

STUDIES ON *EHRlichia CHAFFEENSIS* AND *BORRELIA LONESTARI*,
TICK-BORNE AGENTS TRANSMITTED BY *AMBLYOMMA AMERICANUM*

by

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(Under the Direction of Susan E. Little)

ABSTRACT

Over the past decade, the lone star tick (LST), *Amblyomma americanum*, has become recognized as an important vector of several known or suspected zoonotic agents in the southern United States. Among these are *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis, *E. ewingii*, and agent of human and canine ehrlichiosis, and *Borrelia lonestari*, the putative agent of “southern tick-associated rash illness” (STARI). White-tailed deer (WTD) are a primary host for all motile LST stages and serve as the principal reservoir host for *E. chaffeensis*. However, studies that reveal the course of *E. chaffeensis* infection in reservoir hosts are scarce. Reports of *E. ewingii* and *B. lonestari* in LST from the southern U.S. exist but additional reports would add to an understanding of the distribution of these bacteria. In addition, *B. lonestari* has not yet been isolated in culture, making the development of specific diagnostic assays to differentiate STARI from Lyme disease problematical. This dissertation presents studies which investigated these three tick-borne agents. Findings in Chapter 3 demonstrated that the course of primary infection with *E. chaffeensis* via different routes of needle inoculation did not differ. Chapter 4 showed

that WTD with a primary infection of *E. chaffeensis* were not protected from secondary infection with a genetic variant. In Chapter 5, inoculation of domestic goats with *E. chaffeensis* did not cause active infection, suggesting that goats are an unsuitable model for *E. chaffeensis* infection. In Chapter 6, a three-year survey of LST from northeastern Georgia found prevalences of *E. chaffeensis*, *E. ewingii*, and *B. lonestari* at 2.0% (8/398), 4.8% (19/398), and 1.0% (4/398), respectively, substantiating the presence of these organisms and risk of human exposure in northeastern Georgia. The detection of *B. lonestari* in LST from northeastern Georgia was critical for obtaining the first culture isolate of *B. lonestari*. The isolation and molecular, immunologic, and ultramicroscopic characterization used to identify the spirochetes are described in Chapter 6. These studies contribute to an understanding of recently recognized bacteria transmitted by LST.

INDEX WORDS: *Amblyomma americanum*, *Borrelia lonestari*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, experimental infection, lone star tick, *Odocoileus virginianus*, white-tailed deer

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

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DEDICATION

This work is dedicated to my mother and father, Ana and Eduardo, who nurtured and encouraged my desires to pursue an extensive academic career.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without gracious assistance and support from many people, including my committee members, technicians, graduate students, friends, and family. My mentor, Dr. Susan Little deserves the greatest acknowledgment for her patience, encouragement, and foremost, her ability to open doors to opportunities that I may not have otherwise experienced. Dr. Randy Davidson was an amazing resource in both wildlife disease and parasitology, and his frequent witticisms were refreshing to my usual cynicism. Dr. Dave Stallknecht was always available for the “quick” culture question, the final word in experiment decisions, and “big-picture” questions. This work could not have been done without the pathology expertise and resources of Dr. Buffy Howerth who took the time to teach me techniques and look at results, despite her incredibly busy schedule. Finally, Dr. Ray Kaplan was an essential link to the practical side of veterinary parasitology. All of my committee members were fundamental in helping me to develop into a veterinary parasitologist.

I am also greatly thankful to the graduate and vet students, research scientists, and technicians, both in the department and at SCWDS, who provided help and motivation in this research and in maintaining some sanity. Among these are friends and colleagues, Michael Yabsley, Gus Moore, Molly Murphy, and Cynthia Tate. In addition, Dana Ambrose and Page Luttrell became close dear friends and provided enormous support, both emotionally and directly towards this research. Finally, I am deeply grateful to my family, and especially John Stokes, now my lifelong companion.

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CHAPTER 1

INTRODUCTION

Amblyomma americanum, the lone star tick (LST), is a three-host ixodid tick prevalent throughout the southeastern and south-central United States. Within the past twenty years, the role of LST has escalated from that of a pest to one of public health significance. While important as a cause of irritation and blood loss during high infestations, LST have also become appreciated in recent years as efficient vectors of emerging human pathogens. In the 1990s alone, three newly-recognized human pathogens, *Ehrlichia chaffeensis*, *E. ewingii*, and *Borrelia lonestari*, which cause human monocytotropic ehrlichiosis (HME), ehrlichiosis in canines and rarely humans, and southern tick-associated rash illness (STARI) in humans, respectively, have been implicated as transmitted by LST (Anziani, et al. 1990, Ewing, et al. 1995, Barbour, et al. 1996, Means and White 1997, Buller, et al. 1999).

Of these three agents, the natural history of *E. chaffeensis* is most completely known. *Ehrlichia chaffeensis* has been isolated from humans and white-tailed deer (WTD), the principal natural reservoir host, on multiple occasions (Dawson, et al. 1991, Lockhart, et al. 1997a, Lockhart, et al. 1997b, Little, et al. 1998, Paddock, et al. 2001, Yabsley, et al. 2003b). Studies of *E. chaffeensis* infection dynamics in WTD, however, are limited. Primary infection in deer has been reported four times via either intravenous injection or tick transmission (Dawson, et al. 1994b, Ewing, et al. 1995,

Kocan, et al. 2000a, Davidson, et al. 2001). In addition, the importance of molecular variation of different genetic types in influencing the virulence and epizootiology of *E. chaffeensis* is not understood. A closely related organism, *E. ewingii* is considered primarily a pathogen of dogs. This organism has been implicated in less than ten human ehrlichiosis cases, primarily in immunocompromised patients (Buller, et al. 1999, Paddock, et al. 2001). *Ehrlichia ewingii* has not yet been isolated in culture, but has been detected by PCR or microscopy in humans, ticks, and various mammals including WTD in the South (Anderson, et al. 1992, Dawson, et al. 1996a, Goldman, et al. 1998, Murphy, et al. 1998, Buller, et al. 1999, Paddock, et al. 2001, Arens, et al. 2003, Childs and Paddock 2003). Until 2003, *B. lonestari* had been identified through molecular detection in ticks, deer, and a human patient (Burkot, et al. 2001, James, et al. 2001, Bacon, et al. 2003, Moore, et al. 2003a, Stegall-Faulk, et al. 2003, Stromdahl, et al. 2003, Yabsley, et al. 2003). Suspected cases of STARI have been widely reported in the South, where classic Lyme disease is rare (Campbell, et al. 1995, Masters and Donnell 1995, Kirkland, et al. 1997, Masters, et al. 1998, Armstrong, et al. 2001).

The studies presented contribute to a growing understanding of the disease agents transmitted by *A. americanum*, particularly *E. chaffeensis* and *B. lonestari*.

Specific aims of the research for this dissertation were as follows:

1. Monitor progression of primary infection with *E. chaffeensis* by comparing the efficacy of intradermal, subcutaneous and intravenous inoculation routes in establishing infection in white-tailed deer;

2. Determine if primary infection with *E. chaffeensis* in WTD precludes subsequent co-infection with a strain that differs by number of tandem repeats present in a variable-length PCR target gene and determine the tissue tropism of *E. chaffeensis* in WTD via *in situ* detection in tissues;
3. Evaluate the suitability of domestic goats as an animal model for *E. chaffeensis* infection;
4. Estimate the prevalence of *E. chaffeensis*, *E. ewingii*, and *B. lonestari* in wild-caught LST, *Amblyomma americanum*, from an endemic area in northeastern Georgia; and
5. Attempt to isolate *B. lonestari*, a previously uncultivated organism and putative etiologic agent of STARI, using a co-culture system in ISE6 tick cells.

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CHAPTER 2

LITERATURE REVIEW

Review of *Amblyomma americanum*

Natural history of *A. americanum*

Amblyomma americanum (Acari: Ixodidae) is a metastriate three-host hard tick found in North America. It shares the genus *Amblyomma* with approximately 100 other species and is more commonly known as the lone star tick (LST) in reference to the distinctive white spot present on the scutum of adult females. Suitable habitats for LST generally consist of second-growth woodland forest, often with mixed hardwoods, and a dense understory that creates the humid, cooler microhabitat ideal for maintaining water balance (Childs and Paddock 2003). A low tolerance for dry, cold climates, higher tolerance for warm climates, and higher moisture requirements puts some limitations on the geographic distribution of LST, with the most ideal habitats occurring throughout the southeastern and south-central United States. However, the reported range extends beyond these more concentrated areas and is bounded on the west by Texas and Iowa, extending east into coastal states as far north as New York and more recently Maine (Means and White 1997, Keirans and Lacombe 1998).

Seasonal variation in the abundance of all three motile tick stages has been reported throughout the geographic range of *A. americanum*, and is dependent on environmental factors such as temperature, day length, and humidity (Haile and Mount,

1987; Davidson, et al. 1994, Jackson, et al. 1996). The activity of adult and nymphal stages is typically highest in the spring, with peak abundance between April and June and declining through the summer, at which time larval abundance rises (Davidson, et al. 1994, Childs and Paddock 2003). In a multiyear study in Georgia, the numbers of questing adults were highest beginning in March, and nearly disappear by August (Davidson, et al. 1994). Nymphal populations followed a similar pattern to adult LST in Georgia, but reached a peak in May, followed by a decline in population during the summer (Davidson, et al. 1994). In contrast, larval tick populations in Georgia increased during summer months and reached peak levels in August and September.

In addition to seasonal and geographic variability in the distribution of *A. americanum*, localized variability within a niche also occurs, where tick abundance, under the influence of a number of environmental and host factors, may be more or less concentrated (Schulze, et al. 2001). One of the most influential host factors is carbon dioxide, a host cue that stimulates questing in adult stages of *A. americanum* and acts as a chemo-attractant for all stages of *A. americanum* (Wilson, et al. 1972b). Carbon dioxide emitted from dry ice has been exploited for field collection of wild LST (Hair, et al. 1972, Grothaus, et al. 1976, Koch and Mcnew 1982). Small changes in carbon dioxide levels are often sufficient for stimulating a behavioral response and make carbon dioxide traps a more efficient and consistent method of collecting adult stages of wild ticks than flagging with a cloth (Wilson, et al. 1972a). Nymphal stages are also effectively collected by dry ice traps, but collection of larvae is often not feasible using this method (Koch and Mcnew 1982).

Lone star ticks are aggressive feeders that use a wide range of vertebrate hosts. Medium to larger size mammals make up the majority of hosts for all three stages, with the size of the host generally increasing with the stage. The most important host species used by each stage varies among different geographic locations. Immature stages of LST feed on mammals such as white-tailed deer (*Odocoileus virginianus*), cottontail rabbits (*Sylvilagus floridanus*), squirrels (*Sciurus* spp.), coyotes (*Canis latrans*), opossums (*Didelphus virginianus*) and raccoons (*Procyon lotor*), as well as birds; small mammals, including rodents, are rarely, if ever, hosts (Kollars, et al. 2000). Adult LST prefer medium to larger mammals and will feed on similar medium-sized hosts as nymphs; wild turkeys (*Meleagris gallopavo*) are commonly parasitized by both nymphal and adult LST (Bishopp and Trembley 1945). For both immature and adult stages, WTD are the preferred host and fawns are commonly infested with *A. americanum* (Kollars, et al. 2000). All stages of LST also feed on humans and are often the most commonly removed ticks from people (Felz, et al. 1996, Felz and Durden 1999, Merten and Durden 2000).

Amblyomma americanum as a vector of bacteria

The aggressive feeding behavior, wide geographic range, diverse host list, and common occurrence on humans, make *A. americanum* a highly suitable vector for a number of zoonotic bacteria. Consequently, the reputation of *A. americanum* has shifted from extreme nuisance to that of an important vector for the agents of several zoonotic diseases, primarily of organisms recently recognized, or “emergent” status. Among the zoonotic bacterial agents transmitted by *A. americanum* are *Ehrlichia*

chaffeensis, the agent of human monocytotropic ehrlichiosis (HME), *E. ewingii*, an agent of human and canine granulocytic ehrlichiosis, and *Borrelia lonestari*, putative agent of southern tick-associated rash illness (STARI).

Amblyomma americanum was suspected to vector *E. chaffeensis* when a correlation between the epidemiology of HME and the distribution of LST was observed (Eng, et al. 1990, Lockhart, et al. 1995). *Ehrlichia ewingii*, first implicated as a strain of *E. canis* responsible for canine granulocytic ehrlichiosis, remained unnamed until 1992 (Ewing, et al. 1971, Anderson, et al. 1992). Transmission of *E. chaffeensis* and *E. ewingii* by *A. americanum* was subsequently demonstrated experimentally in 1995 and 1990, respectively (Anziani, et al. 1990, Ewing, et al. 1995). Evidence of these two *Ehrlichia* spp. in LST has been demonstrated repeatedly since their initial recognition (Lockhart, et al. 1997b, Yu, et al. 1997, Murphy, et al. 1998, Roland, et al. 1998, Steiner, et al. 1999, Ijdo, et al. 2000, Irving, et al. 2000, Whitlock, et al. 2000, Stromdahl, et al. 2001, Steiert and Gilfoy 2002, Goddard, et al. 2003, DeShields, et al. 2004). These organisms are discussed in greater detail later.

Spirochetes were first observed in LST by dark field and direct fluorescent antibody staining during the mid-1980s (Schulze, et al. 1984, Magnarelli, et al. 1986). *Borrelia burgdorferi*, the causative agent of Lyme disease, has been detected in *A. americanum* by fluorescent antibody staining and molecular assay, and has been isolated by culture of LST (Luckhart, et al. 1991, Teltow, et al. 1991, Feir, et al. 1994). Lone star ticks, however, are inefficient vectors for *B. burgdorferi* and are not believed to play a significant role in the transmission of *B. burgdorferi* or the epidemiology of Lyme disease (Piesman and Sinsky 1988, Teltow, et al. 1991, Sanders and Oliver 1995,

Oliver 1996). However, another spirochete, *B. lonestari*, has been identified in LST using molecular assays, and is the suspected causative agent of southern tick-associated rash illness (Barbour, et al. 1996).

In addition to the zoonotic bacteria described above, other bacteria have also been identified in *A. americanum*, although the importance of LST in maintenance of these other bacteria remains questionable. For example, *Coxiella burnetii*, the agent of Q fever, has been reported twice from LST, both times approximately 50 years ago, and there has been no recent evidence implicating LST in Q fever transmission (Cox 1940, Parker, et al. 1943, Childs and Paddock 2003). There is, however, evidence that *A. americanum* is a suitable vector for the causative agent of tularemia, *Francisella tularensis* (Parker, et al. 1943, Taylor and Fulford 1981, Jasinskas, et al. 2000), although the prevalence of this agent in lone star tick populations is apparently low (Childs and Paddock 2003). *Rickettsia* spp., such as *Rickettsia amblyommi* and *R. rickettsii*, the causative agent of Rocky Mountain Spotted Fever, have been reported from LST (Parker, et al. 1943, Goddard and Norment 1986, Childs and Paddock 2003). However, the role of LST as a significant vector of *Rickettsia* spp. and the pathogenicity of *R. amblyommi*, remain unclear.

Review of *Ehrlichia chaffeensis*

Biology and Taxonomy of *Ehrlichia chaffeensis*

Ehrlichia chaffeensis, the etiologic agent of human monocytotropic ehrlichiosis (HME), is a small, gram-negative, tick-transmitted obligate intracellular bacterium (Anderson, et al. 1991, Rikihisa 1999). The first isolation of *E. chaffeensis* was made in

1990 from a 21-year old patient from Fort Chaffee, Arkansas (Anderson, et al. 1991, Dawson, et al. 1991). Retrospective examination of samples from a human ehrlichiosis patient from 1986, which was initially attributed to *E. canis* infection, was later determined to have been caused by *E. chaffeensis* (Maeda, et al. 1987, Paddock and Childs 2003).

Sequence comparison of the 16S rRNA gene of *E. chaffeensis* with other *Ehrlichia* spp. showed that *E. chaffeensis* was most closely related to *E. canis*, the causative agent of canine monocytotropic ehrlichiosis which also invades cells in the monocyte-macrophage system (Dawson, et al. 1991, Dumler and Bakken 1995). Consequently *E. chaffeensis* was placed in the family *Rickettsiaceae*, tribe *Ehrlichieae*. Recent taxonomic revisions using molecular analyses of the 16S rRNA, *groESL*, and surface protein genes confirmed the relatedness to *E. canis* but eliminated the tribe *Ehrlichieae* and transferred the genus *Ehrlichia* to the family *Anaplasmataceae*. *Ehrlichia chaffeensis* remains in a cluster with *E. canis*, as well as *E. ewingii*, the agent of canine granulocytotropic ehrlichiosis, *E. muris*, which also invades monocytes, and *Ehrlichia* (Cowdria) *ruminantium*, the agent of heartwater (Dumler, et al. 2001). Reorganization of the *Rickettsiaceae* also moved *E. phagocytophila*, and its close relatives *E. equi* and the agent of human granulocytotropic ehrlichiosis (HGE), to the genus *Anaplasma* and synonymized them under one name, *Anaplasma phagocytophilum* in the family *Anaplasmataceae*. Also included in the family *Anaplasmataceae* is the genus *Neorickettsia*, which contains several species that cause diseases of human and veterinary importance but which differ significantly with regard to their epizootiology (Dumler, et al. 2001).

Morphologically, *E. chaffeensis* resembles other gram-negative bacteria, with a double membrane and scant peptidoglycan layer, but unlike the genus *Rickettsia*, the bileaflet outer membrane is not thickened (Popov, et al. 1998, Rikihisa 1999). *Ehrlichia chaffeensis* most commonly occurs as cocci, but may be occasionally pleomorphic, especially when grown in tissue culture (Rikihisa 1991). Like others in its genus, *E. chaffeensis* is leukotropic in the mammalian host, specifically invading cells in the mononuclear-phagocytic cell system. The act of invasion is not yet fully understood, however, L-selectin and E-selectin, two proteins on the surface of THP-1 cells (a human monocyte/macrophage cell line) have been implicated in playing a role in THP-1 cell attachment and entry *in vitro* and may consequently have a role *in vivo* (Zhang, et al. 2003a). Upon invasion of host cells, *E. chaffeensis* organisms replicate in host-membrane inclusions termed morulae, which are actually modified early endosomes (Barnewall, et al. 1997, Popov, et al. 1998). The number of morulae within infected cells of human patients may reach 15 but generally only one or two morulae are present (Paddock, et al. 1993, Barenfanger, et al. 1996, Martin, et al. 1999).

Following infection with *E. chaffeensis*, the organism is disseminated systemically through the circulation, causing hematopathologic changes and multi-organ pathology, including anemia, leucopenia, thrombocytopenia, cerebral meningitis, hepatic and gastrointestinal manifestations (Berry, et al. 1999, Nutt and Raufman 1999, Rikihisa 1999, Sehdev and Dumler 2003). Detection of *E. chaffeensis* morulae in numerous tissues, most often in spleen, lymph nodes, and bone marrow, by immunohistochemistry and *in situ* hybridization has been most successful in fatal human cases of HME, and has also been used for determining tissue tropism in

experimentally infected animals (Dumler, et al. 1993a, Dumler, et al. 1993b, Yu, et al. 1993, Dawson, et al. 2001, Sotomayor, et al. 2001). No clinical signs of infection have been shown in white-tailed deer, nor have morulae of *E. chaffeensis* been definitively visualized on direct blood smears of deer (Dawson, et al. 1994b, Little, et al. 1998). It is not known if this is due to low circulating bacteria, sequestration in tissues, or another cause; further characterization of the infection in deer will be necessary in order to understand this phenomenon.

Human monocytotropic ehrlichiosis (HME)

Since 1986, when it was first recognized, human monocytotropic ehrlichiosis (HME) has become an important public health issue in the southeastern United States. HME may cause morbidity and has also been attributed as the cause of death of at least ten patients and can result in severe illness if left untreated (Eng, et al. 1990, Dumler, et al. 1993a, Marty, et al. 1995, Rikihisa 1999). Human monocytotropic ehrlichiosis is most commonly associated with a fever of undetermined origin, typically after a history of tick exposure or bite 2-3 weeks prior. While most patients have a history of exposure to a tick or of a tick-bite, an associated rash is rare; only approximately one-third of patients develop the characteristic erythematous rash, in contrast to the approximately 80% of patients with Rocky Mountain Spotted Fever who develop a rash (Dumler and Bakken 1998). The disease occurs most often in May, June and July, which correlates with adult and nymphal peak activity of the tick-vector, *A. americanum*. Patients are, on average, 44 years old, most often men (75%), and generally reside in rural areas (Rikihisa 1999).

The most common clinical findings in HME are an undifferentiated fever with myalgia, malaise, headache, leukopenia, thrombocytopenia, and elevated liver enzymes. Specific symptoms mimic a flu-like illness and may include arthralgia, anorexia, nausea, respiratory signs and CNS involvement (Rikihisa 1999, Tan, et al. 2001, Paddock and Childs 2003). Since recognition of the disease, the CDC has confirmed at least 1100 cases, mostly occurring in the southeastern and mid-central United States where the ticks, deer and organism are present (Childs and Paddock 2003). Physicians may still be unfamiliar with HME, and diagnosis of the disease does not account for exposure. Thus, because, subclinical infections are often not recognized and the disease is reportable in only 20 states, the incidence of infection may be underestimated.

Risk of exposure increases with outdoor activities (Standaert, et al. 1995, Walker and Dumler 1996), an association that was first demonstrated in a study of a two golfing communities in Tennessee. In that study, serologic evidence of exposure was detected in 12.5% of residents and the incidence was higher in residents living near a wildlife management area where both *A. americanum* ticks and white-tailed deer were present (Standaert, et al. 1995). In 1985, serologic evidence of exposure was also detected in 12% of Army reservists in New Jersey and HME was diagnosed in 11% of febrile patients, compared to one case out of seventy-five from a survey in Georgia (Rikihisa 1999). In a recent survey of children (ages 1-17 years) from the southeastern and south-central U.S., 13% had antibody titers of at least 1:80 to *E. chaffeensis* (Marshall, et al. 2002). Treatment of *E. chaffeensis* is dependent upon administration of appropriate antibiotics, generally tetracycline or doxycycline, and supportive care

(Fishbein, et al. 1994, Rikihisa 1999). Understanding the mechanisms underlying the natural maintenance of *E. chaffeensis* will be critical to determining the risk factors for human infection and ultimately for developing control strategies for human monocytotropic ehrlichiosis.

Amblyomma americanum as a vector of *E. chaffeensis*

All *Ehrlichiae* in the *E. canis* genogroup, as well as most *Rickettsiae*, exploit ticks as a vector for transmission. Speculation that *A. americanum* could be a potential vector for *E. chaffeensis* began with the realization that the distribution of HME cases overlapped with the distribution of LST (Eng, et al. 1990). The development of polymerase chain reaction (PCR) primers, HE1 and HE3, targeting the *E. chaffeensis* 16S rRNA gene, and the use of these primers for detection of *E. chaffeensis* DNA in *A. americanum* from five states, provided a strong indication that LST played an important role in the natural history of *E. chaffeensis* (Anderson, et al. 1993). This was later supported by the temporal appearance of anti-*E. chaffeensis* antibodies in deer from a single geographic location following the introduction of *A. americanum* at that location (Lockhart, et al. 1995). Identification of WTD with anti-*E. chaffeensis* antibodies in areas known to have *A. americanum* activity, and the near absence of seropositive deer in areas lacking *A. americanum*, provided additional evidence implicating LST as the vector (Lockhart, et al. 1996).

