ON THE TRACKS OF ERYTHEMA MIGRANS –

IDENTIFYING NOVEL MECHANISMS AND AGENTS OF TICK-

BORNE DISEASE

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CHAPTER I

INTRODUCTION

Erythema migrans is the clinical hallmark of acute Lyme disease in people caused by the spirochete *Borrelia burgdorferi* s.s. (CDC 1997; Shapiro and Gerber 2000). In endemic areas of the USA (Northeast, upper Midwest and northern California), presence of this skin lesion following the bite of an *Ixodes scapularis* (the black-legged tick) is considered pathognomonic (Nichol et al. 1998) for acute Lyme borreliosis even in the absence of laboratory confirmation (CDC 1997). In the southern United States, a similar skin lesion is associated with the bite of a different tick, *Amblyomma americanum* (the lone star tick, LST), which is also the tick most commonly reported from humans and animals. This presentation is described as southern tick-associated rash illness (STARI) and the etiology remains unknown (Wormser et al. 2005a; Wormser et al. 2005b; Phillipp et al. 2006). Amongst others, *Borrelia burgdorferi*, *Borrelia lonestari*, and *Rickettsia amblyommii*, have been considered possible agents, but no causality has been shown (James et al. 2001; Wormser et al. 2005a; Wormser et al. 2005b; Billeter et al. 2007; Nicholson et al. 2009). However, *A. americanum* is associated with transmission of a number of emerging diseases (Anderson et al. 1991; Buller et al. 1999; Apperson et al. 2008; Reeves et al. 2008). In order to better understand the novel disease agents that may be present in *A. americanum* and develop an animal model for STARI, the following studies were undertaken:

1. **Characterize bacterial diversity in *Amblyomma americanum* with a focus on members of the genus *Rickettsia***

Recent studies investigating the etiology of STARI focused on isolation of pathogens from human samples (James et al. 2001; Wormser et al. 2005a; Billeter et al. 2007). Our approach took a different route by characterizing the bacterial community in wild-caught adult *A. americanum* and comparing these to the community in colony-raised ticks. To better define the microbial communities within LSTs, 16S rRNA gene based analysis using bacteria-wide primers, followed by sequencing of individual clones (n=449) was used to identify the most common bacterial operational taxonomic units (OTUs) present within colony-reared and wild LSTs. OTUs present groups based on the genetic distance between a given set of sequences, which reflects their degree of similarity and provides an estimate of the species richness in a given population (Schloss and Handelsman 2005). The colony-reared ticks contained primarily sequences affiliated with members of the genus *Coxiella* (89%; 81/91), common endosymbionts of ticks, and *Brevibacterium* (11%; 10/91). Similarly, analysis of clones from unfed wild LSTs revealed that 96.7% (89/92) of all the OTUs identified were affiliated with *Coxiella*-like endosymbionts, as compared to only 5.1%-11.7% (5/98 – 9/77) of those identified from fed wild LSTs. In contrast, the proportion of OTUs identified as *Rickettsia* sp. in wild caught ticks increased from 2.2% (2/92) before feeding to as high as 46.8% (36/77) after feeding, and all *Rickettsia* spp. sequences recovered were most similar to those described from the spotted fever group *Rickettsia*, specifically *R. amblyommii* and *R. massiliae*. Additional characterization of the *Rickettsiales* tick community by PCR, cloning, and sequencing of 17 kDa and GltA genes confirmed these initial findings and suggested that novel *Rickettsia* spp. are likely present in these ticks. These data provide insight into the overall community, as well as, the rickettsial community of wild lone star ticks and may ultimately aid in identification of novel pathogens transmitted by *A. americanum*.

2. Define the bacterial community in *Amblyomma americanum* nymphs collected from outside the home of a patient with southern tick-associated rash illness (STARI)

With the documentation of a case of STARI within the area from where we collected LSTs we support our strategy which is based on the thorough characterization of bacteria within *A. americanum* in light of their role as potential human pathogens.

