tularemia are most prevalent in Arkansas, Missouri, and Oklahoma (Calhoun and Alford 1955, Petersen et al. 2008). In addition, no larval ticks tested positive for *E. chaffeensis,* which we suspected since transovarial transmission has not been confirmed for this agent (Long et al. 2003). Because we collected varying

numbers of ticks from individual sites, ranging from 17 to 61, it was not possible to make any interpretations of the relative differences in the percent of ticks infected with various organisms. However, it was interesting that LSTs in northern Mississippi had the highest infection for *E. chaffeensis* and *Rickettsia* spp. while LSTs from the other two regions had the highest rate of infection with *E. ewingii.*

Rickettsia amblyommii is commonly found in LSTs and this was shown again with our study. The infection rate of LSTs with *R. amblyommii* has been reported to be as high as 84% in New York (Mixson et al. 2006). Currently, it is uncertain if human illness occurs from infection with *R. amblyommii;* only one case has led researchers to suspect human disease (Apperson et al. 2008). In addition to *R. amblyommii,* we predicted finding *R. parkeri,* which has been detected in LSTs and has been experimentally transmitted by these ticks (Goddard and Norment 1986, Goddard 2003, Yabsley et al. 2009). However, in this study, of all ticks positive for a *Rickettsia* spp., all but five were subsequently determined to be *R. amblyommii*; the five exceptions were identical to multiple spp. as described above. Which *Rickettsia* sp. these represent is unclear because further attempts to identify the species have been unsuccessful.

To the authors‟ knowledge, this is the first evidence of *B. lonestari, E. chaffeensis, E. ewingii,* and *R. amblyommii* in *A. americanum* in Mississippi as determined by PCR. The majority of the tick-borne disease agents in this study are of veterinary importance and, more importantly, they are of public health significance.

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Table 3.1 Detection of Tick-Borne Agents in Adult *A. americanum* by PCR Assay

Bacterial agent	**No. positive ticks**	**% Positive**
Borrelia lonestari[1]	5/191	2.6
Ehrlichia chaffeensis[1]	7/191	3.7
E. ewingii[1]	12/191	6.3
Francisella tularensis	0/191	0
Rickettsia spp.[2]	83/191	43.5

[1] No. of adult ticks submitted for sequencing: *B. lonestari*= 3; *E. chaffeensis*= 1; *E. ewingii*= 1

[2] 79 adult ticks positive for a *Rickettsia* spp. were submitted for sequencing; 74 were 100% identical to *R. amblyommii*; 5 were 100% identical to multiple *Rickettsia* spp.

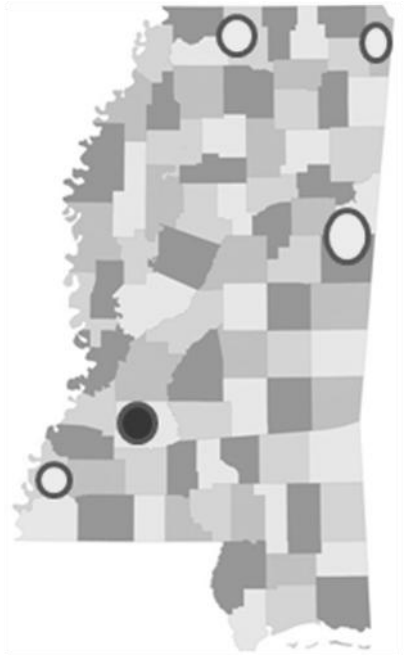

Figure 3.1 *Amblyomma americanum* Collection Sites around Mississippi

-The white dots represent adult *A. americanum* collection sites
-The black dot represents the collection site for larval *A. americanum*