The initial detection of *E. chaffeensis* 16S rDNA in LST relied on PCR assays of pooled adult or nymphal LST and found an overall minimum infection rate (MIR) of less than 0.5% (Anderson, et al. 1993). In a later study, individual ticks assayed by a nested

PCR amplifying an *E. chaffeensis* 16S rRNA gene target had a 12% prevalence of infection, compared to an MIR of 3.5% in pooled ticks. This discrepancy may reflect the presence of increased amounts of PCR inhibitors in tick pools, compared to individual ticks (Lockhart, et al. 1997a). Other surveys have also used a nested PCR to assay wild LST for evidence of *E. chaffeensis*. Using ECB and EG1 outside primers and HE1 and HE3 inside primers on 59 individual *A. americanum* nymphs and adults from Missouri, where HME is endemic, revealed 10.2% and 29% prevalence, respectively, of *E. chaffeensis* (Roland, et al. 1998). Additionally, different primers for initial amplification have also been successfully used for *E. chaffeensis* detection in naturally and experimentally infected ticks (Rechav, et al. 1999, Stromdahl, et al. 2000). A nested PCR protocol has been modified for detection of *E. chaffeensis* RNA by reverse transcription-PCR in order to resolve previous difficulties due to PCR inhibitors and increase PCR data from acquisition-fed ticks (Felek, et al. 2001).

Overall, studies demonstrating transmission of *E. chaffeensis* from LST to deer are sparse. To our knowledge, experimental tick-transmission of *E. chaffeensis* has been successfully reported only once (Ewing, et al. 1995). Ewing et al. were able to successfully transmit *E. chaffeensis* to white-tailed deer by both nymphal and adult *A. americanum*, demonstrating that nymphs also acquired infection while feeding on WTD as larvae (Ewing, et al. 1995). In that study, transmission was confirmed by seroconversion and PCR detection of *E. chaffeensis* in host deer, but PCR assays were not performed on ticks. Transovarial transmission of *E. chaffeensis* in LST does not appear to occur to any significant extent; acquisition of organisms occurs primarily when nymphal stages feed on the major vertebrate reservoir, WTD (Rikihisa 1999).

Organisms are present in adult ticks after molting and serve as the source of *E. chaffeensis* during subsequent feedings on humans, WTD, or other potential hosts (Lockhart, et al. 1997b). However, the movement of the organisms between the vertebrate reservoir host and the tick vector remains incompletely understood.

White-tailed deer: the principal vertebrate reservoir

White-tailed deer were first suspected to be a vertebrate reservoir host for *E. chaffeensis* in 1994, when a study of WTD from 17 states in the Southeast detected 43% seroprevalence in deer for anti-*E. chaffeensis* antibodies (Dawson, et al. 1994a). Successful experimental inoculation of deer soon followed (Dawson, et al. 1994b). In 1997, isolation of *E. chaffeensis* in cell culture from the blood of naturally infected deer provided the first convincing evidence that deer were a major vertebrate reservoir (Lockhart, et al. 1997a). Nucleotide sequencing of these first deer isolates revealed sequences identical to the original Arkansas strain of *E. chaffeensis* (Anderson, et al. 1992, Lockhart, et al. 1997a), providing strong evidence that the organism in deer was indeed the same as the pathogen in humans. Deer likely played a role in the natural history of *E. chaffeensis* well before the index case of HME was detected in 1986, as exemplified by the detection of *E. chaffeensis* in archived deer tissues from 1985 (Little and Howerth 1999). Natural infection of WTD has also been demonstrated repeatedly, by culture isolation, molecular detection, and serologic assay, throughout the southeastern and south-central United States within the last five years (Irving, et al. 2000, Mueller-Anneling, et al. 2000, Yabsley, et al. 2002, Arens, et al. 2003, Yabsley, et

al. 2003a, Yabsley, et al. 2003b)., and these studies indicate that WTD are the principal vertebrate reservoir.

Experimental infection of white-tailed deer has shown that, upon intravenous inoculation with cell culture derived *E. chaffeensis*, deer become rickettsemic and seroconvert by 10 days post-inoculation (Dawson, et al. 1994b, Ewing, et al. 1995, Kocan, et al. 2000a, Davidson, et al. 2001). In these studies, deer were exposed to *E. chaffeensis* by single intravenous inoculation. Whereas *E. chaffeensis* organisms are occasionally observed in blood smears of human patients with HME, morulae are not routinely seen in infected deer. Specific studies evaluating changes in the levels of *E. chaffeensis* circulating in deer following needle inoculation and/or tick transmission have not been performed, nor has the response to multiple exposures of *E. chaffeensis* been evaluated.

Deer have been shown to be capable of maintaining a persistent infection as long as nine months after initial exposure, suggesting that persistently infected deer may be a source of organisms to tick vectors (Davidson, et al. 2001). Studies to date have only evaluated the response of deer to a single exposure of *E. chaffeensis*. However, in nature, deer are more likely exposed multiple times to different strains of *E. chaffeensis* since deer are the primary host for all stages of LST, and different strains have been detected in the same LST population (Patrick and Hair 1978, Yu, et al. 1997, Kollars, et al. 2000, Stromdahl, et al. 2000).

To date, a total of thirteen isolates of *E. chaffeensis* have been reported from deer (Lockhart, et al. 1997a, Lockhart, et al. 1997b, Little, et al. 1998, Yabsley, et al. 2002, Yabsley, et al. 2003a). Analysis of a variable-length PCR target (VLPT) has

revealed distinct differences in the number of tandem repeats present in a nucleotide sequence encoding a 44 kDa immunoreactive protein in *E. chaffeensis* that can be exploited to distinguish different isolates and is explained further below (Paddock, et al. 1997, Sumner, et al. 1999). VLPT has been used to confirm *E. chaffeensis* infection in human patients with a history of tick exposure and fever (Standaert, et al. 2000, Stromdahl, et al. 2000)), to identify infection in ticks (Stromdahl, et al. 2000), and has been used most recently to investigate molecular variation in *E. chaffeensis* strains in naturally infected deer (Yabsley, et al. 2003b). Presence of multiple strains of *E. chaffeensis* in individual naturally infected WTD, and within some WTD populations, suggests that WTD are susceptible to repeated infestations (Yabsley 2003b). The response of deer to experimental inoculation with multiple strains has not been evaluated and would provide more comparable information to infections of WTD in nature.

Other potential reservoir hosts

Although substantial evidence indicates that WTD are the principal reservoir host for *E. chaffeensis*, field evidence indicates that other mammals also may be infected. Anti-*E. chaffeensis* antibodies have been detected in raccoons (*Procyon lotor*) and opossums (*Didelphus marsupialas*) from the eastern United States (Lockhart, et al. 1997b). In addition, domestic dogs and red foxes (*Vulpes vulpes*) have been experimentally infected with *E. chaffeensis* (Comer, et al. 2000, Dawson and Ewing 1992, Dawson, et al. 1996a, Davidson, et al. 1999). Natural infection has been demonstrated in lemurs from a colony in North Carolina and St. Catherine's Island,

Georgia (Williams, et al. 2002, Yabsley, et al. in press), coyotes from Oklahoma and domestic goats in Georgia (Dugan, et al. 2000, Kocan, et al. 2000b). Although antibodies reactive to *E. chaffeensis* have been detected in white-footed mice in Connecticut (Magnarelli, et al. 1997), attempts to detect antibodies in rodents from the Southeast have been unsuccessful (Lockhart, et al. 1998). Subsequent studies suggest that natural infection of mice does not occur because attempts to infect C3H/HeJ mice with isolates from naturally infected deer have been unsuccessful, despite the ability to infect immunocompromised mice with a human isolate (Winslow, et al. 1998, Lockhart and Davidson 1999). Consequently, current investigations should concentrate on understanding the infection dynamics in white-tailed deer, the only known principal reservoir host. However, other naturally infected vertebrate hosts should be considered when interpreting results from WTD.

Importance of a Model for Reservoir Host Research

Because deer are difficult to handle and maintain in captivity, a suitable surrogate model that has shown susceptibility to *E. chaffeensis* may be preferable for experimental infection studies. Anti-*E. chaffeensis* antibodies have been detected in opossums, raccoons and white-footed mice, and the former two may be host to all three stages of *A. americanum*, but PCR detection has not confirmed their ability to support infection (Lockhart, et al. 1997b, Magnarelli, et al. 1997, Comer, et al. 2000). Furthermore, a subsequent serologic survey of eight species of wild rodents in the southeastern United States showed no seroprevalence, thus verifying that rodent are not likely involved in natural maintenance of *E. chaffeensis* (Lockhart, et al. 1998).

Despite the findings in wild rodents, laboratory mice are commonly used models for experimental infection with *E. chaffeensis*. Numerous strains have been used for studies of immunologic response to *E. chaffeensis* (Telford and Dawson 1996, Ganta, et al. 2002, Ganta, et al. 2004) and persistent infection with *E. chaffeensis* has been demonstrated in C3H/HeJ mice (Telford and Dawson 1996). In the latter study, nearly all C3H/HeJ mice inoculated with *E. chaffeensis* cell culture via intracardiac, intraperitoneal, or subcutaneous routes seroconverted by day 15, and *E. chaffeensis* was detected by PCR assay in six mice at least once in eight sample dates (Telford and Dawson 1996). However, a subsequent study to evaluate the potential of using mice as a xenodiagnostic tool was not successful in producing experimental infection, based on seroconversion in the absence of PCR detection (Lockhart and Davidson 1999). Consequently, although mice do not appear to be ideal models for investigating reservoir infection, they may be an appropriate model for investigating the immunology and pathology of human infection (Winslow, et al. 1998, Okada, et al. 2001, Sotomayor, et al. 2001).

Experimental infection of red foxes with *E. chaffeensis* has been successful, but the use of other wildlife would be challenging due to similar management and handling issues (Davidson, et al. 1999). Domestic canids have been experimentally infected with *E. chaffeensis* and natural infection has as been reported in dogs (Dawson and Ewing 1992, Ewing, et al. 1995, Dawson, et al. 1996a, Murphy, et al. 1998, Zhang, et al. 2003b, de Castro, et al. 2004). More recently, beagles were used as a model to investigate persistent infection with *E. chaffeensis* and results suggested that dogs could be carriers of the organism, however, the small number of dogs used (two) limits

the extent to which conclusions can be drawn regarding their use as a model for infection (Zhang, et al. 2003b). The biological differences between white-tailed deer, the principle host in nature, and dogs also suggests that canines may not be an appropriate surrogate for white-tailed deer.

Ultimately, a suitable model to demonstrate the dynamics of vertebrate reservoir infection should be phylogenetically related to deer and have shown susceptibility to infection. Natural infection has been detected in a herd of domestic goats in northern Georgia where seroprevalence was 73.7% and *E. chaffeensis* was isolated from one goat (Dugan, et al. 2000). Since goats are more closely related to deer, compared to other susceptible mammals, and are easier to handle and maintain in enclosed facilities, they may be an appropriate model for reservoir infection.

Molecular variation in *E. chaffeensis*

After cultivation of the first isolate of *E. chaffeensis*, the Arkansas strain, from a patient with HME (Dawson, et al. 1991), numerous additional strains were cultured and/or identified from other sources such as LST, WTD, lemurs, other patients and a goat (Lockhart, et al. 1997b, Little, et al. 1998, Dugan, et al. 2000, Williams, et al. 2002, Yabsley, et al. 2002, Yabsley, et al. 2003a, Yabsley, et al. in press). The availability of various strains, differences in clinical presentation of HME, and widespread geographic range of *E. chaffeensis*, its vector, and its primary host, prompted interest in potential molecular variation among strains (Paddock and Childs 2003). Among the genes identified with discernible variation were the outer membrane protein multigene family (OMP), the variable length PCR target (VLPT), and the 120kDa outer membrane

protein; the latter two genes contain tandem nucleotide repeats that may vary among strains (Sumner, et al. 1999, Popov, et al. 2000, Long, et al. 2002, Cheng, et al. 2003). Molecular heterogeneity, defined by differences in the number of repeats in the VLPT and 120kDa genes among strains, can be detected by PCR amplification.

Thus far, studies on the significance of molecular heterogeneity have not revealed obvious correlations between genetic variants and geographic location (Sumner, et al. 1999, Yabsley, et al. 2003b). Interstrain variation in the VLPT gene, which expresses a 44kDa antigenic protein, has been demonstrated in *E. chaffeensis* variants from LST, human patients, and WTD (Paddock, et al. 1997, Sumner, et al. 1999, Standaert, et al. 2000, Stromdahl, et al. 2000, Liddell, et al. 2002, Cheng, et al. 2003, Yabsley, et al. 2003b). The 120kDa gene encodes an immunogenic protein found on the outer membrane of *E. chaffeensis* and in the matrix of the morula and has also been shown to have genetic variability (Yu, et al. 1997, Popov, et al. 2000, Standaert, et al. 2000, Liddell, et al. 2002, Cheng, et al. 2003, Yabsley, et al. 2003b).

Coinfections with unique genetic variants have been detected in individual WTD and in WTD populations (Yabsley, et al. 2003b). The ability of deer to become infected with multiple strains of *E. chaffeensis* is important in their role as a reservoir host. Individual deer infected with multiple strains of *E. chaffeensis* would provide a source of several different genetic variants for ticks to acquire when feeding, and thereby subsequently increase the diversity of organisms available for transmission to humans, potentially increasing the likelihood that people would be exposed to pathogenic strains.

Other *Ehrlichia* spp. Transmitted by *A. americanum*.

Ehrlichia ewingii

The agent of canine granulocytic ehrlichiosis, *E. ewingii* was initially thought to be a strain of *E. canis* but was shown to invade granulocytic cells, rather than the mononuclear cells that *E. canis* invade (Ewing, et al. 1971). Dogs experimentally infected with the canine granulocytic ehrlichiosis agent provided the source of organism for PCR amplification and sequencing of the 16S rRNA gene that led to its recognition as a unique species (Anderson, et al. 1992). Comparison of the 16S rRNA gene with other *Ehrlichia* spp. demonstrated that *E. ewingii* was most closely related to *E. canis* (98.0 %) and *E. chaffeensis* (98.1%). Antibodies to *E. ewingii* are also known to cross-react with *E. canis* and *E. chaffeensis* (Rikihisa, et al. 1994, Dawson, et al. 1996a, Liddell, et al. 2003).

Amblyomma americanum was identified as the vector of *E. ewingii* by transmission feeding infected adult ticks on susceptible dogs; a similar experiment using *Dermacentor variabilis* was not successful (Anziani, et al. 1990). Since then, evidence of *E. ewingii* has been identified by PCR assay in LST from Missouri, North Carolina, and Oklahoma (Murphy, et al. 1998, Wolf, et al. 2000, Steiert and Gilfoy 2002), and in white-tailed deer, a suspected reservoir host, from Arkansas, Georgia, Kentucky, North Carolina, and South Carolina (Yabsley, et al. 2002, Arens, et al. 2003). In addition, natural infection has been reported in dogs (Anderson, et al. 1992, Dawson, et al. 1996a, Greig, et al. 1996, Ewing, et al. 1997, Goldman, et al. 1998, Murphy, et al. 1998, Kordick, et al. 1999, Goodman, et al. 2003, Liddell, et al. 2003, Hamilton, et al. 2004)) and in humans (Buller, et al. 1999, Paddock, et al. 2001, Hamilton, et al. 2004) in

several states within the range of *A. americanum*. To date, *E. ewingii* has not been isolated in culture.

Anaplasma sp. of WTD

Molecular analysis of *E. chaffeensis* seropositive WTD from an area within the geographic range of *A. americanum* allowed the identification of an *Ehrlichia*-like agent distinct from *E. chaffeensis* (Dawson, et al. 1996b). Nucleotide sequencing and restriction enzyme mapping demonstrated that the agent was closely related to *E. platys*, *E. equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis agent (HGE) agent. Recent analysis of surface protein, 16S rRNA, and *groESL* genes led to taxonomic reorganization in the order *Rickettsiales*. The WTD agent was reclassified as an *Anaplasma* sp., *E. platys* as *A. platys*, and *E. equi*, *E. phagocytophila*, and the HGE were synonymized as *A. phagocytophilum* (Dumler, et al. 2001).

PCR primers targeting a unique region in the 16S rRNA gene were developed for molecular detection of the *Anaplasma* sp. of WTD (Little, et al. 1997) and subsequently used to demonstrate its presence in WTD, mule deer, and black-tailed deer (Foley, et al. 1998, Little, et al. 1998, Brandsma, et al. 1999, Arens, et al. 2003). These findings strengthened suspicions that LST also vectored the novel *Anaplasma* sp. of WTD, and that the organisms might be intracellular, similar to other organisms in the family *Anaplasmataceae*. At that time, however, little was known about the biology of the organism and culture isolation efforts were unsuccessful. The *Anaplasma* sp. of WTD was ultimately isolated from fawns experimentally infected with blood from naturally infected wild WTD using a co-culture system with ISE6 cells, an *Ixodes scapularis*

embryonic tick cell line established by Uli Munderloh (University of Minnesota)
(Munderloh, et al. 2003).

Review of *Borrelia lonestari*

Biology and Taxonomy of *Borrelia* spp.

The Borreliae are spirochetes, motile helically coiled slender bacteria, in the family *Spirochaetacea*, which also contains the genera *Treponema* and *Serpulina* members. *Borrelia* spp. are composed of a protoplasmic cylinder, consisting of the cell cytoplasm, an inner cell membrane, and a peptidoglycan layer, enveloped by an outer membrane (also called an outer sheath) that may contain protrusions or “blebs” (Barbour and Hayes 1986b). Flagella (usually 7 to 20) run between the outer membrane and the protoplasmic cylinder and attach subterminally to the ends of the protoplasmic cylinder. This layering creates the characteristic trilaminar effect seen most distinctly in transmission electron microscopy by negative staining. The length of *Borrelia* spp. spirochetes is variable, ranging between 5 and 25 μm , and occasionally longer, with a width between of 0.2 to 0.5 μm , and a variable wavelength (Schwan, et al. 1999). *Borrelia burgdorferi*, the causative agent of Lyme Disease in North America, falls within this range of length and diameter, has approximately 7-11 flagella, and a variable wavelength (2.2 to 3.3 μm) (Hovind-Hougen 1984, Hovind-Hougen, et al. 1986). The composition of the outer membrane of Borreliae is sufficiently distinct from other bacteria that Borreliae are considered neither gram-negative or gram-positive, although some continue to refer to this genus as being gram-negative (Barbour and Hayes 1986b, Schwan, et al. 1999, Shapiro and Gerber 2000).

Cultivation of *Borrelia* spp.

Borrelia spp. spirochetes are microaerophilic bacteria that are only found in a host-parasite association in nature (Barbour and Hayes 1986b). Some studies also have demonstrated growth under anaerobic conditions (Hardy, et al. 1963, Preac-Mursic, et al. 1991). Initial attempts to isolate *B. burgdorferi* were based on culture methods for the relapsing fever spirochetes. The successful isolation of multiple passages of *B. hermsii* was accomplished using Kelly's medium (Kelly 1971), which was later supplemented to support growth of lower numbers of spirochetes in blood samples (Stoenner 1974). Slight modifications were made to the medium after the first isolation of *B. burgdorferi* sensu stricto from *Ixodes scapularis* (= *I. dammini*) ticks collected from Shelter Island, New York (Burgdorfer, et al. 1982) and the modified media, named Barbour-Stoenner-Kelly (BSK), was used to isolate *B. burgdorferi* from *Ixodes ricinus* in Switzerland (Barbour, et al. 1983). Additional modifications to the original BSK medium included the addition of Yeastolate and removal of glutamine, making BSK II (Barbour 1984). Further improvements led to a standardized medium, BSK-H, that could be distributed for widespread use (Pollack, et al. 1993). Although several other media and substrates, including co-culture systems (Kurtti, et al. 1988, Hechemy, et al. 1992, Konishi, et al. 1993, Tylewska-Wierzbanska and Chmielewski 1997, Speck, et al. 2002), have been successfully used, BSK based media in axenic cultures remains the method of choice for efficient propagation of *Borrelia* spp.

The *Borrelia* spp. have historically been divided into two groups based on the diseases caused: those causing Lyme borreliosis and those causing relapsing fever. Recent recognition of other *Borrelia* spp. and phylogenetic analysis based on the 16S

rRNA and flagellin gene revealed two main groups of *Borrelia* spp., the Lyme borreliosis group (LBG) and the relapsing fever group (RFG), with the members of the RFG further divided into distinctive smaller clusters (Ras, et al. 1996, Rich, et al. 2001, Scoles, et al. 2001). Alternatively, three major groups of *Borrelia* spp. based on flagellin sequences have been proposed whereby the RFG are divided into two distinct groups, the Old World (including *B. duttoni* and *B. crociduridae*) and New World (including *B. parkeri*, *B. hermsii*, *B. miyamotoi*) RFG spirochetes (Fukunaga, et al. 1996).

Regardless of whether one considers two or three major divisions, specific, distinctive biological characteristics are shared among spirochetes within the groups. The LBG spirochetes, which include the eleven genospecies within *B. burgdorferi* sensu lato, are transmitted by *Ixodes* spp; most RFG spirochetes, including *B. hermsii* and *B. parkeri*, are transmitted by soft ticks in the family *Argasidae* (Schwan, et al. 1999). However, several species, including *B. theileri*, *B. miyamotoi*, and *B. lonestari*, which are more closely related to the RFG, are distinct in that they are transmitted by hard (ixodid) ticks (Rich, et al. 2001). The LBG spirochetes also cause a lower density spirochetemia in infected humans than the RFG spirochetes (Barbour and Hayes 1986a).

Southern tick-associated rash illness

In contrast to the Northeast, Midwest, and West Coast, where classic Lyme disease (LD) caused by *B. burgdorferi* sensu stricto is endemic, LD is rare in the southeastern and south-central United States (Barbour and Fish 1993, Oliver 1996). Indeed, to date *B. burgdorferi* sensu stricto has not been isolated in culture from any

human patient in the southern U.S. (Felz, et al. 1999). Although the existence of LD in the South has been questioned, *B. burgdorferi* sensu stricto, *I. scapularis* and rodents such as the white-footed mouse (*Peromyscus leucopus*), which maintain the spirochete in nature in the Northeast and Midwest, are present in the South. *Ixodes pacificus*, which transmits *B. burgdorferi* sensu stricto in the West Coast, is not present in the southern states. In the South, however, several other enzootic transmission cycles exist involving ticks that rarely bite humans (*I. affinis* and *I. minor*), and *I. scapularis* primarily feeds on lizards, which are incompetent hosts for *B. burgdorferi* sensu stricto; thus, the *B. burgdorferi* infection rate of *I. scapularis* in the South is low (Spielman, et al. 1984, Oliver 1996, Oliver, et al. 2003).

Despite the presence of *B. burgdorferi* sensu stricto but the lack of evidence confirming classic LD in the southeastern and south-central United States, patients with erythema migrans (EM) with or without LD-like symptoms are reported in the southern states (Campbell, et al. 1995, Masters and Donnell 1995, Kirkland, et al. 1997, Masters, et al. 1998, Felz, et al. 1999, Armstrong, et al. 2001). A distinct difference in these cases, however, is the association of EM and LD-like symptoms with the bite of *A. americanum*. These cases are also clinically distinct from classic LD by differences by serologic testing. Tests for specific anti-*B. burgdorferi* antibodies are negative or patients show an atypical western blot profile that is not consistent with *B. burgdorferi* infection (Felz, et al. 1999). In addition, attempts to isolate *Borrelia* sp from suspected LD patients in the South have been unsuccessful to date (Campbell, et al. 1995, Masters, et al. 1998, James, et al. 2001). These observations have led clinicians to suspect a syndrome distinct from LD and have designated the syndrome Southern

Lyme, southern tick-associated rash illness (STARI), or Master's disease (Campbell, et al. 1995, Masters and Donnell 1995, Kirkland, et al. 1997, Masters, et al. 1998, Armstrong, et al. 2001).

Individuals with STARI typically present with a mild flu-like illness that includes malaise, headache, fatigue, and occasionally fever, and which can be successfully treated with antibiotics (Campbell, et al. 1995, Masters and Donnell 1995, Kirkland, et al. 1997, Masters, et al. 1998). To date, cases of STARI have been reported from numerous states in the geographic range of *A. americanum*, including Georgia, Maryland, Missouri, North Carolina, and South Carolina (Campbell, et al. 1995, Masters and Donnell 1995, Armstrong, et al. 1996, Kirkland, et al. 1997, Masters, et al. 1998, Felz, et al. 1999, James, et al. 2001).