In August of 2008, an *Amblyomma americanum* nymph was removed from a man in Payne County, Oklahoma. Subsequently, the individual developed a mildly pruritic expanding circular rash at the site of the tick bite consistent with the clinical definition for southern tick-associated rash illness (STARI). Along with the skin lesion, headache and mild fatigue were reported. Upon treatment with doxycycline the rash resolved. Within a week of appearance of the rash, *A. americanum* nymphs were collected from the property where the tick was acquired and analyzed before and after feeding using a 16S rDNA-wide PCR approach followed by phylogenetic analysis of sequences; fed ticks were further evaluated by specific PCR for *Borrelia* spp., *Rickettsia* spp., and *Ehrlichia* spp. Within the bacterial community identified from unfed nymphs, the greatest proportion of 16S rDNA sequences (22/70 clones, 31.4%) were members of the genus *Rickettsia*. The majority of rickettsial clones from unfed nymphs (18/22) were most closely related to *Rickettsia amblyommii*. The remaining 4 sequences were most closely associated with *Rickettsia* sp. described from other ixodid tick species. Upon feeding, the percentage of clones affiliated with the genus *Rickettsia* increased to 51.3% (40/78 clones), with all sequences showing closest association with *Rickettsia amblyommii*. Targeted PCR analysis of individual fed nymphs revealed a 12% prevalence (6/50) for *Rickettsia amblyommii* and 2% prevalence (1/50) for *Ehrlichia chaffeensis*. No *Borrelia* spp. or other *Ehrlichia* spp. were identified in these samples. These data support previous work documenting frequent infection of *A. americanum* with *Rickettsia* spp. and confirm the presence of *R. amblyommii* in all life stages.

3. Development of an animal model for southern tick-associated rash illness (STARI)

The development of an animal model has the potential to fill the gap between information gathered from STARI patients and potential pathogens found in LSTs from areas where STARI is known to occur. It provides the opportunity to test putative agents identified in the tick for their ability to induce lesions in the NZW rabbit.

To develop an animal model for STARI, wild-caught adult LSTs from an area where STARI is known to occur were allowed to feed on New Zealand white rabbits; colony-raised ticks were fed on additional rabbits as negative controls. Skin was evaluated daily for evidence of erythema migrans, and serum, whole blood, and skin biopsy samples were collected at 0, 7, 14, and 21 days post tick infestation (DPTI) and evaluated for evidence of infection via PCR using specific primers targeting known tick-borne pathogens as well as 16S rDNA bacteria-wide PCR followed by sequencing. A total of 4 distinct, circular expanding erythematous lesions 3.0-3.5 cm in diameter developed on rabbits infested with wild caught LSTs; lesions did not develop on rabbits that received colony-raised ticks. PCR revealed that wild-caught ticks contained both *Borrelia lonestari* and *Rickettsia amblyommii*, putative agents of STARI, but no specific pathogens were identified from the skin biopsies, a finding consistent with reports from human STARI patients.

SUMMARY

These three studies provide thorough microbial characterization of the tick of interest, *A. americanum*, a field investigation of ticks at the site of a STARI case, and the establishment of an animal model for STARI. Taken together, they allow a greater understanding of the etiology of STARI and provide valuable tools to be used in future research aimed at identifying the causative agent, development of diagnostic tools and appropriate treatment recommendations, and understanding the natural history of the disease agent.

Although the clinical presentation of STARI has been recognized for more than two decades, the causative agent remains unknown. There can be no improvement in diagnostic testing, refinement of treatment recommendations, or understanding of the effects of chronic disease associated with STARI, if any, as long as the etiology remains unresolved.

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CHAPTER II

LITERATURE REVIEW

I. ERYTHEMA MIGRANS FOLLOWING A TICK BITE

1. CLINICAL APPEARANCE OF ERYTHEMA MIGRANS

In human patients, Lyme disease, which is caused by the spirochete *Borrelia burgdorferi*, is classified into 3 different stages: early localized, early disseminated, and late stage (Embers et al. 2004). In the early (acute) stage, 60 to 80% (Foley et al. 1997) of patients present with what is considered to be the clinical hallmark of this disease: a circular inflammatory lesion, known as erythema migrans (EM) (Steere et al. 1983a; CDC 1997; Shapiro and Gerber 2000). Following dissemination, secondary EMs are reported in 2 to 18% of patients (Egberts et al. 2008; Embers et al. 2004). The erythematous outer circle with common central clearing and regular margins is also described as 'bull's eye lesion' or 'target lesion'. As the spirochetes disseminate though the connective tissue along collagen fibers, possibly spanning skin folds, EM lesions can appear circular or oval (Dandache and Nadelman 2008). In a minority of cases clear, cloudy, or hemorrhagic vesicles develop at the bite site (Dandache and Nadelman 2008).