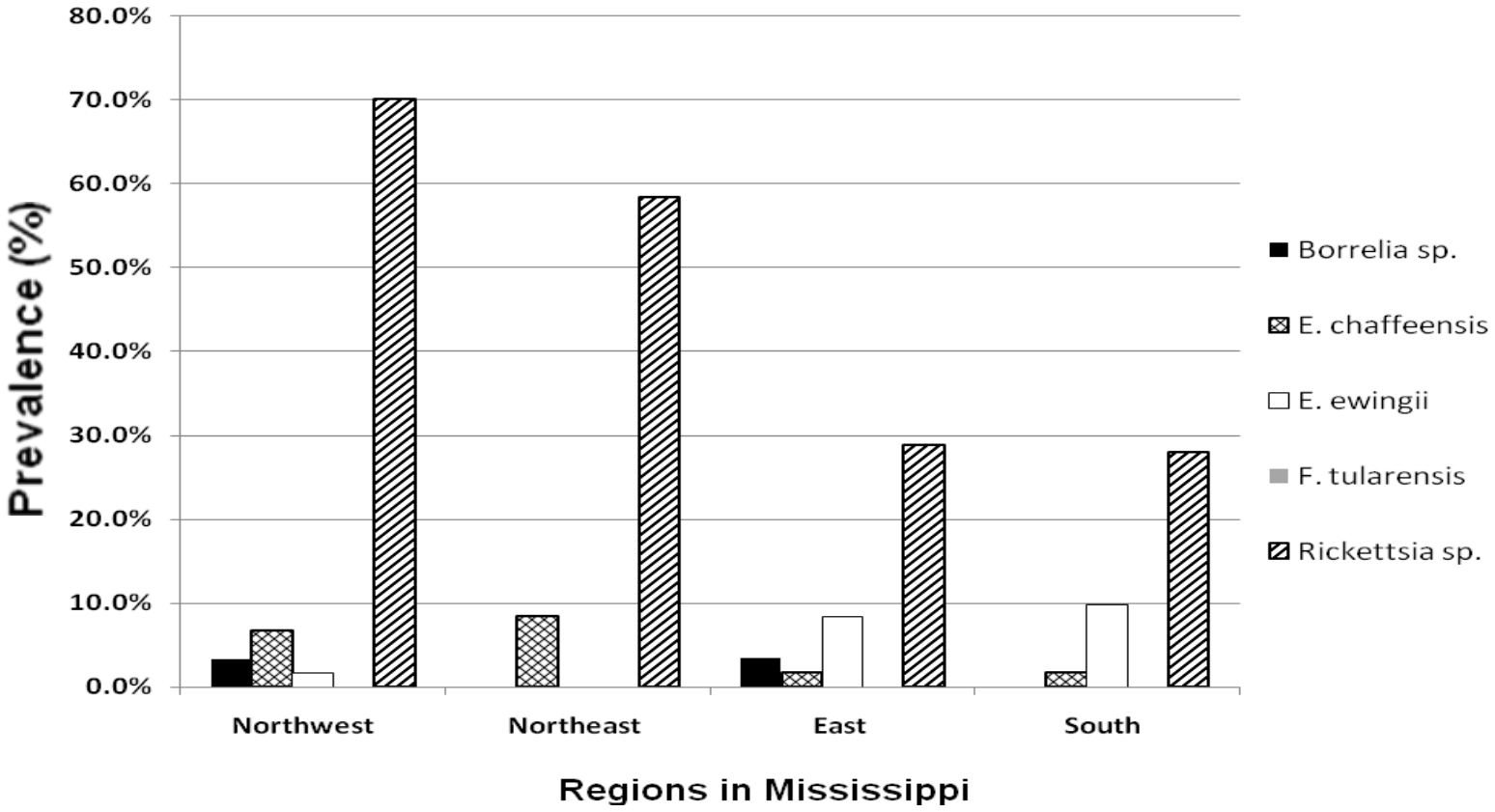

Figure 3.2 Tick-Borne Agents in Specific Sites in Mississippi[1]

[1] Total number of adult ticks per location: 60 (Northwest); 17 (Northeast); 54 (East); 61 (South)