Identification of *Borrelia lonestari*

Spirochetes were first reported from LST in the mid-1980s by visualization using dark-field microscopy and immunofluorescence (Schulze, et al. 1984). A *Borrelia* sp. was proposed as the causative agent of STARI based on clinical similarities to acute LD and microscopic evidence of spirochetes detected in biopsy samples from affected patients (Schulze, et al. 1984, Kirkland, et al. 1997, Masters, et al. 1998). However, although *B. burgdorferi* had been found in *A. americanum*, it was known that LST were not competent vectors of *B. burgdorferi* (Piesman and Sinsky 1988, Luckhart, et al. 1992, Mukolwe, et al. 1992). In 1996, a novel *Borrelia* sp. was identified in *A. americanum* by sequence analysis of the 16S rRNA and flagellin genes (Barbour, et al. 1996). Lone star ticks collected from Texas, Missouri, New York, and New Jersey

showed evidence of spirochetes by direct immunofluorescence using a fluorescein labeled polyclonal anti-*B. burgdorferi* antibody; PCR assays and sequence analysis demonstrated sufficient differences in nucleotide sequences to justify classifying a new species, tentatively named *B. lonestari* (Barbour, et al. 1996).

Subsequent studies using PCR assay to amplify *B. lonestari* flagellin and 16S rRNA gene sequences detected the putative agent of STARI in LST, WTD, and a human patient (Feir, et al. 1994, Burkot, et al. 2001, James, et al. 2001, Bacon, et al. 2003, Moore, et al. 2003b, Stegall-Faulk, et al. 2003, Stromdahl, et al. 2003). The patient, a 74-year old man with evidence of erythema migrans at a site of LST attachment and a history of travel to Maryland and North Carolina, was negative for antibodies to *B. burgdorferi*. However, *B. lonestari* DNA sequences were amplified from cell culture media in which a skin biopsy sample was placed (James, et al. 2001). The molecular detection of *B. lonestari* in LST removed from humans provided further evidence suggesting that humans are exposed to *B. lonestari* through the bite of *A. americanum* (Stromdahl, et al. 2003).

Molecular characteristics of *B. lonestari*

A comparison of flagellin sequences amplified from numerous *B. lonestari* strains from ticks, WTD, and a human has revealed nearly 100% interstrain identity, with the exception of a three base pair insert present at positions 330 to 332 in several sequences. A single nucleotide difference was reported at position 348 (Barbour, et al. 1996), but few other differences have been detected (Bacon, et al. 2003). The significance of the 3-bp insert is not clear. As of 2003, only three 16S rRNA gene

sequences were available for *B. lonestari* (= *B. barbouri*), none of which were 100% identical with each other due to differences in four base positions (Barbour, et al. 1996, Rich, et al. 2001, Bacon, et al. 2003).

Phylogenetic analysis of the flagellin and 16S rRNA gene sequences places *B. lonestari* in a monophyletic group with *B. theileri*, an agent of bovine borreliosis that is transmitted primarily by *Boophilus* sp. ticks, *B. miyamotoi* from *I. persulcatus* in Japan, and a *B. miyamotoi*-like spirochete found in *I. scapularis* from the northeastern United States (Fukunaga, et al. 1995, Fukunaga, et al. 1996, Rich, et al. 2001, Scoles, et al. 2001). Species within this clade are more closely related to the relapsing-fever spirochetes than the Lyme disease spirochetes, but are transmitted by hard (ixodid) ticks rather than soft (argasid) ticks (Fukunaga, et al. 1996, Scoles, et al. 2001). The close relatedness of *B. lonestari* and *B. theileri*, which share 97 and 98% similarity in the 16S rRNA and flagellin genes, respectively, suggests that *B. lonestari* may share other biological characteristics with *B. theileri*, such as the capability for transovarial transmission (Smith and Rogers 1998, Rich, et al. 2001). Consequently, there has been speculation that *A. americanum* and *B. lonestari* represent a host shift from *Boophilus* spp. and *B. theileri* because *Boophilus* spp. historically fed on WTD prior to the eradication of *Boophilus* sp. these ticks from North America (Rich, et al. 2001).

Current understanding of the natural history of *Borrelia lonestari*

Because *B. lonestari* has only recently been recognized, little is known regarding the natural history of this organism. However, some information can be inferred from the knowledge of other *A. americanum*-vectored bacteria, such as *E. chaffeensis*

(Lockhart, et al. 1997b, Lockhart, et al. 1998, Lockhart and Davidson 1999). The host preference of LST for WTD, and the molecular evidence of *B. lonestari* in LST and WTD, suggests that this organism may use a maintenance cycle similar to that of *E. chaffeensis*. In addition, the geographical distribution of suspected cases of STARI is similar to that of HME (Childs and Paddock 2003). At this time, it is suspected that *B. lonestari* is maintained in nature in a cycle involving LST and WTD (Burkot, et al. 2001, Stromdahl, et al. 2001, Bacon, et al. 2003, Childs and Paddock 2003, Moore, et al. 2003b, Stegall-Faulk, et al. 2003, Stromdahl, et al. 2003). Obtaining a live isolate of *B. lonestari* is an essential first step for future research in order to identify new diagnostic targets, determine the importance of *B. lonestari* as a human pathogen, and reveal the natural history of this organism.

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CHAPTER 3

EXPERIMENTAL INFECTION OF WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) WITH *EHRlichia chaffeensis* BY DIFFERENT INOCULATION ROUTES¹

¹ Varela, A.S., Stallknecht, D.E., Yabsley, M.J., Moore, V.A., Davidson, W.R., and Little, S.E. 2004. *Journal of Wildlife Diseases*. 39:881-886. Reprinted here with permission from publisher.

ABSTRACT

The infection dynamics of the tick-transmitted organism *Ehrlichia chaffeensis* were investigated in white-tailed deer (*Odocoileus virginianus*), using different routes of inoculation. Six deer were each inoculated with 5.4×10^6 DH82 cells infected with *E. chaffeensis* (Arkansas strain) by three different routes: intravenous ($n = 2$), subcutaneous ($n = 2$), and intradermal ($n = 2$). Two control deer were inoculated with uninfected cells. Infections were monitored for 54 days and were continued in one deer from each *E. chaffeensis* inoculated group for an additional 31 days. All deer inoculated with *E. chaffeensis* seroconverted ($= 1:64$) and became 16S rDNA polymerase chain reaction and/or cell culture positive by post-inoculation day 15. There was no apparent difference in susceptibility to infection between deer inoculated by different routes for the first 50 days based on detection of *E. chaffeensis* infection by PCR assay of blood ($P = 0.90823$) or culture isolation ($P = 0.91372$). These results demonstrate infection of deer by intradermal and subcutaneous routes for the first time.

Key words: *Ehrlichia chaffeensis*, experimental infection, *Odocoileus virginianus*, white-tailed deer

Since first recognized in 1991, *Ehrlichia chaffeensis* has emerged as an important tick-transmitted human disease agent in the United States. An obligate intracellular bacterium, *E. chaffeensis* is the causative agent of human monocytic ehrlichiosis (HME). *Ehrlichia chaffeensis* is transmitted via the lone star tick, *Amblyomma americanum*; white-tailed deer (*Odocoileus virginianus*) serve as the principal vertebrate reservoir host (Dawson et al., 1994b; Ewing et al., 1995). White-tailed deer were first identified as a potential reservoir host for *E. chaffeensis* in 1994, when anti-*E. chaffeensis* antibodies were detected in 43% of deer from 17 states and experimental infection was demonstrated through PCR and seroconversion (Dawson et al., 1994a, b).

To our knowledge, intravenous (IV) inoculation has been the only route used in needle inoculation of deer with *E. chaffeensis* (Dawson et al., 1994b; Ewing et al., 1995; Kocan et al., 2000; Davidson et al., 2001). Because *E. chaffeensis* is naturally transmitted through the bite of a lone star tick, intravenous injection of organism directly into blood may not suitably illustrate the true course of infection. Experimental transmission by feeding *E. chaffeensis* infected ticks on deer has been successfully accomplished (Ewing et al., 1995), but captive deer are difficult to handle and controlled tick-feeding studies are challenging and often impracticable, compared to needle inoculation. Subcutaneous (SQ) and intradermal (ID) inoculations are also easy to administer, but unlike IV inoculation, SQ and ID routes may more closely mimic transmission via tick feeding because they do not introduce organisms directly into a major vein, but rather near the surface of the skin. Here we describe the course of *E. chaffeensis* infection in deer following IV, SQ, and ID inoculation.

The Arkansas strain of *Ehrlichia chaffeensis* was cultivated in the continuous canine macrophage cell line, DH82, grown in 75 cm² culture flasks supplemented with minimal essential media (MEM) with 10% fetal bovine serum (FBS). Cultures were harvested by detaching the cell monolayer when gross cytopathic effect (CPE) was evident. Collected cells were counted using a hemocytometer, and the percent infected in the *E. chaffeensis* inoculum determined using a direct fluorescent antibody (FA) test specific for *E. chaffeensis*. Cells were diluted in MEM with 10% FBS for a total of 5.4×10^6 *E. chaffeensis* infected or noninfected cells and used to inoculate deer. Noninfected DH82 control cells were grown and harvested in the same manner.

Eight, 3-mo-old, captive-reared white-tailed deer from Clarke County, Georgia (USA; 33°95.19 N, 83°36.60 W) were kept in a climate-controlled animal housing facility at the College of Veterinary Medicine, University of Georgia. Prior to inoculation, all deer were determined to be seronegative to *E. chaffeensis* and *Anaplasma phagocytophilum* (the agent of human granulocytic ehrlichiosis) by indirect fluorescent-antibody test (Dawson et al., 1991), and free of detectable *E. chaffeensis*, the HGE agent and the *Ehrlichia*-like agent of deer, by nested polymerase chain reaction (PCR) (Little et al., 1998). Six deer were inoculated with 5.4×10^6 *E. chaffeensis* (Ark) infected DH82 cells by one of three routes: intravenous (IV; n=2), subcutaneous (SQ; n=2), and intradermal (ID; n=2). Two deer served as negative controls and were inoculated intravenously with uninfected DH82 cells. Blood samples were collected for serology, nested PCR (EDTA tube), and complete blood count (CBC; EDTA tube) immediately prior to inoculation, and on days 8, 15, 22, 29, 36, 43, and 50 post-inoculation (DPI) for the first 54 days of the trial. One deer exposed to *E. chaffeensis* from each inoculation

group was maintained for an additional 31 days and euthanatized on DPI-85. Blood samples from these three deer were collected for nested PCR on DPI-61, 68, 78, and 85; the other three deer were removed for use in a separate study. Blood samples for culture isolation of *E. chaffeensis* were collected on all of the above days except DPI-22 and 36. All deer were inoculated and blood samples collected while under manual restraint. Deer were monitored for clinical signs of infection throughout the study period.

For serology, deer were pre-screened for the presence of *E. chaffeensis*-reactive and HGE agent-reactive antibodies at a serum dilution of 1:64 using *E. chaffeensis* and HGE antigen slides obtained from Focus Technologies (formerly MRL Diagnostics, Cypress, California). After inoculation, anti-*E. chaffeensis* antibodies were measured by an indirect fluorescent-antibody test as previously described (Dawson et al., 1991). Samples were screened at a serum dilution of 1:64 and positive samples were serially diluted 2-fold. For both pre-and post inoculation samples, a 1:50 dilution of fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G was used as the conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland).

DNA was extracted from 100 µl EDTA-anti-coagulated whole blood using a GFX Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey). Extracted DNA was used as the template to test for the presence of *Ehrlichia* spp. 16S rDNA in a nested polymerase chain reaction (Little et al., 1998). *Ehrlichia*-wide primers ECC and ECB were used with 10µl template DNA in a primary reaction, and 1µl of these products were amplified using *E. chaffeensis* specific primers HE1 and HE3 in a secondary reaction. For detection of the HGE agent and *Ehrlichia*-like deer agent in pre-screening samples, primers GA1UR and GE9F were used in the secondary

reaction. Amplified products were visualized on ethidium bromide stained 2% agarose gels via UV trans-illumination. To prevent contamination, DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate laboratories. A negative water control was included in DNA extraction and for each set of reactions in primary and secondary PCR.

For isolation of *E. chaffeensis*, 5-7 ml of aseptically collected EDTA-anti-coagulated whole blood was transferred into sterile 50-ml centrifuge tubes containing 30 ml of ACE lysing buffer (150 mM NH₄Cl, 0.7 mM KH₂PO₄, 3 mM EDTA- Na₂). Tubes were gently inverted to lyse red blood cells and then centrifuged at 2,500 rpm for 10 min to obtain a white-blood cell (WBC) pellet. Supernatant was discarded and the pellet was washed in 15 ml of fresh ACE lysing buffer and centrifuged again. The washed WBC pellet was re-suspended in 1ml of cell growth medium (MEM supplemented with 10% FBS) and overlaid on a confluent culture of DH82 cells in a 12.5-cm² flask with 5 ml medium. Cultures were given fresh medium twice weekly and monitored for evidence of CPE, or for a maximum of 45 days. Cultures showing CPE and cultures negative after 45 days were harvested with a cell scraper and tested by direct FA test as previously described (Lockhart et al., 1997b).

Ehrlichia chaffeensis was isolated from the blood of all deer at least twice during the initial 54 day study period and as late as day 85 in both SQ and IV inoculated deer maintained for the 31 additional days (Table 3.1). Positive cell cultures evident by gross CPE and negative cultures showing no CPE for 45 days were consistently confirmed using the direct FA test. Polymerase chain reaction evidence of circulating *E. chaffeensis* was found at least three times in all inoculated deer during the initial 54 day

trial but was only detected in the SQ inoculated deer beyond day 50 of the trial. There was no apparent difference in conversion to positive by PCR detection in blood or cell culture isolation of *E. chaffeensis*, between the ID, IV, or SQ groups of inoculated deer for the first 54 days of the infection.

All six deer exposed to *E. chaffeensis* by SQ, ID, or IV routes seroconverted (= 1:64) by DPI-15 and remained seropositive in weekly tests during the first 50 days of the study. Antibodies (1:128) were detected in one IV and one ID inoculated deer on DPI-8. Peak geometric mean antibody titer (GMT = 1,024) for IV and SQ routes occurred on DPI-29 and 36, respectively, and on DPI-36 and 43 for deer inoculated intradermally (Figure 3.1). Deer that were inoculated with uninfected DH82 cells were consistently seronegative, culture negative, and PCR negative. No clinical (such as decrease in appetite, lethargy, fever) or hematological abnormalities were observed in any deer during the trial. All deer were seronegative and PCR negative for all organisms tested for pre-screening.

In previous studies of *E. chaffeensis*, needle injection of organisms into experimental vertebrate hosts, such as white-tailed deer or dogs, has relied on the IV route (Dawson and Ewing, 1992; Dawson et al., 1994b; Kocan et al., 2000; Davidson et al., 2001; Felek et al., 2001). Natural transmission of *E. chaffeensis*, however, occurs through the bite of a lone star tick (*Amblyomma americanum*) (Anderson et al., 1993; Lockhart, et al., 1997b), a route that may be more closely simulated by intradermal or subcutaneous inoculation. However, our data show no difference in the time course of *E. chaffeensis* rickettsemia between the artificial routes used; IV, ID, or SQ inoculation

routes should all be appropriate for investigating the infection dynamics of *E. chaffeensis* in white-tailed deer.

In a previous study investigating the persistence of *E. chaffeensis* infection in intravenously inoculated deer, detection of *E. chaffeensis* organisms was intermittent over the 9-month study period, evident by inconsistent PCR results and culture isolation of organisms (Davidson et al., 2001). Other experimental studies also detected *E. chaffeensis* inconsistently in needle-inoculated deer over time (Dawson et al., 1994b; Ewing et al., 1995). Similar findings were observed in this study for deer, regardless of inoculation route. Although *E. chaffeensis* was regularly detectable by at least PCR assay through DPI22, evidence of circulating organisms became sporadic in some deer after that time. This was most noticeable in one intradermally inoculated deer that remained blood PCR negative on all sample days beyond DPI22, and in a single IV and single ID deer that were negative on at least three sampling dates before *E. chaffeensis* was again detected by PCR of blood. Culture isolation was similarly variable and was not consistent with PCR results. The intradermally inoculated deer that was sampled only for 50 days was also negative for one sample day, DPI36, despite showing evidence of *E. chaffeensis* on all other sample days.

Although infected deer in this study were monitored for only 54 or 85 days, findings presented in this study lend further support to existence of a recrudescent rickettsemia in deer (Davidson et al., 2001) and typical of ehrlichial infections in other vertebrate reservoirs (Rikihisa, 1991). The findings that antibodies were present (=1:64) after DPI15 in all deer exposed to *E. chaffeensis* throughout the 85-day study, and that antibody titers rose and fell one to two mo post-infection (Figure 3.1), are similar to the

serologic response previously described for deer experimentally infected with a high dose inoculum via the intravenous route (Davidson et al., 2001). Because this was observed in deer inoculated by SQ, IV, and ID routes, it is likely that this phenomenon occurs independent of the means by which these organisms reach the blood.

Discrepancies between PCR detection and cell culture isolation, such as those observed in this study, have been previously encountered (Ewing et al., 1995; Davidson et al., 2001). One reason for this may be inherent difficulty in culturing ehrlichiae (Walker and Dumler, 1996). Although DH82 cells, a canine macrophage cell line, are commonly used to isolate *E. chaffeensis* in both human cases of HME and infected deer (Dawson et al., 1991; Dawson et al., 1994b; Dumler et al., 1995; Ewing et al., 1995; Lockhart et al., 1997a; Lockhart et al., 1997b; Little et al., 1998; Davidson et al., 2001), variable success has been encountered when using DH82 cells for isolation from experimentally infected deer (Dawson et al., 1994b; Ewing et al., 1995). The possibility that PCR assay may have detected non-viable *E. chaffeensis* in blood samples must also be considered. In comparison to cell culture, PCR only tests 10ul of extracted blood, whereas 5-7 ml of blood are used to isolate *E. chaffeensis* in cell culture. Thus, the chances of including infected monocytes, the blood cell that *E. chaffeensis* invades, are greater in cell culture assays.

Results from this study show that IV, SQ, and ID routes of inoculation will cause infection in the principal vertebrate host of *E. chaffeensis*, white-tailed deer, suggesting that any of these routes is suitable for future studies investigating infection in deer. However, results of cell culture isolation and PCR detection should be interpreted with caution, considering the limitations of both assays. Comparison of the course of

infection in deer via tick transmission is essential to determine whether any of the artificial routes are a suitable model to understanding natural infection in white-tailed deer.

The authors would like to acknowledge numerous personnel at the Southeastern Cooperative Wildlife Disease Study as well as the Animal Care employees at the University of Georgia for indispensable assistance in this project. This work was funded by NIH Grant R15A147413-01.

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Table 3.1. Detection of *E. chaffeensis* by polymerase chain reaction and cell culture isolation^a for six white-tailed deer inoculated by three different routes.^b

Route ^c	WTD number	Day Post-Inoculation										
		8	15	22	29	36	43	50	61	68	78	85
SQ	119	+ (-)	+ (-)	+	- (+)	-	- (+)	+ (-)	- (-)	+ (+)	+ (+)	+ (+)
	118	+ (+)	+ (+)	+	+ (c) ^d	+	+ (+)	- (-)	ND	ND	ND	ND
ID	116	+ (+)	+ (-)	+	- (+)	-	- (+)	- (c)	- (-)	- (-)	- (-)	- (-)
	121	+ (+)	+ (-)	+	+ (c)	-	+ (+)	+ (-)	ND	ND	ND	ND
IV	122	+ (+)	+ (+)	+	+ (c)	-	- (-)	- (-)	- (-)	- (-)	- (-)	- (+)
	123	+ (+)	+ (+)	+	+ (+)	+	+ (-)	- (+)	ND	ND	ND	ND

^a Results of cell culture isolation are shown in parentheses, when attempted.

^b Two control deer (# 82 , 117) were negative on all tests through DPI 50 at which time they were removed from the study.

^c SQ = subcutaneously, ID = intradermally, IV = intravenously.

^d c = culture contaminated; ND= not done (deer were removed for separate study).

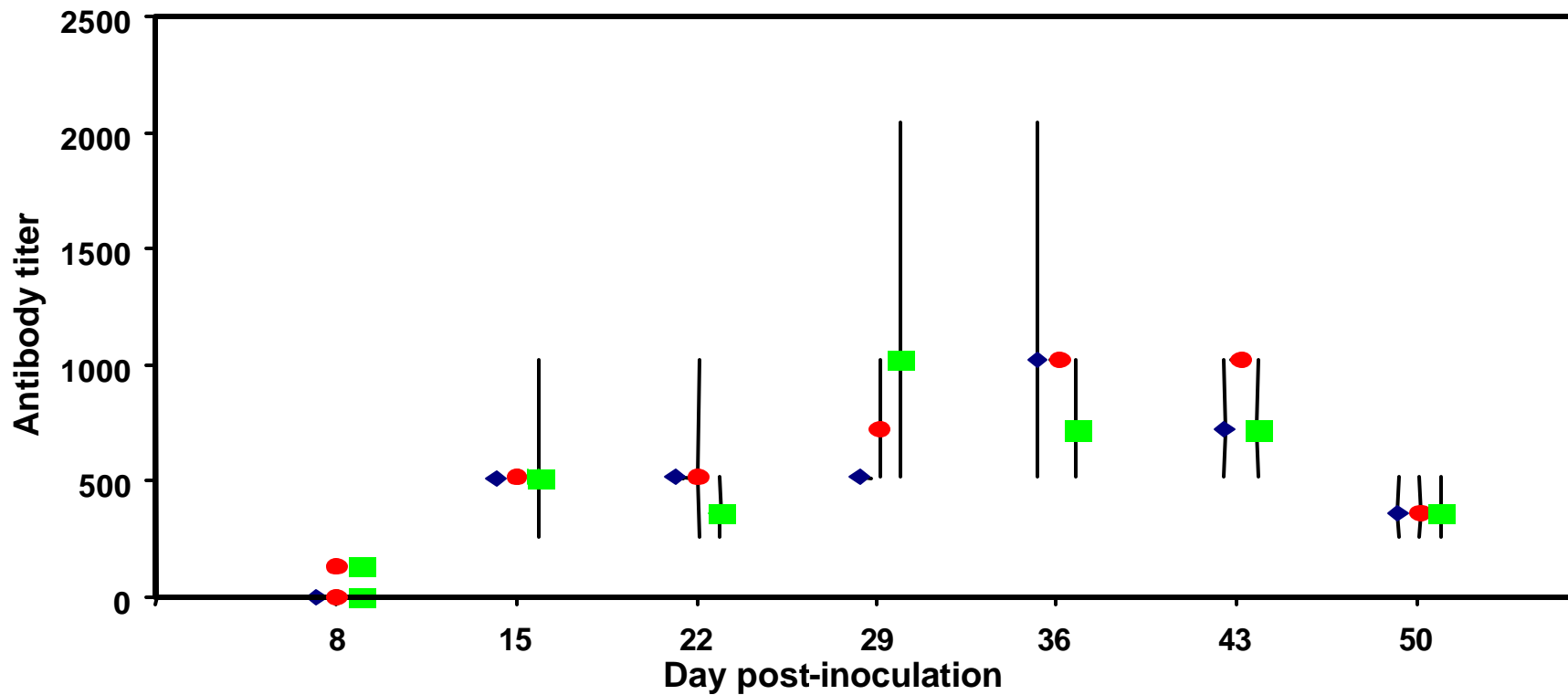


Figure 3.1. Indirect fluorescent antibody (IFA) titers for six white-tailed deer inoculated subcutaneously (n=2; ◆), intradermally (n=2; ●), and intravenously (n=2; ■). For DPI-15 through DPI-50, geometric mean titers (GMT) for both deer are shown as symbols for each inoculation group, except for the SQ group on DPI-15 when only one deer sample was available for titering; the other SQ deer was seropositive at 1:64 but a titer was not determined. Individual deer titers, if different from GMT, are shown as standard error bars. For DPI-8, GMTs were not calculated because all deer were negative except one ID and one IV inoculated deer; symbols at DPI-8 represent results for individual deer.