Interestingly, EM lesions following a tick bite have repeatedly been reported from areas in North America outside Lyme borreliosis endemic foci (predominantly located in the Atlantic coastal United States and the upper Midwestern States) (CDC 2008). These lesions resemble those from patients with diagnostic evidence of infection with *B. burgdorferi* and are associated

with mild systemic symptoms, but have not been associated with chronic disease. In a 1999 study based on 23 patients from an endemic area (Georgia and South Carolina) presenting with EM, 70% of patients (16/23) failed to show laboratory confirmation of infection with *B. burgdorferi*, and 90% of the patients associated the site of skin lesion with a tick bite. However, ticks were removed and were not available for identification and laboratory analysis. In the southern United States, the most common tick associated with bites in humans is *Amblyomma americanum*, the lone star tick (Felz et al. 1999).

2. HISTOLOGICAL CHARACTERIZATION OF ERYTHEMA MIGRANS

Histologically, the central area of an EM lesion caused by *B. burgdorferi* is characterized by fibrin deposits with infiltrating erythrocytes, nuclear fragments, small vessel telangectasia, and edema (accompanied by dilated blood and lymph vessels) (Steere et al. 1983b; De Koning 1993) in the upper dermis. A variable degree of lymphocellular infiltrates, combined with histiocytes, scattered plasma cells and mast cells can be found in both superficial and deep plexuses of the dermis. The density of inflammatory cells is increased in perivascular regions and between interstitial collagen fibers; neutrophils tend to be significantly more abundant than plasma cells (De Koning 1993). Mild acanthosis and spongiosis, foci of parakeratosis, vacuolar alteration and keratinocyte necrosis may be noted in the epidermis. However, the basal layer remains greatly unaffected with only occasional scattered lymphocytes. Eosinophils are rare and most commonly abundant in the interstitium (Steere et al. 1983b; Böer et al. 2007). The peripheral area or "leading edge" of the EM lesion is characterized by lymphohystiocytic inflammation with occasional nuclear fragments in the upper and mid layers of the dermis (Steere et al. 1983b). The subcutis may be involved, especially in foci with high cellular infiltrates (Böer et al. 2007; Moguelet 2007).

Based on this microscopic description, EM lesions can be difficult to differentiate from similar conditions affecting the skin, such as tumid lupus erythematosus, deep gyrate erythemas, and certain B cell lymphomas (Böer et al. 2007; Moguelet 2007). Accordingly, diagnosis of

erythema migrans is essentially based on the macroscopic evaluation of the lesion with later microscopic characterization. Special stains or electron microscopy have been used to locate spirochetes within tissue sections, but such approaches can be difficult to interpret in clinical samples. When present, spirochetes are mainly found close to and between collagen fibers. Infrequently, *B. burgdorferi* is observed within or close to a vessel (De Koning 1993).

3. DISEASES ASSOCIATED WITH ERYTHEMA MIGRANS IN NORTH AMERICA

a. Lyme disease

The first report of erythema migrans accompanied by flu-like symptoms in the United States was published in 1970 and a spirochetal etiology was suspected based on previous reports from Europe (Scrimenti 1970). Subsequent work identified this condition as Lyme disease, and a spirochete in adult *Ixodes scapularis* (Oliver et al. 1993) ticks was designated as the causative agent in 1982 (Burgdorfer et al. 1982). In 1984, this organism was named *Borrelia burgdorferi*, in honor of Willy Burgdorfer (Johnson et al. 1984).

This new form of inflammatory arthritis and accompanying skin rash, suspected to be transmitted by an arthropod vector, was first reported from Lyme, Connecticut in 1975 (Steere et al. 1977). A surveillance system for Lyme disease was initiated by the CDC in 1982, and by 1992 a standardized case definition for the reportable disease had been implemented nationwide (Orloski et al. 2000). According to the current CDC case definition, a diagnosis of Lyme borreliosis is confirmed when a) a physician recognizes an EM (at least 5 cm in diameter) in a patient with a known exposure (having been in appropriate tick habitats within 30 days prior to onset of EM) in an area where disease is endemic, or b) a physician recognizes an EM and confirms laboratory evidence (culture or serology) of infection in patients without a known exposure to an area where disease is endemic or c) a physician recognizes at least one late manifestation of Lyme borreliosis and confirms laboratory evidence of infection (CDC 2007).

b. STARI

Southern tick-associated rash illness (STARI) is also known as southern Lyme disease or Masters' disease in honor of the physician in southeast Missouri who recognized and described this phenomenon in a large number of patients (Masters et al. 1998a; Masters et al. 1998b). Patients present with an erythema migrans at the site of a tick bite. The tick species involved in STARI is *Amblyomma americanum*, the lone star tick (LST), which has repeatedly been shown to be an incompetent vector of *B. burgdorferi* (Piesman and Sinsky 1988; Mather and Mather 1990; Mukolwe et al. 1992a; Oliver et al. 1993; Sanders and Oliver 1995; Piesman and Happ 1997). The first reports of *A. americanum* associated with a rash in a human patient were published in 1984 (Schulze et al.1984). To date, reports of STARI are geographically correlated to the distribution of the LST, mainly from the south-central and southeastern United States (Campbell et al. 1995; Masters and Donnell 1995; Kirkland et al. 1997; Masters et al. 1998a; Masters et al. 1998b; Masters et al. 2008).