CHAPTER IV

TICK-BORNE DISEASE AGENTS IN VARIOUS WILDLIFE FROM MISSISSIPPI

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Abstract

Because tick-borne diseases are becoming increasingly important throughout the world, monitoring their causative agents in wildlife may serve as a useful indicator of potential human exposure. We assessed the presence of known and putative zoonotic, tick-borne agents in four wildlife species in Mississippi. Animals were tested for exposure to or infection with: *Ehrlichia chaffeensis, E. ewingii, Borrelia lonestari, Rickettsia* spp., *Anaplasma phagocytophilum,* and *Francisella tularensis*. Whole blood and serum were tested from white-tailed deer (WTD; *Odocoileus virginianus*) and feral swine (*Sus scrofa*); serum was tested from raccoons (*Procyon lotor*) and opossums (*Didelphis virginiana*). We used polymerase chain reaction to detect all agents in blood, while an indirect fluorescent antibody assay was used to detect antibodies to *E. chaffeensis*, *B. lonestari*, and *R. parkeri* (spotted fever group (SFG) rickettsiae) antigens in serum. Molecular evidence of infection to *E. chaffeensis*, *B. lonestari*, and *A. phagocytophilum* was detected only in WTD. Antibodies to

E. chaffeensis antigen was detected in 43.9% of WTD, 32.8% of swine, 42.1% of raccoons, and 15.8% of opossums. Serologic evidence of exposure to *B. lonestari* antigen was found in 19.3% of WTD, 6.9% of swine, and 5.3% of raccoons, but not in opossums. Interestingly, the percent of animals with antibodies reactive to SFG rickettsiae (*R. parkeri* antigen) was highest in raccoons (73.7%) and opossums (57.9%). These results support the role of WTD as reservoirs for *E. chaffeensis, B. lonestari*, and *A. phagocytophilum,* as well as provide additional evidence for exposure of raccoons and opossums to *E. chaffeensis.* Finally, we provide new data that some feral swine have antibodies to these agents. Thus, in general, these four wildlife species are exposed to tick-borne disease agents in Mississippi, suggesting that ticks carry and have the potential to transmit the agents to humans in the state.

Key Words Wildlife, Mississippi, tick-borne disease, *Anaplasma phagocytophilum, Borrelia lonestari, Ehrlichia chaffeensis, Ehrlichia ewingii, Francisella tularensis, Rickettsia* spp.

Tick-borne diseases of medical and veterinary importance are becoming increasingly important throughout the world, where many tick species are expanding their range and wildlife are serving as reservoirs for tick-borne disease agents. While there are numerous tick vectors and tick-borne bacteria that cause human disease, several are of greater significance in the southeastern United States, including Mississippi. In a study by Goddard et al. (2003), ticks were collected throughout Mississippi and tested for spotted fever group (SFG) rickettsiae, *Ehrlichia chaffeensis,* and *Borrelia* species. Of these ticks tested by

immunofluorescent antibody staining (FA), 20% were found to be infected with SFG rickettsiae, 22% with *E. chaffeensis*, and none were positive for *Borrelia* spp. Only 15% of the ticks that were positive by FA staining had molecular evidence of SFG rickettsiae; no other ticks were positive by polymerase chain reaction (PCR) for an agent. To our knowledge, evidence of tick-borne agents in ticks from Mississippi is limited to that study. However, studies of wildlife in the southeastern states have found that seroprevalence to *E. chaffeensis* can be as high as 100% in white-tailed deer (WTD), 46% in raccoons, and 8% in opossums (Paddock and Childs 2003, Yabsley et al. 2008). In addition, seropositive deer and raccoons have been detected specifically in Mississippi (Dawson et al. 1994a, Yabsley et al. 2003). Because Mississippi is home to several tick species of medical importance and tick-borne diseases are not uncommon here, evidence of tick-borne agents in wild hosts may be correlated to potential human exposure to these agents.