CHAPTER 4

PRIMARY AND SECONDARY INFECTION WITH *EHRlichia CHAFFEENSIS*
IN WHITE-TAILED DEER ¹

¹ Varela, A.S., Stallknecht, D.E., Yabsley, M.J., Moore, V.A., Howerth, E.W., Davidson, W.R., and Little, S.E. Submitted to *Vector Borne and Zoonotic Diseases* 5/21/2004 (see references under Dugan et al, submitted).

ABSTRACT

White-tailed deer (*Odocoileus virginianus*) are the principal reservoir host for *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis (HME). Because white-tailed deer maintain a long-term infection with *E. chaffeensis* and because deer can be naturally exposed to multiple strains of *E. chaffeensis*, we evaluated the response to secondary infection of *E. chaffeensis* in deer. For primary infection, six white-tailed deer were injected with 5.4×10^6 DH82 cells infected with the Arkansas strain of *E. chaffeensis* (Ark) and two control deer were injected with noninfected DH82 cells. On post-infection day 54, three *E. chaffeensis* (Ark) infected deer and one naïve deer were injected with 4.2×10^6 cells infected with strain WTD-6045B *E. chaffeensis*, which differs from the Arkansas strain by number of nucleotide repeats in the variable length PCR target (VLPT) gene; three other Arkansas strain infected deer were injected with noninfected DH82 cells. All animals were monitored for 31 additional days. All deer in the primary infection became positive by PCR amplification of the 16S rRNA or VLPT genes and/or cell culture by DPI-8. PCR amplification of the VLPT gene on whole blood, cell culture, and tissues detected primary and/or secondary strains in all deer exposed to both primary and secondary strains; in one deer, the primary strain was cultured from the lymph node. Our results suggest that the secondary strain was circulating in blood at higher levels, although both strains were present. In conclusion, this study provides evidence that primary infection of deer with *E. chaffeensis* does not protect against subsequent exposure and confirms that deer can be simultaneously coinfectd with at least two different strains of *E. chaffeensis*.

INTRODUCTION

Ehrlichia chaffeensis, causative agent of human monocytic ehrlichiosis (HME), cycles between the primary vertebrate reservoir host, white-tailed deer (*Odocoileus virginianus*), and the lone star tick (*Amblyomma americanum*) vector in nature (13). While nearly every state in the United States has reported HME, the majority of cases occur in the southeastern and south-central U.S. where lone star ticks and white-tailed deer are abundant. In both deer and humans, *E. chaffeensis* invades mononuclear cells where the bacteria replicate in morulae (2). Experimentally infected white-tailed deer demonstrate no evidence of clinical disease but do maintain infection for as long as nine months (4). In contrast, clinical signs in humans vary. People infected with *E. chaffeensis* may develop HME with signs ranging from a mild flu-like illness limited to fever, malaise, and myalgia to severe multi-systemic disease and even death (12, 13, 21). Chronic infection with *E. chaffeensis* has been reported only once in an elderly patient and is otherwise considered extremely rare (11). Differences among *E. chaffeensis* strains that could account for variable clinical presentations of HME have not been fully evaluated.

Numerous isolates of *E. chaffeensis* have been collected including sixteen from white-tailed deer, a domestic goat, and several lemurs (10, 14, 15, 25, 26, 27, 29). The widespread geographic range of *E. chaffeensis* and the variation in clinical presentation among human patients have contributed to increased interest in the potential strain variation in this organism. Antigen-expressing genes in *E. chaffeensis* that appear to vary between isolates include the outer membrane protein multigene family, the 120-kDa immunodominant surface protein gene, and the variable-length PCR target (VLPT)

(3, 20, 23). A recent investigation of the molecular heterogeneity of *E. chaffeensis* isolates demonstrated that white-tailed deer populations and individual deer may be coinfecting with more than one genetic variant (28). In contrast, in areas where the related bacterium *Anaplasma marginale*, is endemic in cattle, multiple genotypes occur in a herd but individual animals are infected with one genotype, a phenomenon attributed to infection exclusion. Some evidence of infection exclusion, where primary infection with one genotype excludes subsequent infection with another genotype of the same species, has also been proposed for strains of *A. phagocytophilum* (8, 9).

The ability of deer to become infected with multiple strains of *E. chaffeensis* is important in their role as a reservoir host. Individual deer infected with multiple strains of *E. chaffeensis* would provide a source of several different genetic variants for ticks to acquire when feeding, and thereby subsequently increase the diversity of organisms available for transmission to humans, potentially increasing the likelihood that people would be exposed to pathogenic strains. Results of serology and PCR for deer infected with the Arkansas strain by different routes of inoculation were reported previously (24). Here we describe the response of infected deer to inoculation with a second, distinct isolate. The purpose of this study was to determine, through experimental infection, whether white-tailed deer exposed to one strain of *E. chaffeensis* remain susceptible to subsequent infection with a second, distinct variant.

MATERIALS AND METHODS

***Ehrlichia chaffeensis* inocula.** For primary infection of white-tailed deer fawns, the Arkansas strain (Ark) of *Ehrlichia chaffeensis* was grown in 75 cm² culture flasks of

DH82 cells, a continuous canine macrophage cell line, in minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS). Infected cultures were harvested by detaching the cell monolayer when gross cytopathic effect (CPE) was evident. Noninfected DH82 control cells were grown and harvested at the same time. Collected cells were counted using a hemocytometer, and approximately 90% of cells were determined to be infected with *E. chaffeensis* (Ark) by a direct fluorescent antibody (FA) test. Cell inocula were diluted for a total of 5.4×10^6 *E. chaffeensis* (Ark) infected or noninfected DH82 cells and inoculated into deer within 6 hours of collection.

For secondary infection, a genetic variant of *E. chaffeensis* was chosen from wild white-tailed deer isolates based on PCR amplification of the VLPT gene. Because molecular characterization of the VLPT gene in *E. chaffeensis* (Ark) has revealed four imperfect repetitive sequence motifs, we chose an *E. chaffeensis* strain (WTD-6045B) with five repeats isolated from a wild deer in Greene County, Arkansas (27, 28).

Ehrlichia chaffeensis (WTD-6045B) was cultivated using the same procedure as used for the primary (Ark) strain above; harvested cells were collected when CPE was evident and at least 70% of cells were determined to be infected by direct FA.

Noninfected control cells were grown concurrently. Cell inocula were diluted to a total of 4.2×10^6 *E. chaffeensis* (WTD-6045B) infected or noninfected DH82 cells and inoculated into deer within 6 hours of collection.

Experimental design. Eight, 3-month-old, weaned, captive-reared white-tailed deer from Clarke County, Georgia were kept in a climate-controlled animal housing facility at the College of Veterinary Medicine, University of Georgia. Prior to injection of *E.*

chaffeensis, all deer were determined to be seronegative to *E. chaffeensis* and *Anaplasma phagocytophilum* by indirect fluorescent-antibody test (IFA) using commercial antigen slides (Focus Technologies, Cypress, CA) (5), and free of detectable *E. chaffeensis*, *A. phagocytophilum* and the *Anaplasma* sp. organism of deer by nested polymerase chain reaction (PCR) (14).

A summary of the study design is provided in Figure 4.1. For primary infection, six deer were injected with 5.4×10^6 *E. chaffeensis* (Ark) infected DH82 cells by one of three routes: intravenous (IV; n = 2), subcutaneous (SQ; n = 2), and intradermal (ID; n = 2). Two deer served as negative controls and were injected intravenously with noninfected DH82 cells. Blood samples were collected on 0, 8, 15, 22, 29, 36, 43, and 50 days post-inoculation (DPI) during the first 54 days of the trial for serology, nested PCR, complete blood count, and culture isolation (all days except DPI-22 and 36).

On DPI-54 of the primary infection, three deer that had been previously exposed to *E. chaffeensis* (Ark) (n=1 IV, 1 SQ, 1 ID) were injected with 4.2×10^6 DH82 cells infected with *E. chaffeensis*, WTD-6045B strain, via the same route as used for primary infection. One naïve deer previously used as a negative control in the primary infection was intravenously injected with the same *E. chaffeensis* (WTD-6045B) inoculum and served as a positive control. The remaining three *E. chaffeensis* (Ark) infected deer (n= 1 IV, 1 SQ, 1 ID) were injected with 4.2×10^6 noninfected DH82 cells via the same route as used for primary infection. Blood samples from these deer were collected for serology, nested PCR, and culture isolation on 3, 7, 10 (11 for culture), 14, 18, 24, and 31 days post-secondary infection (DPS). All deer were euthanized 31 days post-secondary infection and tissues (spleen, bone marrow, and mandibular, inguinal, and

prescapular lymph nodes) collected for PCR assay of the VLPT gene; inguinal lymph node was also collected and processed for culture isolation.

Serology. For both primary and secondary infections, antibodies reactive to *E. chaffeensis* were measured using an indirect fluorescent-antibody test as previously described (5). Briefly, antigen was prepared from DH82 cells infected with either *E. chaffeensis* Arkansas or WTD-6045B (secondary infection) strains. Infected DH82 cells were suspended in phosphate-buffered saline (PBS) and spotted on 12-well ethanol-cleaned slides. Slides were air-dried, stored at -70°C, and then thawed and fixed in acetone for 15 minutes prior to use. Deer serum samples were first screened at a dilution of 1:64 and positive samples were serially diluted two-fold until no fluorescence could be detected at the highest dilution tested. After incubation with diluted sera, slides were washed in PBS and distilled water (dH₂O) and incubated with a 1:50 dilution of fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G as the conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Unbound conjugate was washed with PBS and dH₂O, slides dried and then examined for fluorescence under UV illumination.

DNA extraction. DNA was extracted from 100µl EDTA-anti-coagulated whole blood or approximately 3 mm² sections of tissue using a GFX Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions. For *E. chaffeensis* isolates cultured in DH82 cells, 20µl of cell culture

material was extracted using Instagene matrix (Biorad, Hercules, CA) according to the manufacturer's directions.

PCR assays. Extracted DNA was used as the template for PCR assays to amplify two gene targets specific for *E. chaffeensis*: the 16S rDNA and a variable length PCR target (VLPT). For 16S rDNA, a 389-bp sequence was amplified by nested PCR using *Ehrlichia*-wide primers ECC and ECB with 10µl template DNA in a primary reaction, and *E. chaffeensis* specific primers HE1 and HE3 with 1µl of primary products in a secondary reaction (14). Amplified products were visualized on ethidium bromide stained 2% agarose gels via UV trans-illumination.

Amplification of the VLPT gene was performed in a nested PCR assay following a previously described protocol (24). External primers FB5A and FB3A were used with 2.5µl of DNA template from whole blood and tissues in a primary reaction and 1µl of product was amplified in a secondary PCR using internal primers FB5 and FB3. For assay of cultures, the VLPT gene was amplified in a single run PCR reaction using 2.5µl of template DNA with primers FB5 and FB3. Amplified products of either 4 tandem repeats (459 bp) corresponding to the Arkansas strain, or 5 tandem repeats (549 bp) corresponding to strain WTD-6045B were visualized on ethidium bromide stained 3% agarose gels via UV trans-illumination.

To prevent contamination, DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate laboratories. A negative water control was included in DNA extraction and for each set of reactions in primary and secondary PCR.

Isolation of organisms. *Ehrlichia chaffeensis* was re-isolated from deer as previously described (16). Cultures were monitored until evidence of CPE was seen, or for up to 45 days, and then harvested and tested by direct fluorescent antibody (FA) assay as previously described (16). Harvested cells from flasks with evident CPE and a positive FA were tested by PCR assay of the VLPT gene to determine the strain(s) present.

***In situ* hybridization and immunohistochemistry.** *In situ* hybridization was used to evaluate tissues for presence of *E. chaffeensis* as previously described (8). A digoxigenin-labeled riboprobe based on the 16S rRNA gene was constructed using a digoxigenin labeling kit and T7 RNA Polymerase (Roche, Indianapolis, IN) on nested PCR products generated using *Ehrlichia* genus-wide external primers Ec261F and Ec412R, and internal primers T3-270F and T7-398R on *E. chaffeensis* DNA (8). Probes were tested for reactivity to *E. chaffeensis* by Southern hybridization and for extent of labeling by dot-blot analysis. *In situ* hybridization was performed on formalin-fixed paraffin-embedded spleen, bone marrow, mandibular lymph nodes, prescapular lymph nodes, and inguinal lymph nodes from deer with both primary and secondary infection.

Immunohistochemistry was performed using hyperimmune serum from goats injected with *E. chaffeensis* infected DH82 cells to detect *E. chaffeensis* in formalin-fixed paraffin embedded spleens and mandibular lymph nodes from deer. Hyperimmune goat serum (1:1024 anti-*E. chaffeensis* antibody titer) and serum from a goat injected with noninfected DH82 cells was prepared for immunohistochemistry by adsorption with DH82 cells, followed by salt precipitation using sodium sulfate and then dialyzing

overnight at 4°C. Tissue sections were sequentially deparaffinized, rehydrated, and then steamed for 20 min with 0.01M saline sodium citrate (SSC) for antigen retrieval. Tissue sections were blocked with Dako protein block (Dako, Carpinteria, CA) followed by incubation for 2 hr at room temperature with primary anti-*E. chaffeensis* antibodies diluted 1:1600 in PBS, and subsequently washed and incubated with anti-goat biotinylated immunoglobulins at room temperature for 20 min (BioGenex, San Ramon, CA). Unbound immunoglobulins were washed and tissues incubated at room temperature with streptavidin-alkaline phosphatase (BioGenex, San Ramon, CA) for 20 min, washed, and reacted with naphthol/fast red substrate (Dako, Carpinteria, CA). Slides were counterstained with Mayer's hematoxylin. Positive and negative controls, consisting of paraffin-embedded *E. chaffeensis* infected and noninfected DH82 cells, were included with each set of tissues tested by both *in situ* hybridization and immunohistochemistry. For immunohistochemistry, a negative antibody control consisting of dialyzed, adsorbed serum from an uninfected goat was included for each tissue section.

RESULTS

Serology. As previously described (25), all deer seroconverted (=1:64) by DPI-8 of the primary infection, attained peak antibody titer (range 1:512 to 1:2048) between DPI-29 and 43, and remained seropositive on all time points tested during primary infection. Deer in the primary infection developed antibodies reactive to both the primary *E. chaffeensis* (Ark) strain and the secondary (WTD-6045B) strain on all days tested through DPI-85 (Fig. 2A). Deer injected with the secondary strain on DPI-54 still had

detectable antibodies to the primary strain throughout all sample days for the remainder of the trial, except for one seronegative deer on DPI-57; antibodies reactive to the secondary strain in injected deer rose slightly throughout the secondary infection trial but did not exceed 1:512 in any deer (Fig. 2B). The naïve deer that was injected only with *E. chaffeensis* WTD-6045B was seropositive by DPI-10 for antibodies (1:128) reactive to both the WTD-6045B strain and the Arkansas strain. Antibodies against the Arkansas strain in this deer remained at 1:128 on all days tested throughout the 31 days of infection, while antibodies against the injected (WTD-6045B) strain were 1:256, 1:64, and 1:128 for DPI-18, 24 and 31, respectively.

PCR assays. Deer in the primary infection were positive by 16S rDNA PCR on as many as six of eight days sampled (24) until initiation of the secondary infection (DPI-54) and on as many as three of seven days sampled thereafter. All deer injected with the secondary (WTD-6045B) strain were positive by 16S rDNA PCR on blood collected 14 and 18 DPS and on at least one other sample day (Table 4.1). VLPT analysis revealed only the Arkansas strain in deer from the primary infection trial (data not shown), whereas only the secondary strain, WTD-6045B, was detected by VLPT analysis of deer exposed to the secondary strain (Table 4.1). The control deer injected only with WTD-6045B was positive by 16S rDNA PCR on 7, 10, 14, 18, and 31 DPI; VLPT analysis revealed the presence of only this strain on all days tested including DPI-24.

PCR amplification of the VLPT gene in tissues detected the primary strain (Ark) most often in the lymph nodes of deer from the primary infection, whereas the primary

and/or secondary strains were detected by VLPT PCR assay in spleen, bone marrow, or lymph nodes of deer exposed to both strains (Table 4.2). All tissues tested from the naïve deer infected with the WTD-6045B strain were positive only for that strain.

Culture. The Arkansas strain of *E. chaffeensis*, but not WTD-6045B, was cultured from at least one deer inoculated with only the Arkansas strain on DPS-11, 14, 24, and 31, which corresponded to DPI- 65, 68, 78, and 85 (24; data not shown). All three deer inoculated with the secondary strain on DPI-54 (DPS-0) were culture positive for the primary and/or secondary strains on DPS-11, 14, 24, and 31 (Table 4.1). The deer inoculated only with strain WTD-6045B was culture positive for that strain on all days tested, except on blood taken 3 days post-inoculation, when the culture was lost to contamination.

In situ hybridization and immunohistochemistry. Intracytoplasmic structures were detected in cells of the mononuclear phagocyte system by in situ hybridization in the spleen of a deer infected with the Arkansas strain, and in the spleen and prescapular lymph node of a deer infected with both the Arkansas and WTD-6045B strains of *E. chaffeensis* (Fig. 4.3A). Immunohistochemical staining demonstrated similar intracytoplasmic structures that were positive for *E. chaffeensis* antigen in the mandibular lymph nodes of two deer, that were singly infected with either the Arkansas strain or WTD-6045B (Fig. 4.3B). All tissues that were positive for in situ hybridization signal or immunohistochemical staining were also positive by PCR amplification of the VLPT gene.

DISCUSSION

In this study, primary infection of white-tailed deer with one strain of *E. chaffeensis* (Ark) did not confer protection against a second infection with a genetic variant of *E. chaffeensis* (WTD-6045B) that differed in the number of tandem repeats present in the VLPT gene. Deer exposed to the secondary strain showed evidence of both strains in blood culture and tissues by VLPT PCR, although only the secondary strain was found by direct PCR of whole blood. Naïve deer that were exposed to only the primary strain had detectable levels of only the primary strain at varying points throughout the 85-day trial, indicating that the same genetic variant was maintained for nearly three months of infection and that organisms were present at the time of exposure to the secondary strain.

The results suggest that infection with *E. chaffeensis* does not confer immunologic cross-protection to distinct strains in white-tailed deer. Despite detectable levels of antibodies, deer remained susceptible to secondary infection. The additional detection of the primary strain in tissues and blood (as shown by cell culture), but not in direct PCR of blood from deer exposed to both strains, suggests that the primary strain was circulating below the detection level for routine blood PCR. The presence of the primary strain in tissues supports persistence of the organism during chronic infection (4).

These results suggest that the phenomenon of “infection exclusion”, which is recognized in cattle infected with *Anaplasma marginale*, does not appear to exist with *E. chaffeensis* in white-tailed deer (8, 9). In epidemics of *A. marginale* infection, multiple

strains may be present in a herd but individual animals are infected with only one strain (19). Conversely, in naturally infected white-tailed deer from areas where *E. chaffeensis* is endemic, multiple strains may be present both in the population and also in individual animals (29).

The inability of *in situ* hybridization or immunohistochemistry to detect more organisms in deer tissues may either reflect a low level of organism present in tissues at this late point of infection, a lower sensitivity of *in situ* hybridization and immunohistochemistry for detecting organisms in tissue sections, compared to PCR assays of tissues, or both. Detection of organisms in tissues using immunohistochemistry and *in situ* hybridization has been successful in human patients infected with *E. chaffeensis* (8) and in mice experimentally infected with the HF strain, an ehrlichia closely related to *E. chaffeensis* (17, 22). However, these previous studies were performed on fatal human cases, and within 9 days of infection in clinically ill mice, when the levels of circulating organisms would be expected to be high. In a previous experimental infection of white-tailed deer with *E. chaffeensis*, immunohistochemistry performed on deer at DPI-31 revealed structures resembling morulae in dendritic reticular cells and macrophage-type cells of lymph nodes (6). Similar to that earlier study (6), in the current study, the positive control deer that was injected with a comparable dose of the secondary strain only and also was euthanatized 31 days post-infection, had only one cell of the mononuclear-phagocyte system in the mandibular lymph node which contained a structure that stained positively with anti-*E. chaffeensis* antibody by immunohistochemistry. Subsequent *in situ* hybridization on this same tissue section slide did not reveal any positive staining.

In conclusion, this study demonstrates that white-tailed deer, the principal natural reservoir host of *E. chaffeensis*, are susceptible to secondary infection with a genetic variant that differs in the number of tandem repeats in the VLPT gene. Our results suggest that deer infected with multiple strains would increase the number of genetic variants available for acquisition by ticks feeding on individual deer in the population, and consequently increase the potential for transmission of pathogenic strains to humans. Future studies that investigate infection dynamics of *E. chaffeensis* in white-tailed deer should evaluate response to secondary exposure with the same genetic variant as well as multiple strains that differ by VLPT or other surface antigens, in order to more fully evaluate the potential for maintenance of various strains in nature.

ACKNOWLEDGMENTS

The authors gratefully thank Molly Murphy, Cynthia Tate, numerous personnel at the Southeastern Cooperative Wildlife Disease Study, and Animal Care employees at the University of Georgia for assistance in assays and handling deer. This work was funded by NIH Grant R15A147413-01.

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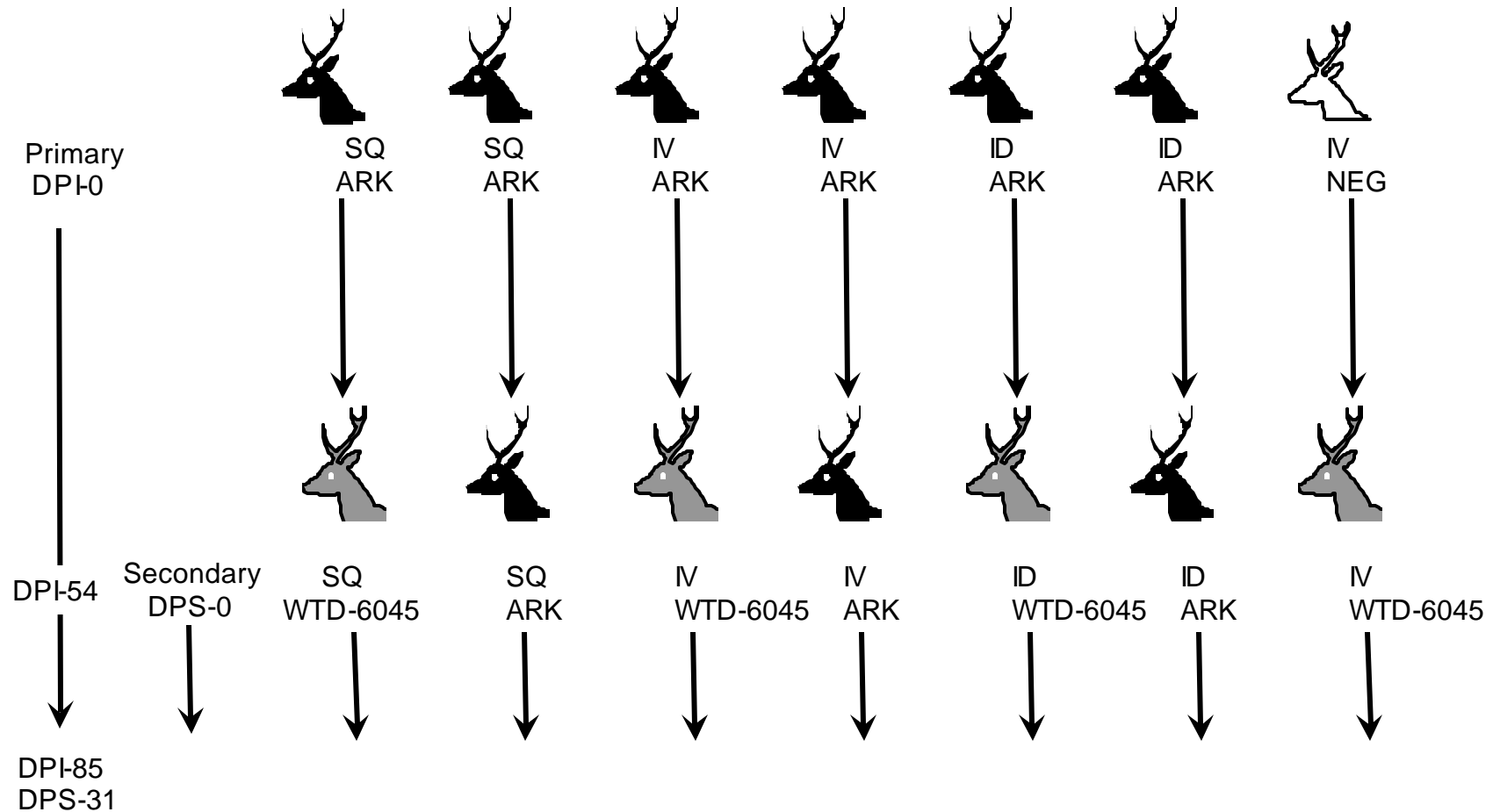


Figure 4.1. Experimental design for primary and secondary infection of WTD. In the primary infection, six deer were infected with the Arkansas strain of *E. chaffeensis* (□); two deer were uninfected controls (■). For the secondary infection, three Arkansas infected deer (▨) and one naïve deer (■) were injected with the WTD-6045B strain; the remaining Arkansas infected deer were injected with uninfected inoculum. All deer were maintained for a total of 85 days.