The etiologic agent of STARI has yet to be determined. Pathogens that have been associated with patients or individual ticks associated with STARI cases are *Borrelia lonestari* (James et al. 2001), a relapsing fever group spirochete (Barbour et al. 1996), and *Rickettsia amblyommii*, a member of the spotted fever group (Billeter et al. 2007). However, thus far clinical microbiological studies have not confirmed involvement of these agents in the etiology of the phenomenon (Wormser et al. 2005a; Wormser et al. 2005b; Nicholson et al. 2009) and *B. burgdorferi* s.s. has never been cultured from patients with EMs in the southern US (Campbell et al. 1995; Master and Donnell 1995; Kirkland et al. 1997; Felz et al. 1999).

Patient histories of frequent tick bites as well as histopathological and clinical data do not support hypersensitivity reaction as the cause for the skin rash (Masters and Donnell 1995; Masters et al. 2008). Occasionally, mild fever, fatigue, muscle ache and head ache accompany the rash. Close resemblance of these symptoms with symptoms associated with human Lyme disease as well as the extension of the distribution of *A. americanum*, which used to generally be restricted to the southern US, northwards into areas where Lyme disease is endemic, further complicates the issue. Indeed, cases of STARI might be amongst cases reported as acute Lyme

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disease based on geographic association and presence of an EM (CDC 1997; Paddock and Yabsley 2007). The majority of reports of Lyme borreliosis are based on EM alone (Bacon et al. 2008). At this time, STARI remains a clinical diagnosis (Masters and Donnell 1995).

II. LYME BORRELIOSIS CAUSED BY *B. BURGDORFERI* SENSU STRICTO

1. *BORRELIA BURGDORFERI*

Borrelia burgdorferi is a spirochete, 10 to 30 μm long, with multiple endoflagella and 7 to 11 periplasmic flagella that run from either end of the bacteria lengthwise, allowing it to travel through viscous solutions (Grubhoffer et al. 2005; Fikrig and Narasimhan 2006). The complete genome sequence of *Borrelia burgdorferi* has been published (Fraser et al. 1997) and is known to consist of one linear chromosome (910,725 bp, 853 ORFs) and up to 21 linear and circular plasmids (Casjens et al. 2000). The extrachromosomal linear and circular plasmids are key genetic elements and represent more than 40% of the genome (Casjens et al. 2000; Fikrig and Narasimhan 2006). Loss of plasmids quite frequently takes place during long-term culture and cloning of the spirochete. Consequences of the plasmid loss are variable, depending on the nature of changes in the profile of proteins expressed. Outer-surface membrane proteins (OMPs) are of interest due to their potential role in diagnostic and vaccine development and have been thoroughly investigated and characterized (Fraser et al. 1997; Bauman 2004).

One such OMP is VlsE, a Vmp (variable membrane protein)-like sequence expressed protein of approximately 35 kDa. VlsE is a surface lipoprotein encoded on linear plasmid 28-1 (lp28-1). Antigenic variations occur when recombination between 15 silent vls cassettes and the transcriptionally active vlsE occur (Zhang 1997). The VlsE has a central variable domain with six invariable regions (IR_{1-6}) and six variable regions that are affected by recombination events (Liang 1999). Epitopes of one of these invariable regions, IR_6, is highly immunogenic, is exposed on the VlsE but not the spirochetal surface and, therefore, not accessible to antibodies. Based on these observations a role as a decoy epitope has been proposed (Liang et al. 1999). A synthetic

peptide (C6) based on this IR_6 region has been prepared for routine diagnostic testing in form of an ELISA that detects both IgG and IgM antibodies and shows high sensitivity and specificity (Mogilyansky et al. 2004).

2.   *IXODES SCAPULARIS / IXODES PACIFICUS*

Borrelia burgdorferi is transmitted by *Ixodes scapularis,* the black-legged tick (Steere et al. 1978) in the eastern United States or *Ixodes pacificus*, in the western US (Burgdorfer et al. 1985). Both species are three-host ticks and take approximately 2 to 3 years to complete their life cycle.