The lone star tick, *Amblyomma americanum,* is the most common tick in the Southeast, including Mississippi. This tick serves as the primary vector for *E. chaffeensis*, the cause of human monocytic ehrlichiosis (HME), *E. ewingii*, a causative agent of human granulocytic ehrlichiosis (HGE), and *B. lonestari*, the putative agent of “southern tick-associated rash illness” (STARI). It also vectors the rickettsias *Rickettsia amblyommii* and *R. parkeri* which have been associated with human disease, and *Francisella tularensis*, the agent of tularemia (Paddock and Yabsley 2007, Goddard and Varela-Stokes 2009). *Amblyomma maculatum,* the Gulf Coast tick, is primarily found in the Gulf and Atlantic Coasts. It is the

primary vector for *R. parkeri,* the most recently recognized human disease agent that causes, what has been called, "American Boutonneuse Fever" (Sumner et al. 2007). *Ixodes scapularis,* the black-legged tick, is the vector for *Anaplasma phagocytophilum,* which causes human granulocytic anaplasmosis (HGA) (Hodzic et al. 1998). Our objective was to evaluate four wildlife species, namely WTD (*Odocoileus virginianus*), feral swine (*Sus scrofa*), raccoons (*Procyon lotor*) and opossums (*Didelphis virginiana*), as potential hosts for these agents in Mississippi.

Between 2007-2008, we obtained 153 samples from WTD, feral swine, opossums, and raccoons. Only whole blood was collected from some WTD and feral swine while serum was obtained from all animals. DNA was extracted from whole blood using an Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, Piscataway, NJ) following the manufacturer"s protocol. We tested all extracted samples for the presence of the six tick-borne agents by PCR. A single species-specific assay was used to amplify a segment of the *msp2* gene of *A. phagocytophilum* (Michalski et al. 2006). Nested genus-wide assays were used to amplify a segment of the *flaB* gene for *Borrelia* spp. (Moore et al. 2003), the *fopA* gene for *Francisella* spp. (Fulop et al. 1996), and the 17-kDa antigen gene for *Rickettsia* spp. (Paddock et al. 2004). For both *Ehrlichia* spp. a species-specific nested PCR assay was used to amplify a segment of the 16S rRNA gene (Anderson et al. 1992, Dawson et al. 1994b). All products were electrophoresed in a 2% agarose gel stained with ethidium bromide and visualized under

ultraviolet light. PCR products from representative positive samples were purified and sent to MWG Biotech (Huntsville, AL) for sequencing.

Indirect fluorescent antibody assays (IFA) were used to test for antibodies reactive to *E. chaffeensis*, *B. lonestari*, and *R. parkeri* (SFG rickettsia) antigens. Slides were coated with antigen consisting of: *E. chaffeensis* grown in DH82 cells; *B. lonestari* grown in ISE6 cells; and *R. parkeri* grown in Vero cells. Sera were screened at a 1:64 dilution. Positive and negative control sera were included on all slides.

Molecular evidence of DNA from at least one agent was found in 25% of deer (Table 4.1). Two deer were co-infected with *E. chaffeensis* and either *A. phagocytophilum* or *B. lonestari.* No feral swine were positive by PCR for any of the tick-borne agents targeted. *Francisella tularensis* was not detected in any animal tested, which was not surprising considering the general lack of evidence of tularemia in the animals examined. All submitted sequences were 100% identical to published sequences for respective organisms in National Center for Biotechnology Information (NCBI).

Wildlife with antibodies to *B. lonestari* ranged from 0-19.3%, 15.8-43.9% for *E. chaffeensis*, and 8.8-73.7% for SFG rickettsiae (*R. parkeri* antigen) (Table 4.1). WTD had the highest seroprevalence to *B. lonestari* and *E. chaffeensis*, but had the lowest seroprevalence to SFG rickettsiae (*R. parkeri* antigen). Interestingly, raccoons had the highest seroprevalence to *R. parkeri* antigen.