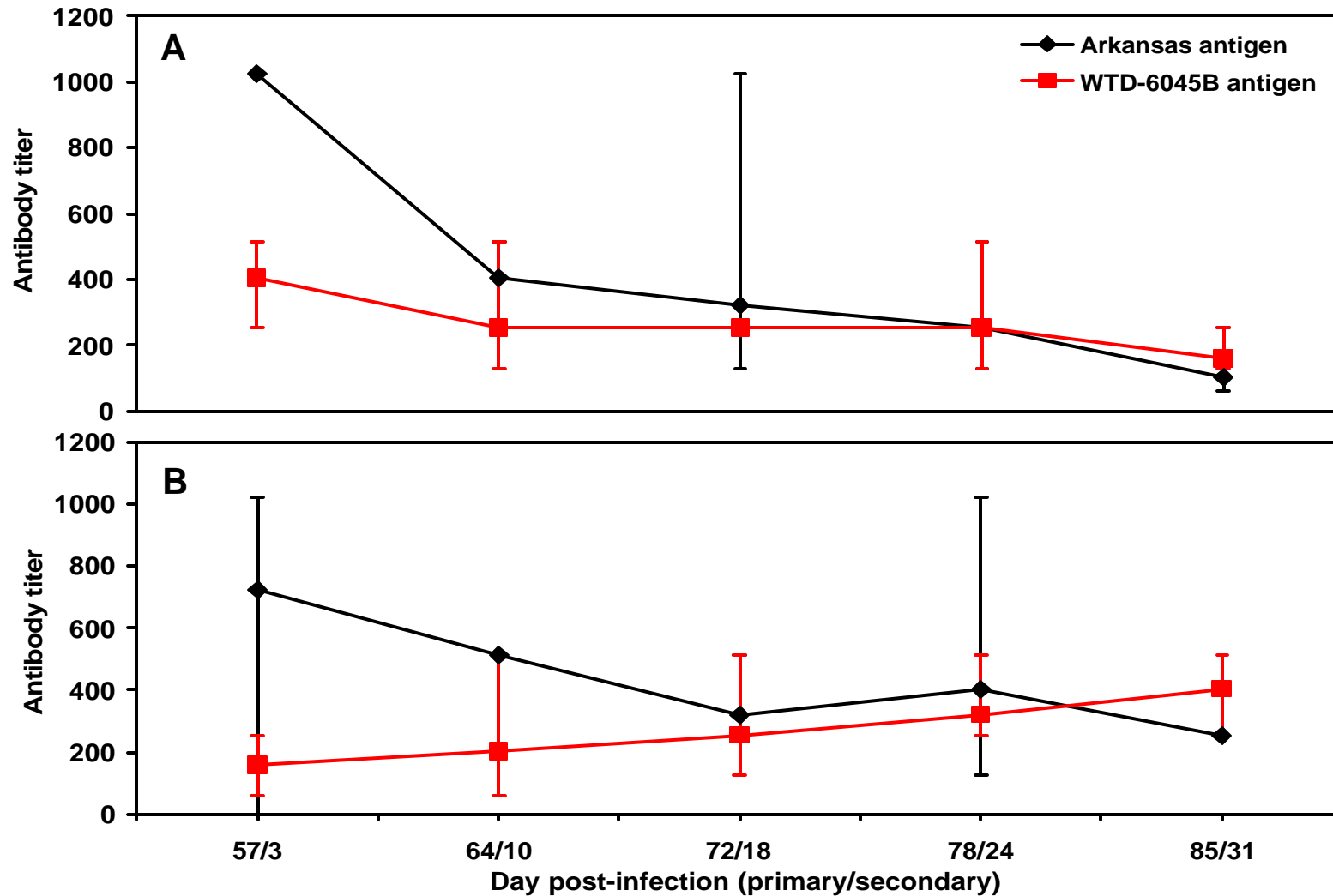


Figure 4.2. IFA determined for WTD injected with the primary (Arkansas; A) and secondary (WTD-6045B; B) strains of *E. chaffeensis* against primary (■) and secondary (◆) strain antigen, measured during the secondary trial. Points and error bars to minimum and maximum y titers represent geometric mean for the three WTD at each time point.

Table 4.1. Detection of *E. chaffeensis* by culture isolation and PCR assay of 16S rDNA and VLPT; identification of strains by number of VLPT repeats is shown in parentheses (Arkansas = 4 repeats, WTD-6045B = 5 repeats).

DAY POST-SECONDARY INFECTION (DPS) ^c								
	WTD ^d	3	7	10/11 ^a	14	18	24	31
PCR ^b	SQ	-	-	+ (5)	+ (5)	+ (5)	- (5)	-
	ID	-	-	-	+ (5)	+ (5)	+ (5)	+ (5)
	IV	- (5)	+ (5)	+ (5)	+ (5)	+ (5)	+ (5)	+ (5)
CULTURE	SQ	+	-	+ (5)	+ (5)	ND	+ (5)	+ (5)
	ID	-	-	+ (4)	+ (4)	ND	+ (4,5)	+ (5)
	IV	C	+ (4,5)	+ (5)	+ (5)	ND	+ (4)	+ (4,5)

^a PCR sample collected on DPS-10; Culture sample collected on DPS-11.

^b Nested 16S rDNA PCR results are indicated by +/-; VLPT results are indicated by the number of repeats (parentheses); in some cases, only one pcr assay was successful

^c *E. chaffeensis* WTD-6045B was detected by PCR and isolated in the primary exposure, positive control WTD on all sample days except DPS-3.

^d Route of inoculation for each deer in study.

C = contaminated culture; ND = not done.

Table 4.2. Detection of *E. chaffeensis* strains by PCR amplification of the VLPT gene in tissues of WTD following primary and secondary infection. Number of repeats corresponds to the Arkansas strain (4) or the WTD-6045B strain (5).

	SPLEEN	BONE MARROW	MANDIBULAR LN	PRESCAPULAR LN	INGUINAL LN
PRIMARY					
SQ (Ark)	4	-	4	-	-
ID (Ark)	-	-	4	4	-
IV (Ark)	-	-	4	-	-
IV (WTD-6045B) ^a	5	5	5	5	5
SECONDARY					
SQ (WTD-6045B)	5	5	4,5	4	-
ID (WTD-6045B)	5	5	-	4,5	5
IV (WTD-6045B)	5	-	5	5	5

^a SQ (Ark), ID (Ark), IV (Ark) were necropsied 85 DPI; all other deer were necropsied on 31 DPS (SQ, ID, IV WTD-6045B) and 31-DPI (IV WTD-045B).

CHAPTER 5

ATTEMPTED INFECTION OF GOATS WITH
*EHRlichia CHAFFEENSIS*¹

Varela, A.S, Dugan, V.G, Stallknecht, D.E., Hurd, C.C., and Little, S.E.. Study submitted to *Vector Borne and Zoonotic Diseases* (in reference Dugan et al 2004).

ABSTRACT

White-tailed deer (WTD), *Odocoileus virginianus*, are the principal reservoir host for the tick-borne bacterium, *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis. However WTD are difficult to maintain in captivity and experimental infections using WTD are labor-intensive. Thus, it would be beneficial to use an alternative animal model to study infection in vertebrate hosts. Because natural infection with *E. chaffeensis* has been reported in domestic goats and goats are commonly used as experimental animals, we evaluated whether domestic goats could be artificially infected with *E. chaffeensis*, and thus be a suitable animal model for further investigations of *E. chaffeensis* infections. Experimental infection of goats via three routes of inoculation failed to produce active infection in goats, although goats were did produce detectable anti-*E. chaffeensis* antibodies. These results suggest that goats may not be appropriate models for future investigations of *E. chaffeensis* using needle inoculation.

INTRODUCTION

A critical element in understanding the maintenance of *Ehrlichia chaffeensis*, the tick-borne agent of human monocytotropic ehrlichiosis (HME), in nature, is evaluating the infection dynamics of this bacterium in the reservoir host. The principal reservoir host and the primary vector for *E. chaffeensis* are white-tailed deer (WTD), *Odocoileus virginianus*, and lone star ticks (LST), *Amblyomma americanum*, respectively. In addition to WTD, *E. chaffeensis* has also been reported from other wild animals,

including raccoons, opossums, coyotes, and lemurs (Lockhart et al. 1997, Comer et al. 2000, Kocan et al. 2000).

A limited number of studies have utilized experimental infections of WTD to investigate acute and persistent infection with *E. chaffeensis* (Dawson et al. 1994, Ewing et al. 1995, Davidson et al. 2001). Although various mouse strains, dogs and foxes have also been experimentally infected with *E. chaffeensis*, few other animals have been evaluated for their suitability as models for reservoir host infection (Dawson and Ewing 1992, Telford and Dawson 1996, Davidson et al. 1999, Lockhart and Davidson 1999). Evidence of *E. chaffeensis* infection has been reported in a domestic goat herd by culture isolation and serologic response suggesting that goats may be susceptible to experimental infection with *E. chaffeensis*. (Dugan et al. 2000). Because studies using captive deer are challenging and labor-intensive, an alternative animal model is desirable. The purpose of this study was to evaluate the suitability of goats as an animal model for *E. chaffeensis* infection.

MATERIALS AND METHODS

Experimental animals

Ten domestic Nubian-cross goats (approximately 3-month-old, seven males, three females) and eight WTD (approximately 3-month-old, six males, two females,) were used. All animals were housed in climate controlled, indoor pens (1-4 animals per pen) in the Animal Resources Facility at the College of Veterinary Medicine, University of Georgia. Prior to beginning the study, all animals were found to be free of tick

infestation and negative for *E. chaffeensis* and *Anaplasma phagocytophilum* infection by both indirect fluorescent antibody (IFA) assay and nested PCR testing.

Experimental infection

The Arkansas (Ark) strain of *E. chaffeensis* was propagated in DH82 cells and flasks harvested as previously described (Dawson and Ewing 1992). Goats and WTD were inoculated by one of three routes: intravenous (IV; n = 2 goats, 2 WTD), subcutaneous (SQ; n = 2 goats, 2 WTD), and intradermal (ID; n = 2 goats, 2 WTD). Two additional goats were challenged weekly for three weeks with fresh *E. chaffeensis* (Ark) organisms via intradermal inoculation. Two goats and two WTD were inoculated with uninfected DH82 cells serving as negative control animals. Blood collection was performed as described in trial one on days 0, 3, 6, 8, 15, 22, 27, 29, 36, and 43, and assayed for evidence of *E. chaffeensis* infection by IFA, PCR, and cell culture techniques. At 43 DPI, goats were euthanized and complete necropsy examination performed; sections of spleen from goats were harvested and frozen for PCR testing.

Serology

Weekly serum samples were tested for the presence of antibodies reactive to *E. chaffeensis* by an indirect fluorescent antibody test (IFA) as previously described (Dawson et al. 1991). Briefly, *E. chaffeensis* (Ark) infected DH82 cells grown in culture were harvested, resuspended in culture medium, and placed onto each well of a 12-well Teflon coated slide. The slides were air-dried for 1 h and stored at -70°C. Before IFA testing, slides were thawed at room temperature and fixed in acetone for 15 minutes

each. Serum samples were initially screened at dilutions of 1:64 in 0.01M phosphate buffered saline (pH 7.4), with positive samples further tested in serial two-fold dilutions until maximum positive titer was established. Fluorescein isothiocyanate-labeled rabbit anti-goat and rabbit anti-deer immunoglobulin G antibody conjugates (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) were used for IFA testing of the goat and deer sera, respectively; each were diluted 1:50 in phosphate buffered saline.

Prescreening of goats and deer for antibodies to *A. phagocytophilum* was carried out as described above except commercial antigen slides (Focus Technologies, formerly MRL Diagnostics, Cypress, CA) were used as antigen in the IFA assay.

Nested PCR

Nested PCR tests were performed on weekly whole blood samples from goats and deer and on spleen from goats collected at necropsy. The GFX Genomic DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) was used to extract DNA from tissue samples or from 100 µl of EDTA-anti-coagulated blood for PCR testing, following the manufacturer's instructions. Each DNA pellet was resuspended in 50 µl of molecular biology grade water. Five µl of extracted DNA was tested by nested PCR for the presence of a 389 base pair sequence specific to *E. chaffeensis* 16S rDNA as previously described using outside primers ECC and ECB (Dawson et al. 1994) and inside primers HE1 and HE3 (Little et al. 1998). Products were separated by gel electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and UV transillumination. To insure accuracy, both negative and positive controls were

included in each step of the assay. To prevent contamination, DNA preparation, amplification, and analysis were performed in separate laboratories.

Culture isolation

Blood obtained on 0, 3, 8, 15, 29, and 43 DPI was cultured for *E. chaffeensis* as previously described (Dawson et al. 1994). Briefly, 5 to 7 ml of EDTA blood was transferred into a sterile centrifuge tube with 25 ml of ACE lysing solution (150 mM NH_4Cl , 0.7 mM KH_2PO_4 , 3 mM EDTA-Na_2), and the tube was gently inverted. After 5 min at room temperature, the suspensions were centrifuged at $160 \times g$ for 5 min, the supernatant was discarded, and the cells washed twice more in lysing solution. The leukocyte pellet was suspended in 1 ml of DH82 growth medium consisting of MEM supplemented with 5-10% fetal bovine serum and inoculated into 12.5 cm² tissue culture flasks (Becton Dickinson & Co., Franklin Lakes, New Jersey) containing DH82 cells with 5 ml DH82 growth medium were inoculated with the suspension. Cultures were maintained by replacing fresh medium twice weekly. Cultures were tested by a direct fluorescent antibody test as previously described (Dawson et al. 1991) if cytopathic effect (CPE) was observed, or at approximately 45 days if CPE was not observed. To confirm FA results, both positive and negative cultures were assayed by nested PCR. For these assays, DNA was extracted using Instagene™ Matrix (Biorad, Hercules, CA) using 20 µl of cell culture material according to the manufacturer's instructions and tested by nested 16S rDNA PCR assay for *E. chaffeensis*, as previously described above (Dawson et al. 1994; Little et al. 1998).

RESULTS

Serology

All eight goats exposed to *E. chaffeensis* seroconverted by 8 DPI with titers ranging from 1:128 to 1:512. Peak antibody response was detected on 15 DPI with both IV inoculated goats attaining titers of 1:1024 and 1:2048, and a 1:1024 titer detected in one ID inoculated goat and one goat given weekly ID injections. By 43 DPI all but three exposed goats had undetectable antibodies; two goats had titers of 1:64 while the goat in the SQ inoculation route held a titer of 1:128. Seroconversion of infected WTD was first detected in ID and IV inoculated animals on 8 DPI (1:128 titer). All WTD seroconverted (>1:128) by 15 DPI. There was no difference in antibody titers between deer in the three groups of inoculation routes (Varela et al. 2003). Peak geometric mean antibody titer for all deer during the 43-day trial occurred on 36 DPI (1:912). The negative control animals remained seronegative throughout the study.

PCR

All goats were PCR negative throughout the infection trial. Deer inoculated by all routes of infection were PCR positive on at least 2 and as many as 7 separate sample collection days of the 43-day trial (Varela et al. 2003). Both negative control goats and WTD also remained negative throughout the trial.

Isolation of organisms

Ehrlichia chaffeensis was not isolated from blood of any inoculated goats, but was

routinely isolated in culture attempts from WTD (Varela et al. 2003). All negative control animals were culture negative throughout both trials.

DISCUSSION

The results of this study demonstrated that experimental infection of goats and WTD with *E. chaffeensis* did not result in active infection in goats, although all positive control WTD became infected. Using young, 3-month-old goats, which were the same age as positive control deer failed to produce any detectable infection in goats. This suggests that goats are not susceptible to *E. chaffeensis* infection via needle inoculation by IV, SQ, or ID routes. The results were somewhat unexpected because of evidence of natural infection in goats from *E. chaffeensis* endemic areas (Dugan et al. 2000). Furthermore, evidence of active infection in WTD by *E. chaffeensis* (Ark), suggests that the absence of infection in goats was not a result of organism viability or lack of infectious dose.

It is unclear why goats failed to become infected. Goats in this study were bottle-raised in our tick-free facilities and experimentally inoculated at an early age. In addition, all animals used were seronegative for *E. chaffeensis* reactive antibodies and were PCR negative for *E. chaffeensis* DNA. Thus, pre-existing immunity due to previous exposure is unlikely and should not account for the lack of active infection in goats. In this study, all three routes attempted successfully infected WTD (Varela et al. 2003), and needle inoculation has been used successfully to infect young domestic dogs and red foxes with *E. chaffeensis* (Dawson and Ewing 1992; Davidson et al. 1999). However, the ability of *E. chaffeensis* organisms to establish a patent infection in

goats may depend on an immunologic response or other factors associated with tick feeding.

Serologic, molecular, and isolation results from the WTD mirror the course of infection documented in previous experimental infections studies and reaffirm that WTD are reservoir hosts which support persistent *E. chaffeensis* infections (Dawson et al. 1994; Ewing et al. 1995; Davidson et al. 2001; Varela et al. in press). All deer seroconverted by day 15 post infection and remained seropositive throughout each trial. Organism was detected in deer by PCR of whole blood from 7 to 56 DPI, and from lymph node, spleen, and bone marrow from one deer at necropsy. Furthermore, *E. chaffeensis* was routinely isolated in culture attempts from WTD in trial 2 (Varela et al. 2003). Previous isolation work with WTD detected *E. chaffeensis* approximately 25 - 37 days after inoculation of DH82 cells with blood or tissue homogenates (Lockhart et al. 1997). All culture results in both of the trials reported here were also detected in this time frame, with observable CPE and positive FA results between 12 to 29 days after inoculation. All FA positive cultures had observable CPE.

Future studies to evaluate domestic goats as an experimental model for *E. chaffeensis* infection should include experimental trials involving tick-transmitted exposure of naïve goats. However, the unsuccessful infection of goats through multiple routes of infection in this experimental trial indicates that domestic goats may not be susceptible to experimental infection by needle inoculation. Thus, despite clear evidence of natural infection (Dugan et al., 2000), goats do not appear to be a useful experimental model for *E. chaffeensis* reservoir host infection.

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CHAPTER 6

DISEASE AGENTS IN *AMBLYOMMA AMERICANUM*
FROM NORTHEASTERN GEORGIA

¹ Varela, A.S., Moore, V.A., and Little, S.E. 2004. *Journal of Medical Entomology*.

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ABSTRACT

Amblyomma americanum (lone star tick) is known or suspected to vector several organisms that are implicated as human pathogens, including *Ehrlichia chaffeensis*, *E. ewingii*, and *Borrelia lonestari*. These three agents have also been detected in white-tailed deer (*Odocoileus virginianus*). Because northeastern Georgia has a high abundance of both lone star ticks and white-tailed deer, and one of these organisms, *E. chaffeensis*, is already known to be endemic in the area, we assayed individual adult *A. americanum*, collected during the spring of 2001, 2002, and 2003, for these three organisms. A total of 400 ticks were dissected and tissues assayed by PCR using *Ehrlichia* species-specific and *Borrelia* genus-wide primers. Of ticks tested 2.0% (8/398) had evidence of *E. chaffeensis*, 4.8% (19/398) had evidence of *E. ewingii*, and 1.0% (4/398) had evidence of *B. lonestari*. *Borrelia* sp. spirochetes were also visualized by an indirect fluorescent antibody test, using an anti-flagellin monoclonal antibody (H9724), in a total of 10.7% (32/300) of ticks tested in 2003. These results reconfirm the presence of *E. chaffeensis* and establish evidence of *E. ewingii* and *B. lonestari* in questing adult *A. americanum* ticks from northeastern Georgia. Detection of at least two of the three organisms in ticks collected each year suggests that people in northeastern Georgia are at risk of infection with these organisms.

KEY WORDS

Amblyomma americanum, *Borrelia lonestari*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, Georgia

INTRODUCTION

Over the last decade, *Amblyomma americanum* (lone star tick) has become recognized as an important vector of several human disease agents (Childs and Paddock 2003). The expanding geographic distribution, aggressive feeding behavior, and broad host range of *A. americanum* establishes this tick as an effective avenue for the transmission and maintenance of a number of pathogenic bacteria of medical and veterinary significance. *Amblyomma americanum* is the primary vector of *Ehrlichia chaffeensis*, the agent of human monocytic (monocytotropic) ehrlichiosis (HME) (Ewing et al. 1995); is also known to vector *E. ewingii*, an agent of canine and human granulocytic (granulocytotropic) ehrlichioses (Anziani et al. 1990); and is a suspected vector of *Borrelia lonestari*, the putative agent of “southern tick-associated rash illness” (STARI) (Barbour et al. 1996, James et al. 2001). *Amblyomma americanum* is also a capable, but less critical vector of *Francisella tularensis*, the agent of tularemia (Hopla and Downs 1953, Taylor et al. 1991), and has been shown to harbor *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever (Parker et al. 1943; Goddard et al. 1986), and *Coxiella burnetii*, the agent of Q fever (Cox 1940, Parker et al. 1943).

Lone star ticks occur in large numbers throughout the southeastern and south-central United States, ranging from Texas northward to Iowa, and east along the Atlantic Coast to New England. Recent surveys recorded *A. americanum* as far north as New York and Maine (Ginsberg et al. 1991, Means and White 1997, Keirans and Lacombe 1998), which extends their northern border beyond what was historically believed to be New Jersey. White-tailed deer are considered to be the primary host for all stages of *A. americanum* (Bishopp and Trembley 1945, Patrick and Hair 1977, Koch

1988, Means and White, 1997). However, *A. americanum* is a relatively indiscriminate feeder uses a wide variety of hosts; all stages feed on both medium and large-sized mammals, immatures will also feed on birds but rarely small mammals (Means and White 1977, Kollars et al. 2000). Humans are common hosts to both immature and adult stages (Felz et al. 1996, Means and White 1997). In Georgia and South Carolina, *A. americanum* has been incriminated as the most common tick removed from humans, and has an apparently increasing abundance that may be attributed to growing regional deer populations (Felz et al. 1996, Merten and Durden, 2000).

Ehrlichia chaffeensis has been detected by polymerase chain reaction (PCR) from *A. americanum* in numerous states throughout the south-central, southeastern, and mid-Atlantic United States (Anderson et al. 1993, Lockhart et al. 1997b, Yu et al. 1997, Burket et al. 1998, Irving et al. 2000, Roland et al. 1998, Stromdahl et al. 2000, Stromdahl et al. 2001, Steiert and Gilfoy 2002, Whitlock et al. 2000). In addition, this organism has been found in white-tailed deer, the primary reservoir host, throughout this range (Lockhart et al. 1997b, Little et al. 1998, Little and Howerth 1999, Yabsley et al. 2003) and evidence of infection has been repeatedly identified in deer from northeastern Georgia (Lockhart et al. 1995, 1996, 1997a, 1997b, Little and Howerth 1999).

Ehrlichia ewingii has also been identified in *A. americanum* ticks from Missouri, North Carolina, and Oklahoma (Murphy et al. 1998, Wolf et al. 2000, Steiert and Gilfoy 2002), and in white-tailed deer, a suspected reservoir host, from Arkansas, Georgia, Kentucky, North Carolina, and South Carolina (Yabsley et al. 2002). In addition, natural infection has been reported in dogs (Anderson et al. 1992a, Dawson et al. 1996a,

Goldman et al. 1998, Murphy et al. 1998) and in humans (Buller et al. 1999, Paddock et al. 2001) in several states within the range of *A. americanum*.

Borrelia lonestari has been identified in *A. americanum* (Armstrong et al. 1996, Barbour et al. 1996, Burkot et al. 2001, Bacon et al. 2003, Stegall-Faulk et al. 2003, Stromdahl et al. 2003) and white-tailed deer (Moore et al. 2003). Confirmed and suspected human cases of STARI have been reported from several southeastern and south-central states, including Georgia (Campbell et al. 1995, Masters et al. 1995, 1998, Kirkland et al. 1997, Felz et al. 1999, James et al. 2001). Recently, *B. lonestari* was isolated directly from wild lone star ticks collected from Clarke County, in northeastern Georgia (Varela et al. 2004), providing substantial confirmation that *A. americanum* also harbors this pathogen.

Although the presence of *E. chaffeensis* has been established in ticks from northeastern Georgia, there has been no record of *E. ewingii* or *B. lonestari* in ticks from this area despite evidence of these organisms in deer (Lockhart et al., 1997b, Yabsley et al., 2002, Moore et al., 2003). The objective of this study was to confirm the presence and determine the prevalence of these three disease agents in *A. americanum* ticks from northeastern Georgia over a three-year sampling period and to evaluate the occurrence of co-infection with multiple pathogens in individual ticks.

MATERIALS AND METHODS

Tick collection. Adult *A. americanum* ticks were collected from Whitehall Experimental Forest, an 800-acre research and teaching site at the University of Georgia, consisting of mixed pine and hardwood forest in Clarke County, Georgia (USA; 33°57'N, 83°22'W).

In 2001 and 2002, additional tick collections were made on the Moore and Ambrose estates in Clarke and Oconee (USA; 33°50'N; 83°26'W) Counties, respectively. Ticks were collected from March through May, corresponding with the peak adult activity for *A. americanum* in Georgia (Davidson et al. 1994) using CO₂ (dry ice) traps (Gladney 1978, Koch and McNew 1982). Briefly, traps were constructed from approximately 0.3 m² (1 ft²) pieces of cardboard lined along the periphery with masking tape facing upward. Dry ice was placed in the center of the traps and allowed to sublime several hours. Ticks were removed from masking tape and *A. americanum* adults were maintained at 94% humidity until processed for assays.

Sample preparation. Individual adult *A. americanum* were sectioned through a coronal plane using sterile #11 scalpel blades under a dissection microscope. Tissue contents were scraped and removed using the scalpel blade and a plastic toothpick for each tick and were placed in 100µl 0.1% DEPC (diethyl pyrocarbonate). Samples were frozen at either -70°C or -20°C until extraction.

For ticks collected in 2003, 5-10µl of tissue contents from the 100µl 0.1% DEPC suspension was also smeared on two microscope slides pre-cleaned with 100% ethanol for indirect fluorescent antibody testing (IFA). Smears were allowed to air dry and then stored -70°C until assayed.

Polymerase Chain Reaction. DNA template for PCR assays was prepared using the Instagene Matrix (Biorad Laboratories, Hercules, CA) (2001 samples) and GFX genomic blood DNA purification kit (Amersham Biosciences Corp., Piscataway, NJ)

(2002 and 2003 samples) on 50µl of tick tissue suspension in 0.1% DEPC. To evaluate the samples for amplifiable DNA, samples were tested for the presence of tick mitochondrial 16S rDNA using primers 16S +2 and 16S -1 in a previously described protocol with the substitution of 1.25 units of Taqbead Hot Start Polymerase (Promega, Madison, WI) per 50µl for the manual hot start (Black and Piesman 1994). A nested PCR assay which included negative (water) and positive controls was used for detection of all three organisms. The 16S rRNA gene was targeted for detection of *Ehrlichia* spp., using 10µl of DNA template in a primary PCR reaction with external primers ECC and ECB, which target all *Ehrlichia* spp. (Anderson et al. 1992b, Dawson et al. 1994). For secondary PCR amplification, 1 µl of primary product was used in reactions with species-specific primers HE1 and HE3 (Dawson et al. 1994), and EE72 and HE3 (Dawson et al. 1996b) for the detection of *E. chaffeensis* and *E. ewingii*, respectively. An additional nested PCR assay that targets the variable length PCR target (VLPT), a gene specific to *E. chaffeensis* which encodes an immunoreactive protein, was also used to screen ticks for *E. chaffeensis* and to additionally differentiate isolates based on the number of tandem repeats as previously described (Sumner et al. 1999). Briefly, primers FB5 and FB3 were used in a primary PCR reaction with 2.5 µl DNA template and primers FB5A and FB3A were used with 1 µl of primary product in the secondary PCR reaction.

External primers FLALL and FLARL and internal primers FLALS and FLARS, which amplify a region of the *flaB* gene of all species in the genus *Borrelia*, were used in a nested PCR assay as previously described to detect *Borrelia* spp. (Moore et al. 2003). Primary PCR was performed using 10 µl of DNA template, and 1 µl of primary product

was used in the secondary PCR reaction. Amplified products from all PCR assays were separated by electrophoresis on 2% or 4% (VLPT gene target products only) agarose gels stained with ethidium bromide and visualized by UV transillumination. DNA extraction, primary and secondary PCR reactions, and product visualization were performed in separate, designated laboratory areas to minimize risk of PCR contamination. All *flaB* *Borrelia* spp. amplicons and a representative amplicon of *E. ewingii* were sequenced to establish and confirm identity, respectively. Products were purified and concentrated using a Microcon 100 microconcentrator (Amicon, Inc., Beverly, Mass.) and submitted for sequencing to MWG-BIOTECH (High Point, NC). Sequences were aligned using ClustalX multiple alignment program and compared to GenBank sequences for the *flaB* gene of *Borrelia* spp.

Indirect fluorescent Antibody Test. Ticks collected in 2003 also were screened for evidence of *Borrelia* spp. by an indirect fluorescent antibody test (Luckhart et al. 1991) using the *Borrelia* genus-wide anti-flagellin monoclonal antibody (MAb) H9724 (kindly provided by Tom Schwan, Rocky Mountain Laboratories, MT) (Barbour et al. 1986). Briefly, slides with tissue smears were fixed in acetone and incubated with undiluted H9724 monoclonal antibody for 25 min at 37°C, washed twice for 5 min in phosphate buffered saline (pH 7.2, PBS) and rinsed for 5 min in distilled water. After drying, slides were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse (1:30) for 25 min; the wash steps were repeated with a final rinse in 0.00066% erichrome black T counterstain for 10 min to minimize auto-fluorescence from tick tissues. A positive control slide consisting of *B. burgdorferi* (Guilford strain) live culture mixed with tick

tissue from laboratory-reared adult *A. americanum*, and a negative control slide consisting of tick tissues from laboratory-reared adult *A. americanum* were included in each assay of wild *A. americanum* ticks tested. Control slides were subjected to the same IFA procedures. Slides were examined on a Nikon Eclipse E-200 compound microscope under ultraviolet fluorescence.

RESULTS

Amblyomma americanum ticks from Clarke County had evidence of all three disease agents during the three year sampling period, 2001 to 2003 (Table 5.1). Amplifiable tick mitochondrial DNA was detected in all but two ticks assayed over the three year period. In 2001, 6.1% (3/49) and 4.1% (2/49) had DNA consistent with *E. chaffeensis* by amplification of the VLPT and 16S rDNA gene, respectively. In 2002, 4% (2/50) of ticks were positive for *E. chaffeensis* by amplification of VLPT, but all ticks were negative by 16S rDNA PCR. In 2003, 1.0% (3/299) and 0.67% (2/299) had evidence of *E. chaffeensis* by detection of the VLPT and 16S rRNA genes, respectively. *Ehrlichia chaffeensis* strains with 3 (n=1), 4 (n=6), and 5 (n=1) tandem repeats in the VLPT gene were detected in ticks (data not shown). *Ehrlichia ewingii* 16S rDNA was not detected in ticks collected in 2001, but was detected in 8.0% (4/50) of ticks from 2002 and 5.0% (15/299) of ticks from 2003 (Table 5.1). One tick sampled in 2002 was co-infected with both *E. ewingii* and *E. chaffeensis*; four tandem repeats were detected in the VLPT gene for *E. chaffeensis* in this co-infected tick.

PCR amplification and sequencing of the flagellin B gene detected *B. lonestari* in 4.1% (2/49) of ticks from 2001, 0% (0/50) in 2002, and 0.67% (2/299) of ticks sampled

in 2003 (Table 5.1). Sequences from the four positive ticks (Genbank Accession nos. AY552533-AY552536) were identical to published Genbank sequences for *B. lonestari* (Table 5.2). A 3-bp insert (5'-AGA-3') at positions 330-332 that has been reported in several *B. lonestari* sequences (Moore et al. 2003) was also present in three of the four amplicons. All four sequences contained an adenosine at base position 348, where an adenosine or a guanine base has been reported in published *B. lonestari* sequences from ticks (Barbour et al. 1996, Burkot et al. 2001, James et al. 2001).

Spirochetes that reacted to the H9724 *Borrelia* genus-wide MAb were detected by IFA in 10.7% (32/300) of ticks assayed in 2003, including both of the ticks with DNA evidence of *B. lonestari*. Ticks that were PCR negative but IFA positive had comparable fewer spirochetes, often seen as small clumps (Fig. 5.1A), than ticks that were PCR positive. In the two PCR positive ticks, large numbers of spirochetes were seen throughout the tissues (Fig. 5.1B).

DISCUSSION

DNA from at least one of three tick-borne bacterial pathogens was detected in 7.8% of adult *A. americanum* collected in northeastern Georgia from 2001 to 2003. The overall prevalence of *E. chaffeensis*, *E. ewingii*, and *B. lonestari*, for the three year study ranged between 1.0% (*B. lonestari*) and 4.8% (*E. ewingii*). The prevalence of *E. chaffeensis* was 1.0% and 2.0% for the two DNA targets assayed, a lower prevalence than the 7.6%-15% that has been reported in previous studies that tested individual ticks (Lockhart et al. 1997b, Ijdo et al., 2000, Whitlock et al. 2000, Stromdahl et al. 2001, Steiert and Gilfoy 2002). Of 50 adult *A. americanum* ticks collected from Whitehall

Experimental Forest from 1993 to 1995, 12% had DNA corresponding to *E. chaffeensis* (Lockhart et al. 1997b). The prevalence of *E. chaffeensis* by VLPT assay alone was 6.1%, 4.0%, and 1.0% in ticks collected in this study over successive years. However, the limited time period considered and our relatively small sample size, particularly for 2001 and 2002, preclude any interpretation of this change in prevalence. Continued annual monitoring using larger tick sample sizes and analyzing data in combination with environmental variables may identify trends in prevalence, if present.

Amplification of the VLPT gene in *A. americanum* demonstrated variants of *E. chaffeensis* with 3, 4, and 5 tandem repeats, all of which have been previously reported from *A. americanum* and white-tailed deer (Sumner et al. 1999, Stromdahl et al. 2000, Stromdahl et al. 2001, Yabsley et al. 2003). The 4-repeat variant was the strain most often detected in this study; prior to this work, the 4-repeat variant has been the only strain detected in this location (Yabsley et al. 2003). The 3-repeat strain has been detected from a human patient in Georgia, and all three strains have been detected from white-tailed deer in other areas of Georgia (Sumner et al. 1999, Yabsley et al. 2003). Thus, our findings establish the presence of multiple VLPT-based strains of *E. chaffeensis* in a single population of lone star ticks.

The prevalence of *E. ewingii*, although variable (0% - 8.0%), was comparable to those reported from previous studies of wild lone star ticks. In adult *A. americanum* ticks assayed from North Carolina, the prevalence was 0.65% in 1999, whereas in Missouri, where *E. ewingii* infection has been diagnosed in humans, the prevalence was 5.4% in 2000 (Buller et al. 1999, Wolf et al. 2000, Paddock et al. 2001, Steiert et al. 2002). Because *E. ewingii* has been detected in white-tailed deer in Georgia (Yabsley

et al. 2002), and *A. americanum* is a known vector capable of transmitting this organism (Anziani et al. 1990), its presence in lone star ticks in our study is not surprising. Interestingly, both *E. chaffeensis* and *E. ewingii* DNA was detected in one tick, suggesting that humans could be exposed to both pathogens through the bite of a single lone star tick.

We also found *B. lonestari* DNA in 1.0% of ticks over the three sampling years. Previous studies have reported a wide range of prevalence, including 11% (2/19) in adult *A. americanum* from Alabama (Burkot et al. 2001), a minimum infection rate of 5.6% in pooled nymphal and adult *A. americanum* from Southeast Missouri (Bacon et al. 2003), and a minimum infection rate of 0.84% in *A. americanum* from Tennessee (Stegall-Faulk et al. 2003). In addition, low estimated or actual prevalence of infection (1.2 - 4.3%) was recently reported in *A. americanum* removed from humans in several different states (Stromdahl et al. 2003). Despite our relatively low infection prevalence by PCR, *Borrelia* spirochetes were detected using an anti-flagellin monoclonal antibody in 10.7% of ticks assayed in 2003. Disagreement between detection methods for *B. burgdorferi* has been reported (Kahl et al. 1998, Gustafson et al. 1989), and a higher *B. burgdorferi* infection rate in ticks was previously observed using IFA in comparison to PCR assay (Kahl et al. 1998). Although the anti-flagellin MAb H9724 is *Borrelia*-wide and will not recognize *Treponema*, *Leptospira*, or *Spirochaeta*, other *Borrelia* spp. may also be detected using this antibody (Barbour et al., 1986). However, because PCR amplification of the *flaB* gene is not species-specific, other *Borrelia* spp., such as *B. burgdorferi*, which have been reported from *A. americanum* (Teltow et al. 1991, Luckhart et al. 1992, Feir et al. 1994, Oliver et al. 1998, Stromdahl et al. 2001) would

also be detected using this assay, suggesting that the IFA may be a more sensitive method. The recent isolation of *B. lonestari* from a pool of ten ticks collected from Whitehall Experimental Forest (Varela et al. 2004) demonstrates that viable organism is present in this tick population and suggests that the actual prevalence of infection may be higher than our calculated 1.0% by PCR. Regardless, the detection of DNA consistent with *B. lonestari* and the presence of *Borrelia* spp. in tick tissues of *A. americanum* substantiate the presence of organism in this area of Georgia.

Historically, *A. americanum* was considered a relatively insignificant vector of human disease. In recent years, this tick has become recognized as an important component of the natural history and zoonotic transmission of several newly-recognized pathogens. Understanding the potential of *A. americanum* to transmit disease agents is increasingly important because of the apparent expanding geographic range of lone star ticks and the significance of these pathogens for both immunocompetent and immunocompromised humans (Childs and Paddock 2003). This study highlights the importance of *A. americanum* as a vector of human disease agents, and underscores the need for continued efforts to understand the epidemiology of *Amblyomma*-vectored human pathogens in the Southeast.

ACKNOWLEDGMENTS

The authors would like to thank Dana Ambrose, Grace Chan, Amy Sexauer, Danny Mead, David Stallknecht, Michael Yabsley, and personnel at the Southeastern Cooperative Wildlife Disease Study for technical support and assistance in the

preparation of this manuscript. This work was supported in part by grant R15 AI47413-01 from the National Institutes of Health.

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Table 5.1. Prevalence of *A. americanum* in Clarke County, Georgia from 2001 through 2003, by nested PCR assay.

Year	<i>E. chaffeensis</i>		<i>E. ewingii</i>	<i>B. lonestari</i>
	16S	VLPT	16S	FLA
2001	2/49 (4.1%)	3/49 (6.1%)	0/49 (0%)	2/49 (4.0%)
2002	0/50 (0%)	2/50 (4.0%)	4/50 (8.0%)	0/50 (0%)
2003	2/299 (0.67%)	3/299 (1.0%)	15/299 (5.0%)	2/299 (0.67%)
Total	4/398 (1.0%)	8/398 (2.0%)	19/398 (4.8%)	4/398 (1.0%)

Table 5. 2. *B. lonestari flaB* sequences from ticks from Clarke County, GA with representative Genbank sequences.

Source	Reference	State	Genbank accession no.	NUCLEOTIDE DIFFERENCES ^a			
				330	331	332	348
LST culture isolate	Varela et al. 2004	GA	AY442142	*	*	*	A
LST	Barbour et al. 1996	TX	U26074	*	*	*	G
LST	Barbour et al. 1996	NJ	U26075	*	*	*	A
LST	Burkot et al.2001	AL#1	AF298653	*	*	*	A
LST	James et al.2001	NC/MD#2	AF273671	A	G	A	A
Human	James et al. 2001	NC.MD#1	AF273670	A	G	A	A
WTD	Moore et al. 2003	GA	AF538852	A	G	A	A
WTD	Moore et al. 2003	NC	AF538849	*	*	*	A
LST	Stromdahl et al. 2003	VA	AY237676	*	*	*	A
LST (F2)	This study	GA	This study	A	G	A	A
LST (F18)	This study	GA	This study	A	G	A	A
LST (73)	This study	GA	This study	*	*	*	A
LST (65)	This study	GA	This study	A	G	A	A

LST, lone star tick; WTD, white-tailed deer

^a Numbers correspond to nucleotide positions of *B. lonestari flaB* from U26705; asterisks denote absent base at position.

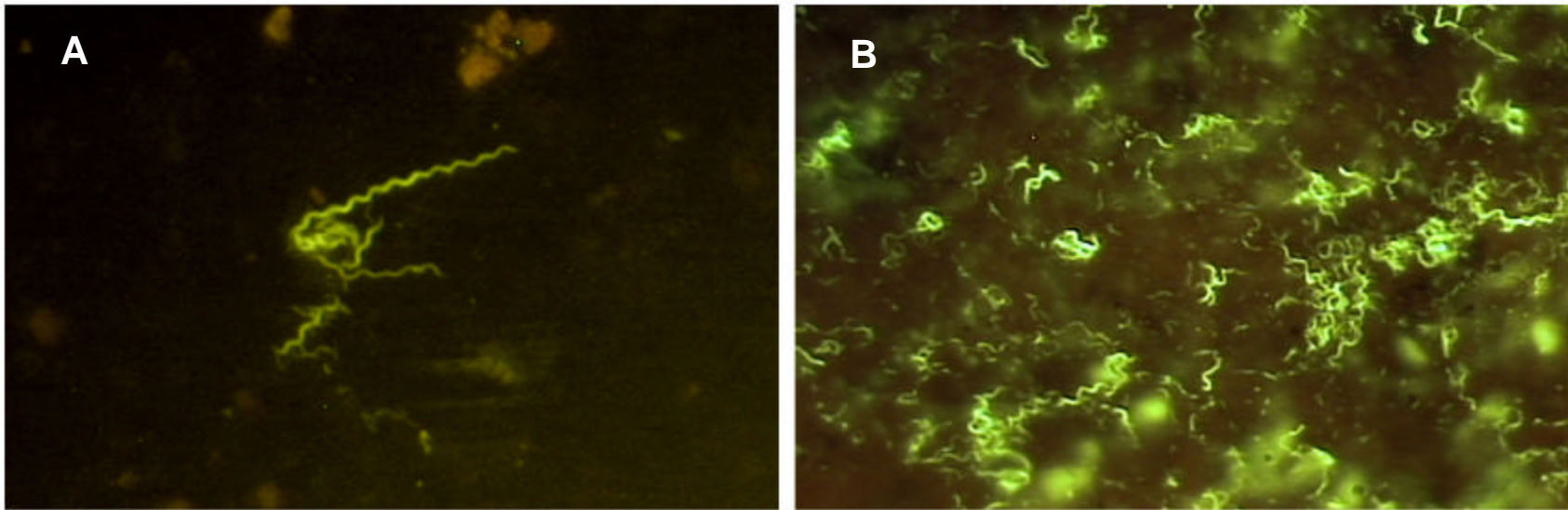


Figure 5.1. Detection of spirochetes by indirect fluorescent antibody staining using anti-flagellin MAb H9724 in *A. americanum* that were PCR positive (A) and negative (B) for *B. lonestari* DNA.

CHAPTER 7

FIRST CULTURE ISOLATION OF *BORRELIA LONESTARI*, PUTATIVE AGENT OF
SOUTHERN TICK-ASSOCIATED RASH ILLNESS¹

¹ Varela, A.S., Luttrell, M.P., Howerth, E.W., Moore, V.A., Davidson, W.R., Stallknecht, D.E., and Little, S.E. 2004. *Journal of Clinical Microbiology*. 42:1163-1169. Reprinted here with permission from publisher.

ABSTRACT

Southern tick-associated rash illness (STARI) is a Lyme disease-like illness described in patients in the southeastern and south-central United States, where classic Lyme disease is relatively rare. STARI develops following the bite of a lone star tick (*Amblyomma americanum*) and is thought to be caused by infection with an “uncultivable” spirochete tentatively named *Borrelia lonestari*. In this study, wild lone star ticks collected from an area where *B. lonestari* is endemic were cocultured in an established embryonic tick cell line (ISE6). The cultures were examined by dark-field microscopy for evidence of infection, and spirochete identity and morphology were evaluated by flagellin B and 16S rRNA gene sequence, by reaction to *Borrelia*-wide and *B. burgdorferi*-specific monoclonal antibodies, and by electron microscopy. Live spirochetes were first visualized in primary culture of *A. americanum* ticks by dark-field microscopy 14 days after the cell culture was inoculated. The sequences of the flagellin B and 16S rRNA genes of cultured spirochetes were consistent with previously reported sequences of *B. lonestari*. The cultured spirochetes reacted with a *Borrelia*-wide flagellin antibody, but did not react with an OspA antibody specific to *B. burgdorferi*, by indirect fluorescent antibody testing. Electron microscopy demonstrated organisms that were free and associated with ISE6 cells, with characteristic *Borrelia* sp. morphology. This study describes the first successful isolation of *B. lonestari* in culture, providing a much needed source of organisms for the development of diagnostic assays and forming a basis for future studies investigating the role of the organism as a human disease agent.

INTRODUCTION

Lyme disease, caused by *Borrelia burgdorferi* sensu lato, is the most common tick-borne disease of humans worldwide and the most frequently reported vector-borne disease in the United States. The hallmark of acute Lyme disease is erythema migrans, which is present in 60 to 90% of patients (13, 45), although several other nonspecific multisystemic symptoms also occur (9). *B. burgdorferi* is maintained in nature through a cycle involving rodent reservoir hosts and *Ixodes* sp. tick vectors (1, 28, 46). The disease is endemic throughout much of the Northeast, mid-Atlantic states, Midwest, and West Coast; however, in much of the South, where *Ixodes* sp. ticks are seldom found on humans (17, 18), epidemiologic evidence and case reporting suggest that classic Lyme disease is relatively rare, despite the presence of *B. burgdorferi* in wild rodent populations and ticks (16, 36, 37, 38, 40).

Since the mid-1980s, physicians have described a Lyme disease-like illness in patients from the southeastern and southcentral United States in which an erythema migrans rash and mild flu-like symptoms develop following the bite of a lone star tick, *Amblyomma americanum* (2, 3, 12, 19, 31, 43). This disease is alternatively referred to as southern tick-associated rash illness (STARI), Master's disease, or southern Lyme disease (23, 30). *A. americanum* has been shown to be an incompetent vector for *B. burgdorferi*, and serologic testing of STARI patients does not support a diagnosis of classic Lyme disease despite microscopic evidence of spirochetes in biopsy samples of affected skin, leading researchers to speculate that another *Borrelia* sp. may be responsible (16, 24, 29, 31, 33, 35, 39, 42, 48, 49). However, all attempts to culture spirochetes from patients with STARI and from ticks have failed.