Although limited by the small sample size, our study results support previous studies. These results showed that WTD had the highest

seroprevalence to *B. lonestari* and *E. chaffeensis.* In addition, WTD had molecular evidence of *B. lonestari*, *E. chaffeensis*, and *A. phagocytophilum*; in fact, two were co-infected. Our results support the role of WTD as reservoirs for these agents (Paddock and Yabsley 2007). Interestingly, other wildlife tested had antibodies to tick-borne agents. For example, raccoons had the highest prevalence of exposure to SFG rickettsiae; feral swine also had some evidence of antibodies to the three tick-borne bacteria. Although raccoons are not typical hosts for *A. maculatum*, they are hosts for *A. americanum*, and more importantly, for *Dermacentor variabilis*, the vector for *R. rickettsii* (agent of Rocky Mountain spotted fever); SFG rickettsiae have previously been detected in *D. variabilis* removed from raccoons (Kollars and Kengluecha 2001). This suggests that seropositive raccoons may actually have been exposed to *R. amblyommii* or *R. rickettsii.* In addition, it is possible that there were other instances of cross-reactivity in the IFAs. For example, an animal positive for *E. chaffeensis,* by serology, could have antibodies against another *Ehrlichia* species.

In the United States, the bulk of *E. chaffeensis* and *E. ewingii* cases have been reported from the Southeast, reflecting the vast number of *A*. *americanum* ticks in this area. In addition, previous studies have generally shown a higher prevalence of *E. chaffeensis* than *B. lonestari* in wild-caught *A. americanum,* which is consistent with the lower seroprevalence in deer to *B. lonestari* (Varela et al. 2004, Mixson et al. 2006). The prevalence of WTD with molecular evidence of *B. lonestari* in our study (3.1%) is slightly lower than a previous report from throughout the South (8.7%), however, that report did not detect *B.*

lonestari in WTD tested from Mississippi (Moore et al. 2003). Because *A. phagocytophilum* is more endemic in the northeastern U.S. and Pacific coast, and appears to be less common in the South-central United States, we were not surprised to find only one animal infected with the agent (Dumler et al. 2005).

Finally, our serologic data showed that the wildlife tested may be exposed to multiple tick-borne agents, and may ultimately play a role in the maintenance of these organisms in nature. Our results demonstrate the presence of *E. chaffeensis, B. lonestari, Rickettsia* spp., and *A. phagocytophilum* in wildlife in Mississippi, where cases of tick-borne disease in humans and animals occur. Future studies to fully evaluate the role of species other than WTD as reservoirs for tick-borne agents are necessary in order to identify potential sentinels for human infection.

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Disclosure Statement

No competing financial interests exist

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Table 4.1 Detection of and Exposure to Tick-Borne Agents in Wildlife by PCR and Serology

	WTD *No. pos (%)*	**Feral swine** *No. pos (%)*	**Raccoons** *No. pos (%)*	**Opossums** *No. pos (%)*
PCR				
A. phagocytophilum	1 (3.1)	0 (0)	N/A	N/A
B. lonestari	1 (3.1)	0 (0)	N/A	N/A
E. chaffeensis	6 (18.8)	0 (0)	N/A	N/A
E. ewingii	0 (0)	0 (0)	N/A	N/A
F. tularensis	0 (0)	0 (0)	N/A	N/A
Rickettsia spp.	0 (0)	0 (0)	N/A	N/A
Total samples	32	18	0	0
Serology[1]				
B. lonestari	11 (19.3)	4 (6.9)	1 (5.3)	0 (0)
E. chaffeensis	25 (43.9)	19 (32.8)	8 (42.1)	3 (15.8)
R. parkeri	5 (8.8)	17 (29.3)	14 (73.7)	11 (57.9)
Total tested	57	58	18	19

[1]Although specific antigens were used to detect antibodies, cross-reactivity exists between species of the same genus, particularly among the SFG rickettsiae.