Molecular evidence of a novel *Borrelia* sp. has been reported from lone star ticks, from white-tailed deer, and from the skin of a patient with STARI, as well as from a lone star tick removed from that patient (2, 10, 11, 23, 32, 47). The organism, tentatively named *Borrelia lonestari*, has been described only by PCR amplification of the flagellin B gene and 16S ribosomal DNA (rDNA). Complete understanding of *B. lonestari* and its role in STARI has been hampered by the inability to culture the etiologic agent. Here, we report the first culture isolation of *B. lonestari* and provide a microscopic and ultrastructural description of the organism in cell culture.

MATERIALS AND METHODS

Tick specimens. Adult *A. americanum* ticks were collected, using dry ice (CO₂) traps (25), from Whitehall Experimental Forest, an 800-acre forest owned by the University of Georgia in Clarke County, Ga., during March and April 2003. This population of ticks had been previously confirmed to harbor *B. lonestari* (A. S. Varela, V. A. Moore, and S. E. Little, unpublished data). The ticks were maintained at 94% humidity in chambers containing saturated potassium nitrate for ~2 months before they were cultured.

Culture isolation. Procedures for the coculture of *B. burgdorferi* in a tick cell line modified from previous studies were used to isolate *B. lonestari* from wild-caught ticks (26, 27, 34). Ten adult *A. americanum* ticks (five female and five male) collected from Whitehall Experimental Forest on 12 March 2003 were washed under sterile conditions by vortexing them for 3 min in successive solutions of 3% hydrogen peroxide, 95% alcohol, 0.1% sodium hypochlorite, and 1X phosphate-buffered saline (PBS; pH

7.2). After being washed, the ticks were placed in a sterile petri dish and individually dissected using fine forceps and a no. 11 scalpel blade. The instruments were sterilized between individual tick dissections with a bead sterilizer, and the scalpel blades were changed between ticks. Tissues from each dissected tick were pooled in a sterile 1.5-ml tube with 1 ml of complete Barbour-Stoenner-Kelly II (BSKII) medium prepared by the College of Veterinary Medicine, University of Georgia, as previously described (6, 7) using bovine serum albumin fraction V (catalog number 81003; ICN Bio medicals, Costa Mesa, Calif.). The medium was supplemented with 6% rabbit serum (catalog number 16120099; Invitrogen, Carlsbad, Calif.), phosphomycin (0.02 mg/ml), rifampin (0.05 mg/ml), amphotericin (2.5 µg/ml), and 1.4% gelatin, hereafter referred to as BSKII/G. The contents of the tube were mixed well by vortexing, transferred to a sterile 4.5-ml snap cap tube with µ3 ml of BSKII/G medium, and maintained at 34°C overnight to test for contamination.

When no evidence of contamination was observed after 24 h, the snap cap tube was centrifuged at 75 x g for 5 min to pellet the contents. After 4 ml of supernatant was removed, the pellet was resuspended in the remaining 0.5 ml of BSKII/G and added to 4.5 ml of L-15B300 medium consisting of L-15B (catalog number 41300; Invitrogen) supplemented with 5% fetal bovine serum (catalog number F0643; Sigma, St. Louis, Mo.), 10% tryptose phosphate broth (catalog number T9157; Sigma), and 0.1% bovine lipoprotein concentrate (catalog number 191475; ICN Biomedicals) (34). This 5-ml suspension was transferred to a 12.5-cm² flask with a confluent culture of ISE6 cells, an embryonic *Ixodes scapularis* tick cell line (kindly provided by Uli Munderloh, University of Minnesota). The culture was fed every 3 to 4 days, and the spent medium was

transferred to a separate sterile tube and centrifuged at $210 \times g$ for 5 min to pellet spirochetes and cells. After all but 0.5 to 1 ml of medium was removed, the pellet was resuspended and saved at -20°C for PCR assay and/or used to prepare slides for dark-field microscopy, for indirect fluorescent antibody (IFA) testing, or for subsequent passages. Subsequent passages of the original isolate were maintained in L-15B300 with 10% BSK-H (catalog number B3528; Sigma); the BSK-H medium was supplemented with 6% rabbit serum and antibiotics as described for the BSKII medium above.

Upon detection of live spirochetes under dark-field microscopy and confirmation of their identity by PCR, samples of the primary culture of the isolate, designated strain LS-1, were passaged into additional flasks of ISE6 cells to maintain the culture and into cell-free culture tubes with BSK/G or L-15B300 with or without supplemental BSK/G; culture aliquots were also frozen in liquid nitrogen for future use. As a positive control for IFA tests, *B. burgdorferi* SH2-82 was cultured in cell-free BSK/G medium and passaged to ISE6 cells with L-15B300 and 10% BSK/G. All cocultures in ISE6 cells were fed twice weekly, and the spent media were assessed by dark-field microscopy for the presence of spirochetes. Samples of cultured tick spirochetes in cell-free tubes were monitored for color change as evidence of growth and harvested after 30 days in culture to check for live spirochetes.

Molecular analysis. DNA was extracted from 100 μl of tick cell culture 3, 14, and 17 days after inoculation into ISE6 cell monolayers, using the GFX Genomic Blood Purification kit (Amersham Pharmacia Biotech, Piscataway, N.J.). The sample collected

on day 3, which contained remnants of the tick tissue inoculum, was tested to determine whether the original inoculum contained *Borrelia* spp. Two DNA targets were amplified and sequenced to identify the organisms: a 330-bp region of the flagellin B gene and a 1,336-bp region of the 16S rRNA gene. The external primers FLALL and FLARL and the internal primers FLALS and FLARS were used in a nested-PCR assay to amplify the *flaB* target as previously described (32). Due to failure to amplify the 16S rDNA target using a previously described protocol (10), we used a heminested-PCR assay to increase amplification of the template. The primary reaction mixture consisted of 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 0.5 μ M primers 8F (5'-AGTTTGATCATGGCTCAG-3') and 16RnaR, 200 μ M each deoxynucleoside triphosphate, 50 mM KCl, 2.5 mM MgCl₂, and 10 mM Tris-HCl (pH 9.0), with 5 μ l of DNA template in a final reaction volume of 100 μ l (10). The same reagent concentrations and 2 μ l of primary product were combined in a secondary reaction using primers 16RnaL and 16RnaR. Thermal cycling for amplification of the 16S rDNA followed the program used by Barbour et al. (10). Water controls were included in each step of each reaction, and DNA extraction, primary and secondary PCR, and gel visualization were performed in separate laboratories to reduce the risk of contamination. Amplicons were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide under UV transillumination.

Amplicons were purified and concentrated using a Microcon 100 microconcentrator (Amicon, Inc., Beverly, Mass.) and submitted to MWG Biotech (High Point, N.C.) for direct sequencing of both forward and reverse strands. The resultant sequences were aligned using the ClustalX multiple-alignment program and directly

compared to published 16S rDNA and *flaB* sequences of *B. lonestari* (= *Borrelia barbouri*) and other *Borrelia* spp. available in GenBank. To further compare the 16S rDNA sequence from our isolate with those of other *Borrelia* spp., phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis program version 2.1. For phylogenetic analysis, sequence alignment was performed using the ClustalX multiple-alignment program on the 16S rDNA fragment sequenced from the LS-1 isolate, corresponding to positions 190 to 1076 of the *B. lonestari* 16S rDNA sequence U23211. The lengths of analyzed 16S rDNA sequences were limited by the amount of sequence data available for *Borrelia theileri* and thus did not include the entire available LS-1 sequence. Accession numbers for all *Borrelia* sp. sequences used in the construction of the phylogenetic tree are provided in Fig. 6.1.

IFA testing. Samples of ISE6 cell cultures infected with LS-1 and separate ISE6 cell cultures infected with *B. burgdorferi* SH2-82 were fixed on microscope slides with acetone and tested by IFA assay for reactivity with a *Borrelia*-wide monoclonal antibody (MAb) against flagellin (H9724) and a *B. burgdorferi*-specific MAb which recognizes OspA (H5332) (both MAbs were kindly provided by Tom G. Schwan, Rocky Mountain Laboratories, Hamilton, Mont.) (5, 8). Slides were incubated with undiluted primary antibody for 25 min and then washed twice in 1X PBS for 5 min each, rinsed in distilled water for 5 min, and allowed to dry. A 1:30 dilution of fluorescein isothiocyanate-labeled goat antimouse secondary antibody was applied, and the slides were incubated for 25 min and subsequently washed in PBS as described above and then counterstained with

eriochrome black T. After drying in the dark, the slides were examined with a compound microscope under UV illumination.

Light and electron microscopy. For measuring the lengths of spirochetes, samples prepared by cytocentrifugation from culture passages were fixed in methanol, stained with Giemsa stain, and examined by light microscopy. For scanning and transmission electron microscopy (SEM and TEM), a sample of spirochetes was prepared from the primary isolate, LS-1, cocultured in ISE6 cells. Approximately 5 ml of medium from the original flask, containing nonadherent ISE6 cells and spirochetes, was initially centrifuged at $83 \times g$ for 1 min. Two milliliters of supernatant was removed for subsequent passage of cell-free spirochetes. The remaining 3 ml was resuspended; 0.5 ml was retained for dark-field examination, PCR, and further passage, and 2.5 ml was centrifuged at $210 \times g$ for 5 min to pellet spirochetes and ISE6 cells. The supernatant was removed, and the pellet was fixed in 2% glutaraldehyde– 2% (para)formaldehyde – 0.2% picric acid in 0.1 M cacodylate-HCl buffer (pH 7.0 to 7.3). The fixed cell suspension was divided for TEM and SEM preparation. For TEM, the portion was centrifuged at $9,550 \times g$, the fixative was removed, and the pellet was resuspended in buffer. This was repeated once, and then the sample was centrifuged a third time, the buffer was removed, and the pellet was resuspended in 50°C molten agar. The agar suspension was centrifuged at $9,550 \times g$ for 5 min and allowed to solidify at 4°C. When solid, the pellet was cut into sections no greater than 1 mm thick, placed in buffer solution, and subsequently postfixed in 1% OsO₄– 0.1 M cacodylate-HCl buffer. The sections were rinsed four times in deionized water for 10 min each time, stained en bloc

with 0.5% uranyl acetate (aqueous) for 1 h, and rinsed with deionized water three times for 2 min each time. After being dehydrated with increasing concentrations of alcohol, the sections were infiltrated with Epon-Araldite (EMS, Hatfield, Penn.) and then polymerized at 75°C prior to examination with a JEOL JSM-1210 transmission electron microscope.

The fixed portion retained for SEM was rinsed three times in 0.1 M buffer for 15 min each time and allowed to adhere to a coverslip as follows. Briefly, a drop of poly-L-lysine (in PBS) was placed onto a 12-mm-diameter round coverslip and allowed to set at room temperature for 1 h and was then rinsed in running water. A drop of washed cell suspension was placed on the coated coverslip, and the coverslip was stored in a humidity chamber to allow the cells to settle. The coverslip was critical-point dried, mounted on an aluminum stub with Electrodag 502 (Ted Pella, Inc., Redding, Calif.) to dry, and overlaid with sputter coat prior to examination with a JEOL JSM-5800 scanning electron microscope. A separate sample for negative staining was taken from a second passage of LS-1 spirochetes in ISE6 cell culture. Medium with nonadherent ISE6 cells and spirochetes was centrifuged at 302 x *g* for 10 min, and the pellet was resuspended in sterile filtered PBS and centrifuged at 302 x *g* for 10 min. The washed pellet was resuspended in Karnovsky's type fixative, fixed at 4°C for 1 h, and centrifuged at 302 x *g* for 10 min. The pellet was washed in sterile filtered PBS, centrifuged at 302 x *g* for 10 min, and resuspended once more with a small (~50- μ l) volume of PBS. A 40- μ l volume of suspension in a drop of deionized water was airfuged (pounds per square inch gauge 10) on a copper grid at 43,110 x *g* for 5 min and stained with 2% phosphotungstic acid (pH 7.0) for 30 s for TEM examination.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *B. lonestari* flagellin gene and the 16S rRNA gene reported in this paper are AY442141, and AY442142, respectively.

RESULTS

Culture isolation. Live spirochetes from the primary culture were first visualized both free in the medium and attached to ISE6 cells under dark-field microscopy 14 days after the flask was inoculated and then twice weekly thereafter from spent cell medium that was removed when the flask was fed. A thawed aliquot that had been frozen for 3 days in liquid nitrogen was proven viable after passage into a confluent monolayer of ISE6 cells. Spirochetes passed into culture tubes with cellfree medium were not viable after 30 days in culture. **Molecular characterization.** *flaB* sequences of cultured spirochetes collected 3, 14, and 17 days postinoculation were identical to each other and to previously published *flaB* sequences of *B. lonestari* (Table 6.1), including the original *flab* sequence of *B. lonestari* described from *A. americanum* collected in New Jersey (10) and *flaB* sequences subsequently described from ticks from Alabama and Missouri and deer from Georgia and North Carolina (4, 11, 32). The 3-bp insert at nucleotides 330 to 332 that has been described from several other *flaB* sequences of *B. lonestari* (23, 32, 47) was not present in our isolate. The 16S rDNA sequence from our isolate also was consistent with sequences previously reported from *B. lonestari* (= *B. barbouri*) (Table 6.2) and differed by 1 base from the 16S rDNA sequence of *B.*

lonestari reported from *A. americanum* from Missouri and by 2 bases from the 16S rDNA sequence of *B. lonestari* reported from *A. americanum* from Texas (4, 10).

Phylogenetic trees constructed using 16S rDNA sequences from our isolate and other *Borrelia* spp. resulted in *B. lonestari* forming a group with *B. miyamotoi* and *B. theileri* that is more closely related to the relapsing fever *Borrelia* spp. than either group is to *B. burgdorferi* and its allies (Fig. 6.1), as previously described (10, 44).

IFA testing. The anti-flagellin MAb H9724, which recognizes all *Borrelia* spp., bound to whole-cell antigen from the LS-1 strain spirochetes (Fig. 2A) as well as to that from *B. burgdorferi* SH-1 spirochetes in ISE6 cell culture. The anti-OspA MAb specific to *B. burgdorferi* recognized *B. burgdorferi* but not the LS-1 isolate (data not shown).

Light and electron microscopy. The morphology of *B. lonestari* was typical for *Borrelia* spp. (7, 15, 20, 21, 22). Spirochetes were seen free and attached to or closely associated with ISE6 cells and other spirochetes and within cells on TEM (Fig. 2B, 3A, and 4B). Under light microscopy, spirochetes were 11 to 25 μm long and 0.23 to 0.26 μm wide. The average length of most spirochetes was 15.48 μm . Several apparently longer spirochetes (37 to 40 μm) were suspected to be in the process of dividing or attached to another spirochete in tandem.

Several spirochetes were intimately associated with ISE6 cells and appeared to be indenting the cell surface; in these areas, the spirochete-ISE6 cell interface seemed less distinct, suggesting that the spirochetes were in the process of entering the cells

(Fig. 6.3A and C). Within ISE6 cells, multiple spirochetes were seen inside membrane-bound vacuoles (Fig. 6.3A). Transverse sections of spirochetes revealed a trilaminar membrane consisting of an outer membrane (sheath), a cell wall, and an inner cytoplasmic membrane, analogous to what has been described for other *Borrelia* spp. (7, 49) (Fig. 6.3C and 6.5C). Periplasmic flagella were seen as bundles on one side of the spirochete, between the outer membrane and the cell wall of the spirochete in transverse sections, and emerging along the submembranous surfaces of organisms in oblique sections. The majority of spirochetes appeared to have a flat, wavelike shape, with widely variable wavelengths (1.5 to 2.36 μm) and amplitudes (0.45 to 0.53 μm). In spirochetes that appeared helical, the spirals were directed counterclockwise (Fig. 6.4B). Flagella were not apparent on SEM, but protrusions resembling cytoplasmic blebs, as well as less distinct membrane-associated debris, were observed on the surfaces of spirochetes (Fig. 6.4A and C). A trilaminar membrane was confirmed on TEM of negatively stained spirochetes, with a prominent outer membrane extending over the end of an organism (Fig. 6.5A). Bundles of 6 to 11 flagella were present in the submembranous space directed parallel to the long axis (Fig. 5B). The widths of flagellar bundles ranged from 0.105 to 0.156 μm .

DISCUSSION

Our cell culture and molecular, immunological, and electron microscopy data all indicate that we have made the first isolation of *B. lonestari* in culture; we have designated this strain LS-1. We were able to cultivate this fastidious organism using a tick cell line and a source of organisms from naturally infected lone star ticks. The

spirochetes were found to possess molecular characteristics consistent with the organism tentatively named *B. lonestari* and morphological and IFA staining properties distinct from those of *B. burgdorferi*. The flagellin B gene sequence of our *B. lonestari* isolate (LS-1) is identical to *B. lonestari flaB* sequences previously reported from lone star ticks and white-tailed deer in that it lacks a 3-base insert at positions 330 to 332 (4, 10, 11, 32). Although the LS-1 isolate differs in this respect from the single *flaB* sequence of *B. lonestari* reported from a human clinical case (23), the significance of this 3-base insert is not clear.

Nucleotide differences have been observed at four base positions in the 16S rRNA gene among the reported sequences of *B. lonestari* (= *B. barbouri*). Our LS-1 isolate 16S rRNA gene sequence matched one of the two published sequences for *B. lonestari* at three of these base positions and both *B. lonestari* sequences at the fourth position (base 522) (10). The published sequence for *B. barbouri* contained a single base difference at position 522, but because this was a partial sequence (355 bp) that did not include bases 698, 1025, and 1201, nucleotide similarity could not be evaluated at those positions (41). While it is important to note them, these differences are minor, and both the 16S rRNA and flagellin B gene sequences of the LS1 spirochetes support the identification of the organisms we have cultured as *B. lonestari*.

We also report here the first ultrastructural description of *B. lonestari*. The LS-1 organisms had typical spirochete morphology and characteristics consistent with *Borrelia* spp. (7, 22). Their lengths, although variable, also fell within the range of *Borrelia* spp. The ends of LS-1 organisms, like those of *Borrelia anserina* and *Borrelia recurrentis*, were tapered, but the tips were relatively blunt in comparison to those of *B.*

burgdorferi; unlike *B. anserina*, strain LS-1 was not regularly waved (15, 22).

Cytoplasmic protrusions or blebs on the surfaces of LS-1 organisms were consistent with protrusions that have been described for other spirochetes (7, 14).

TEM showed multiple sections of spirochetes inside vacuole-like, membrane-bound organelles within the ISE6 cells. Transverse organism sections that shared the same periplasmic flagellar bundle were evidently the same organism; other apparently unassociated sections may have been the same organism or another organism within the same organelle. The presence of multiple membrane-bound organelles within the same ISE6 cell, as well as single spirochetes that appeared to be free within the cytoplasm, suggests that *B. lonestari* may replicate within membrane-bound organelles of cells and then emerge from the organelles and cells. Spirochetes seen in close association with ISE6 cells on SEM, often indenting the host cell membrane, may represent the attachment and subsequent invasion of ISE6 cells, suggesting a possible dependence on the host tick cell. The interactions between these spirochetes and their growth in culture are among many significant life history characteristics that await investigation.

The findings in this study establish the critical foundation necessary for further investigations of *B. lonestari* that were previously hampered by the lack of a live isolate. Although culture isolation of *B. lonestari* from human cases of STARI is necessary to demonstrate a causative link to the illness, our results are an important step toward a greater understanding of STARI and the variable presentation of this Lyme disease-like illness. Cultured organisms are critical for the development of accurate human diagnostic assays; this will allow investigation of the clinical manifestations of infection

in humans, as well as further our understanding of the epidemiology and natural history of *B. lonestari*, including host and vector competence, natural maintenance cycles, and geographical distribution. In addition, an in vitro source of organisms is now available for controlled experiments using animal hosts as models for human infection, as well as tick transmission studies. The knowledge gained from studies using cultured *B. lonestari* will be fundamental in differentiating between *B. lonestari* and *B. burgdorferi* infections and ultimately in revealing the true occurrence of Lyme disease and STARI in the southeastern and south-central United States.

ACKNOWLEDGMENTS

We are grateful to Wayne Roberts and Mary Ard at the University of Georgia, Athens, for assistance with dark-field microscopy and electron microscopy, respectively; Tom G. Schwan, Rocky Mountain Laboratories, Hamilton, Mont., for supplying MAbs; and Uli Munderloh, University of Minnesota, for providing the ISE6 tick cell line. We also thank Grace Chan, Amy Sexauer, and numerous personnel at the Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, for their assistance in field collections and laboratory studies.

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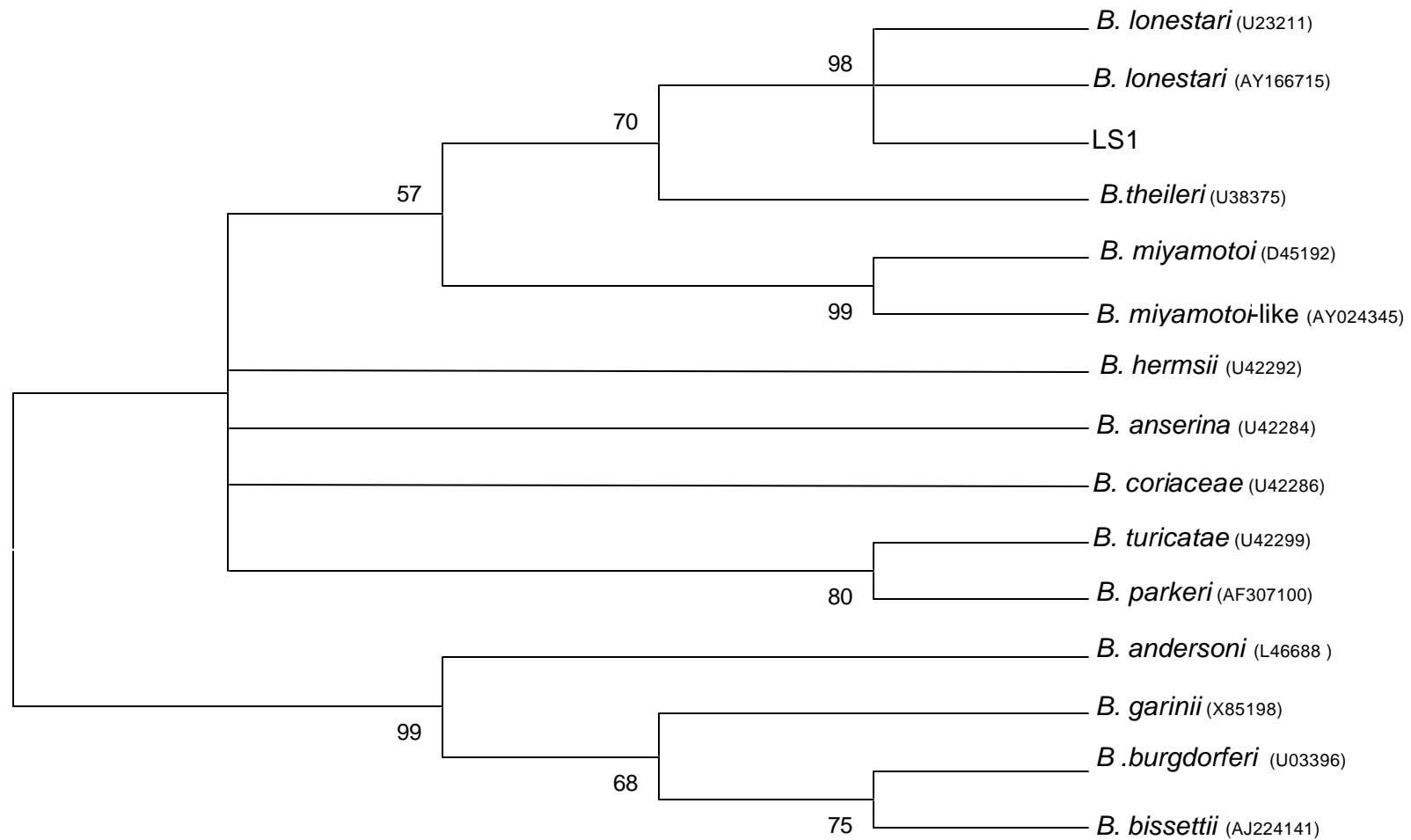


Figure 6.1. Bootstrap consensus (1,000 times) of phylogenetic tree of *Borrelia* sp. 16S rDNA gene sequences generated using maximum-parsimony analysis with close-neighbor interchange. The number at each node indicates the percentage of times that node was supported by bootstrap analysis.

Table 6.1. Comparison of *B. lonestari* flagellin B gene sequences from Genbank with those amplified from our isolate.

Source ^a	State	Genbank accession no.	Nucleotide difference at position ^b			
			330	331	332	348
Cell culture ^c	GA	AV442141	*	*	*	A
LST (4)	MO	AY166716	*	*	*	A
LST (10)	TX	U26074	*	*	*	G
LST (10)	NJ	U26075	*	*	*	A
LST (11)	AL	AF298653	*	*	*	A
LST (11)	AL	AF298654	*	*	*	A
LST (23)	NC,MD	AF273670	A	G	A	A
LST (23)	NC,MD	AF273671	A	G	A	A
LST (32)	NC	AF538851	*	*	*	A
LST (32)	GA	AF538852	A	G	A	A

^a LST, lone star tick; WTD, white-tailed deer.

^b Numbers correspond to nucleotide position of *B. lonestari* *flab* sequence U26704. An asterisk indicates no corresponding base at the position.

^c Sequence of the isolate reported in this study; originally isolated from *A. americanum* from Georgia..

Table 6.2. Comparison of *B. lonestari* (= *B. barbouri*) 16S rRNA gene sequences from Genbank with those amplified from our isolate.

Source ^a	State	Genbank accession no.	Nucleotide difference at position ^b			
			522	698	1065	1201
Cell culture ^c	GA	AY442142	C	T	G	C
LST	MO	AY166715	C	T	A	C
LST	TX	BLU23211	C	C	G	T
LST	MD	U38374	T	NR	NR	NR

^a LST, lone star tick.

^b Numbers correspond to nucleotide position of *B. lonestari* 16S rDNA sequence U23211.

^c Sequence of the isolate reported in this study; originally isolated from *A. americanum* from Georgia.

^d NR, not reported.

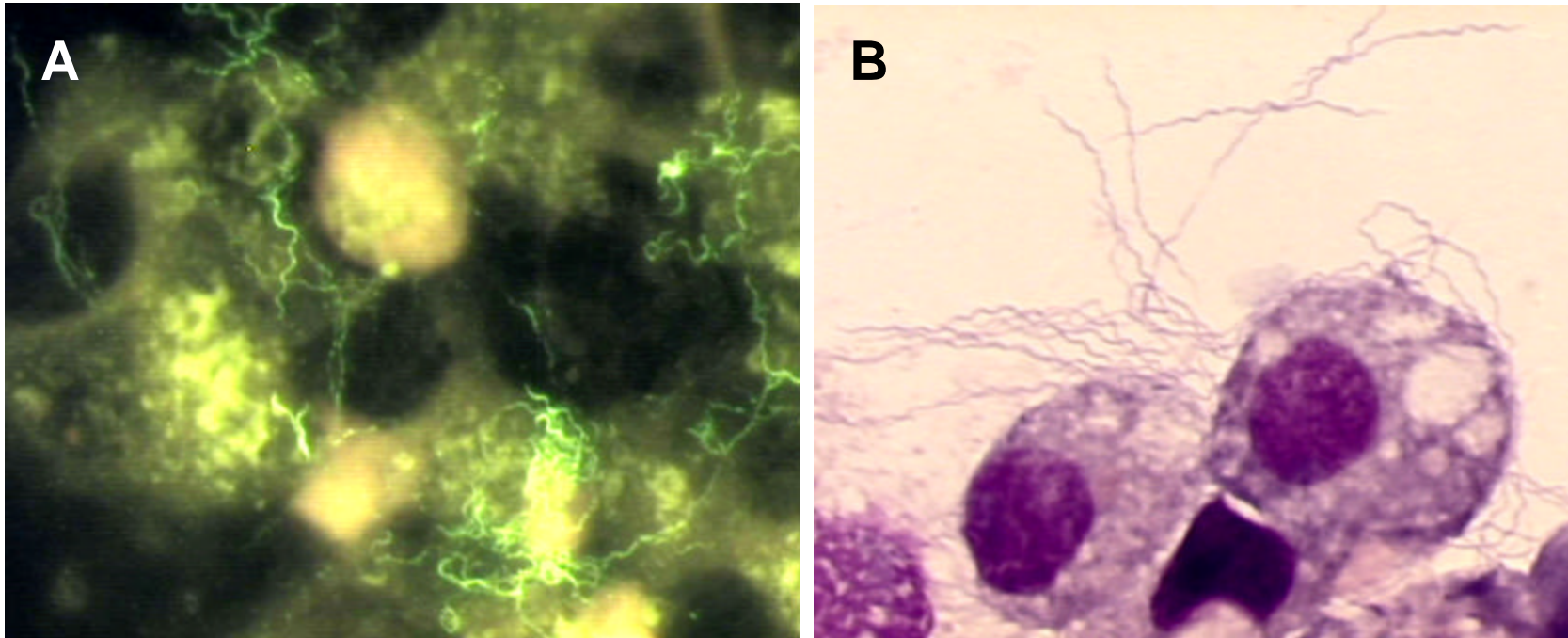


Figure 6. 2. Immunofluorescence antibody and Giemsa staining of cultured spirochetes. (A) Immunofluorescence antibody staining of spirochetes isolated from lone star ticks using *Borrelia*-wide anti-flagellin antibody (H9724). (B) ISE6 tick cell cultures of spirochetes were Giemsa stained; spirochetes are present free and attached to ISE6 cells. Magnification, X1,000.

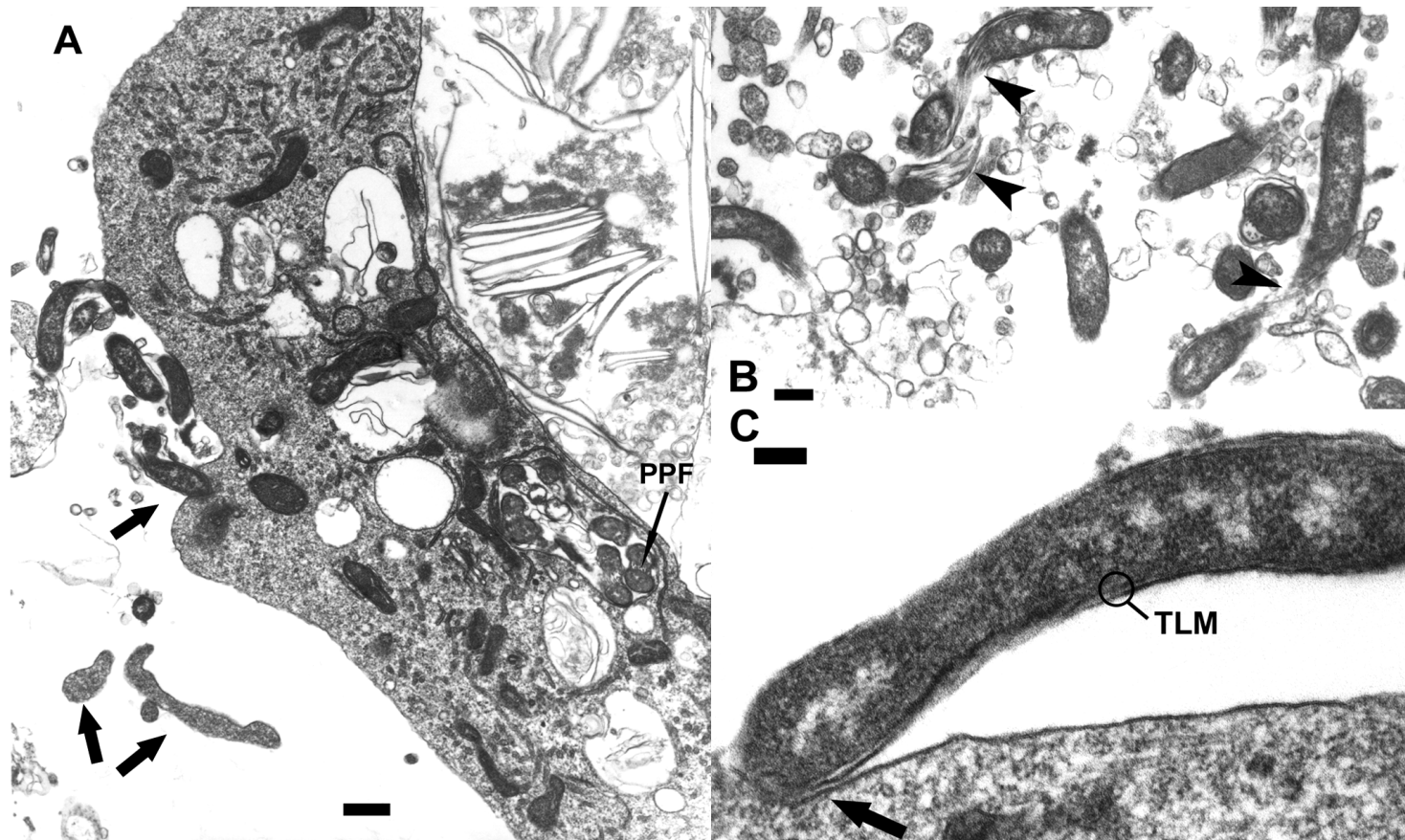


Figure 6. 3. Transmission electron micrographs of cultured spirochetes. (A) Spirochetes are present free and associated with ISE6 cells (arrows). Transverse sections of intracellular spirochetes showing periplasmic flagella (PPF) are evident within membrane-bound vacuoles. (B) Bundles of flagella can be seen running along oblique and transverse sections of spirochetes (arrowheads). (C) Spirochete and ISE6 cell in close association, with loss of resolution at the site of contact (arrow). The trilaminar membrane (TLM) is also seen. Bars, 500 (A), 200 (B), and 100 (C) nm.

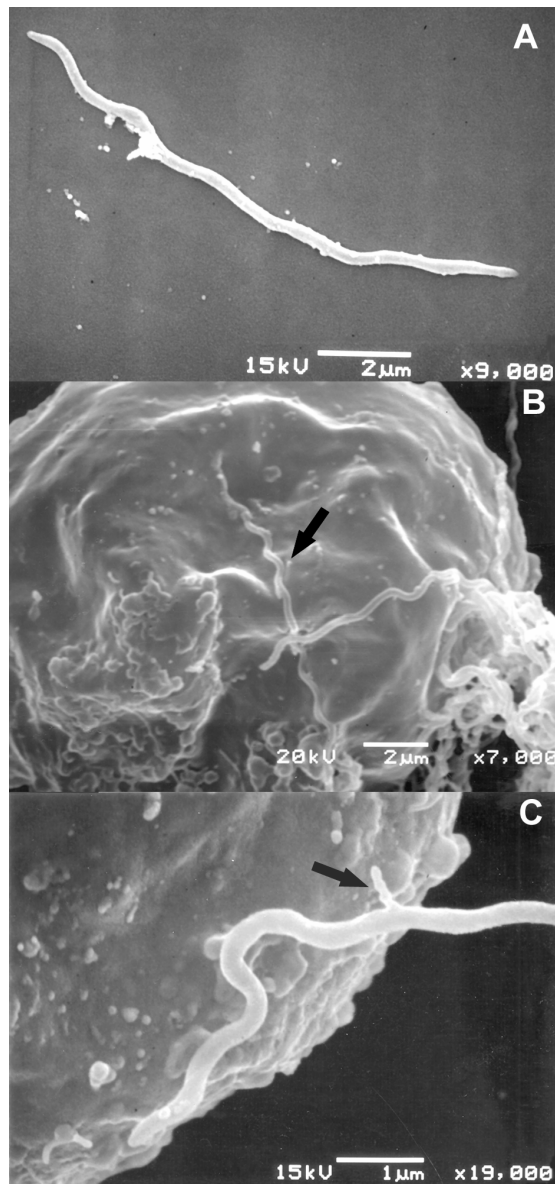


Figure 6.4. Scanning electron micrographs of cultured spirochetes. (A) Single spirochete with characteristic tapered ends, relatively flat wavelength, and a cytoplasmic bleb. (B) The arrow points to a spirochete with a counterclockwise helix (left-handed; moving away from the observer); also evident are spirochetes entangled with each other and closely associated with the ISE6 cell. (C) Greater magnification of a cytoplasmic bleb (arrow).

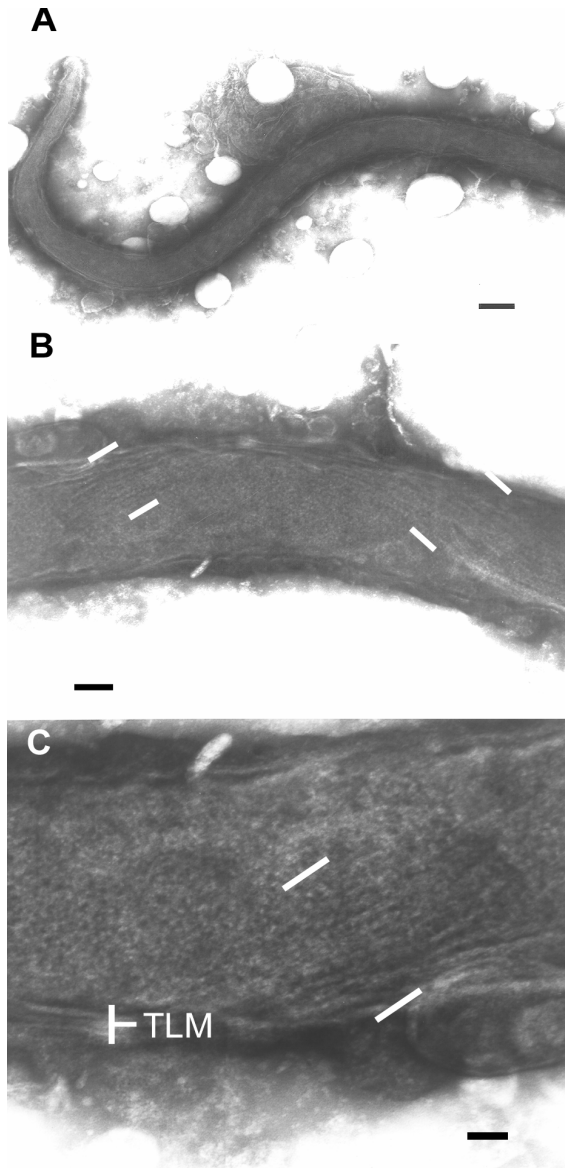


Figure 6.5. Transmission electron micrographs of negatively stained spirochetes. (A) Spirochete with a prominent outer membrane extending over one end. (A and B) Flagella can be seen running along the axis of the spirochete. (B) Flagellar bundles are evident on either side of the curvature of the spirochete (bars). (C) Greater magnification of the spirochete from panel B reveals the trilaminar membrane (TLM) with evidence of multiple flagella running periplasmically and more clearly demonstrates a single flagellar bundle (bars). Bars, 200 (A), 100 (B), and 50 (C) nm.

CHAPTER 8

CONCLUSIONS

CONCLUSIONS

Over the past two decades, the lone star tick (LST), *Amblyomma americanum*, has risen in status as an important vector of several newly recognized zoonotic agents in the United States. Among these zoonotic agents are two bacteria in the family *Anaplasmataceae*: *E. chaffeensis*, the agent of human monocytotropic ehrlichiosis (HME) and *E. ewingii*, an agent of canine and human granulocytic ehrlichiosis. In addition, *A. americanum* harbors *B. lonestari*, the putative agent of southern tick-associated rash illness (STARI), a syndrome similar to acute Lyme disease (Barbour, et al. 1996). An important ectoparasite of a variety of birds and mammals, *A. americanum* is the tick most frequently removed from humans in the southern United States (Felz, et al. 1996, Kollars, et al. 2000, Merten and Durden 2000). Because all stages of LST will feed on humans and because white-tailed deer (WTD) are the primary natural host for all three motile stages of LST, WTD are an ideal reservoir host for LST-vectored zoonotic agents.

To date, WTD are understood to be the principal reservoir for *E. chaffeensis*, and a suspected reservoir of *E. ewingii* and *B. lonestari*. Consequently, evaluating the infection dynamics of *E. chaffeensis* in experimentally infected WTD should contribute to an understanding of the natural maintenance of *E. chaffeensis*. In addition, determining the prevalence of *E. chaffeensis*, *E. ewingii*, and *B. lonestari* in LST from an area where both LST and WTD are plentiful will provide important epizootiological information. Finally, the isolation of *B. lonestari*, the most recently recognized LST-

associated zoonotic agent, will generate essential material for experimental infections and the development of diagnostic tools. The work presented in this dissertation has contributed to these areas and is summarized in the following paragraphs.

Chapter 3: Experimental infection of WTD with *E. chaffeensis* by different routes.

Previous experimental infections of white-tailed deer using needle inoculation of *E. chaffeensis* have used only the intravenous (IV) route; however, natural transmission of *E. chaffeensis* occurs through the bite of a lone star tick (Anderson, et al. 1993; Dawson, et al. 1994b; Ewing, et al. 1995; Lockhart, et al. 1997b; Kocan, et al. 2000a; Davidson, et al. 2001). Because experimentally feeding ticks on WTD is difficult and labor-intensive, we hypothesized that needle inoculation via a route that more closely simulates tick feeding, such as intradermal (ID) or subcutaneous (SQ) injection, may be more suitable, in comparison to intravenous injection, for simulating and studying natural infection dynamics of *E. chaffeensis* in the principal reservoir host. In this study, white-tailed deer were inoculated with *E. chaffeensis* infected DH82 cells by three different routes: IV ($n=2$), SQ ($n=2$), and ID ($n=2$); two control deer were inoculated IV with uninfected cells. Results showed that IV, SQ, and ID routes of inoculation caused infection in white-tailed deer, as measured by serology, PCR, and culture isolation, and demonstrated infection of WTD with *E. chaffeensis* by ID and SQ routes for the first time. No difference was observed in the time course of *E. chaffeensis* rickettsemia between the artificial routes used. Overall, this study showed that experimental infection with *E. chaffeensis* by any of the three routes is suitable for future studies investigating infection dynamics in WTD. Ultimately, additional comparison of the

course of infection via tick transmission will be essential to determine whether any of the artificial routes is a suitable model for natural transmission.

Chapter 4: Primary and secondary *E. chaffeensis* infection of WTD.

Under natural conditions, WTD are exposed to multiple genetic variants of *E. chaffeensis* from infected lone star ticks. In addition, experimentally infected deer are capable of maintaining a long-term infection with a single exposure to *E. chaffeensis* (Davidson, et al. 2001). Because genetic variants of *E. chaffeensis* may differ in pathogenicity to humans, the ability of WTD to become infected with a genetic variants upon secondary exposure should provide insight into the role of WTD as reservoirs for multiple genetic variants. In this study, we evaluated the response in WTD to secondary exposure with a genetic variant of *E. chaffeensis* that differed by the number of tandem repeats present in the VLPT gene. For primary infection, six WTD were injected with *E. chaffeensis* (Ark), a four-repeat strain, and two WTD served as negative controls. On post-infection day 54, three *E. chaffeensis* (Ark) infected deer and one naïve deer were injected with *E. chaffeensis* (WTD-6045B), a five repeat strain, and three other Ark strain infected deer were injected with non-infected DH82 cells; all animals were monitored for 31 additional days. The primary and/or secondary genetic variants were detected in all deer exposed to both variants, as determined by PCR amplification of the VLPT gene on whole blood, cell culture, and tissues. The results of this study demonstrated that WTD, the principal reservoir host of *E. chaffeensis*, are susceptible to secondary infection with a genetic variant that differs in the number of repeats in the VLPT gene. Thus, WTD infected with multiple strains potentially increase

the number of genetic variants available for acquisition by ticks and consequently the risk for transmission of a wide variety of potentially virulent strains to humans.

Chapter 5: Attempted infection of goats with *E. chaffeensis*

White-tailed deer (WTD), *Odocoileus virginianus*, are the principal reservoir host for the tick-borne bacterium, *Ehrlichia chaffeensis*, causative agent of human monocytic ehrlichiosis. However WTD are difficult to maintain in captivity and experimental infections using WTD are labor-intensive. Thus, it would be beneficial to use an alternative animal model to study infection in vertebrate hosts. Because natural infection with *E. chaffeensis* has been reported in domestic goats and goats are commonly used as experimental animals, we evaluated whether domestic goats could be artificially infected with *E. chaffeensis*, and thus be a suitable animal model for further investigations of *E. chaffeensis* infections. Experimental infection of goats via three routes of inoculation failed to produce active infection in goats, although goats were did produce detectable anti-*E. chaffeensis* antibodies. These results suggest that goats may not be appropriate models for future investigations of *E. chaffeensis* using needle inoculation.

Chapter 6: Disease agents in *A. americanum* from Georgia.

Amblyomma americanum is known or suspected to vector several organisms that are implicated as human pathogens, including *E. chaffeensis*, *E. ewingii*, and *B.lonestari*, agents that have also been detected in WTD. Both LST and WTD occur in high numbers in northeastern Georgia, and *E. chaffeensis* has been detected in LST

from this area (Lockhart, et al. 1997b). However, the prevalence of *E. ewingii* and *B. lonestari* was not previously investigated. In this study, a total of 400 individual adult *A. americanum*, collected during the spring of 2001, 2002, and 2003, were assayed by PCR for all three organisms using *Ehrlichia* species-specific and *Borrelia* genus-wide primers. Of ticks tested, 2.0% (8/398) had evidence of *E. chaffeensis*, 4.8% (19/398) had evidence of *E. ewingii*, and 1.0% (4/398) had evidence of *B. lonestari*. *Borrelia* sp. spirochetes were also visualized by IFA, using an anti-flagellin monoclonal antibody (H9724), in a total of 10.7% (32/300) of ticks tested in 2003, suggesting that IFA may be a more sensitive detection assay for *Borrelia* sp. The results of this study reconfirmed the presence of *E. chaffeensis* and established evidence of *E. ewingii* and *B. lonestari* in questing adult *A. americanum* ticks from northeastern Georgia, and underscores the risk of human exposure to these pathogens.

Chapter 7: First culture isolation of *B. lonestari*

Borrelia lonestari, the putative agent of STARI, a Lyme disease-like illness described in the southeastern and south-central United States from patients following the bite of a LST, was first identified 1996 (Barbour, et al. 1996). Unlike *B. burgdorferi*, which is relatively easy to cultivate in BSK-based culture, *B. lonestari* is a fastidious organism and attempts to isolate the organism from patients and other sources have been unsuccessful thus far. Because a live isolate is essential for the development of diagnostic assays to differentiate STARI from classic Lyme disease, and for deeper exploration of the natural history of *B. lonestari*, novel methods for culturing the organism have been considered. This study reports the first successful isolation of *B.*

lonestari. Ten adult LST collected from northeastern Georgia were dissected and tissues removed and placed in a co-culture system using ISE6 cells, an *Ixodes scapularis* embryonic cell line. Spirochetes first observed after two weeks had 16S rRNA and *flaB* sequences that corresponded with previously reported *B. lonestari* sequences. Results of IFA testing were also consistent with a *Borrelia* sp. other than *B. burgdorferi*, and electron microscopy demonstrated spirochetes with morphology matching *Borrelia* spp. The isolate obtained will be essential for filling a critical gap in necessary investigations, such as experimental infections and the development of diagnostic tools.

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