In the northern US adult females feed between September and May to lay eggs in the summer (June to July). Larvae hatch in late summer (late July to August) and feed in the fall to overwinter as engorged larvae or emerged nymphs. Nymphs become active the following spring or early summer, and feed and molt within the same year. In contrast to larvae and adults, nymphs have a low chance of successful overwintering in the engorged stage (Yuval and Spielman 1990). The developmental cycle of *Ixodes* spp. may be skewed in the southern US due to warmer climate, with molting processes occurring earlier in the year, starting with oviposition in late winter to early spring (February to May) (Padgett and Lane 2001).

Adult *I. scapularis* preferentially feed on larger mammals, especially white-tailed deer (*Odocoileus virginianus*) and Columbian black-tailed deer (*Odocoileus hemionus columbianus*) for *I. scapularis* and *I. pacificus*, respectively, but raccoons, horses, cattle, companion animals and people are also potential hosts (Gray et al. 2002; James and Oliver 1990; Westrom et al. 1985). In the Northeast, larvae and nymphs of *I. scapularis* prefer to feed on rodents, especially white-footed mice (*Peromyscus leucopus*)*,* eastern chipmunks (*Tamias striatus*), and shrews (*Blarina brevicauda* and *Sorex cinereus*), whereas immature *I. scapularis* inhabiting the southeastern US prefer to feed on lizards (Spielman et al. 1984; Apperson et al.1993).

Besides its role in the etiology of Lyme borreliosis, *I. scapularis* is established as the vector of two other pathogens, *Babesia microti* and *Anaplasma phagocytophilum*, which cause

potentially lethal febrile diseases in humans, depending on the immune status of the patient (Kjemtrup and Conrad 2000; Dumler et al. 2005; Gray et al. 2010). *Babesia microti* is an apicomplexan parasite and one of the causative agents of human babesiosis (Kjemtrup and Conrad 2000; Gray et al. 2010). Natural reservoirs of this parasite are shrews and rodents, especially white-footed mice (*Peromyscus leucopus*) and meadow voles (*Microtus pennsylvanicus*), which are frequently co-infected with *B. microti* and *B. burgdorferi* (Anderson et al. 1986). The natural reservoir hosts of *A. phagocytophilum*, the causative agent of human granulocytic anaplasmosis, formerly known as *Ehrlichia equi* and the human granulocytic ehrlichiosis (HGE) agent, are ruminants as well as rodents such as shrews, mice and voles (Woldehiwet 2010).

3. NATURAL HISTORY

Natural competent reservoir hosts for *Borrelia burgdorferi* are primarily white-footed mice, chipmunks, and shrews (Brisson et al. 2008). These species are commonly infested by immature *I. scapularis* in the northeastern United States where nymphal stages are implicated as the most important life stage for *B. burgdorferi* transmission (Stafford et al. 1998; Falco et al. 1999). The importance of nymphs is attributed to the correlation between activity of this stage during the summer months when human outdoor activity is increased as well as their small size and decreased likelihood of being discovered prior to transmission of the bacteria compared to adult ticks (Diuk-Wasser et al. 2006).

Although *B. burgdorferi* is known to be circulating in nature in the southeastern US, it is unlikely to be transmitted to people or dogs due to the feeding behavior of its common vector in these areas (Lin et al. 2001; Oliver et al. 2003). The impact of lizards on the distribution of the etiologic agent of human Lyme disease has repeatedly been investigated. Presumably due to host availability, immature *I. scapularis* inhabiting the southeastern US commonly feed on lizards (Spielman et al. 1984; Apperson et al. 1993) which do not serve as reservoir host for *B. burgdorferi. Ixodes scapularis* immatures selectively feed on certain lizard species, including the

southeastern five-lined skink (*Eumeces inexpectatus*), the ground skink (*Scincella lateralis*), the broad-headed skink (*Eumeces laticeps*), and the eastern glass lizard (*Ophisaurus ventralis*) (Appersen et. al 1993). Furthermore, *I. scapularis* is infrequently associated with human tick bites in the southern US (Luckhart et al. 1992; Felz et al. 1996).

Competent reservoir hosts for *B. burgdorferi* in the southern US include cotton mice (*Peromyscus gossypinus*), cotton rats (*Sigmodon hispidus*), and eastern woodrats (*Neotoma floridana*) (Oliver et al. 2003). Other *Ixodes* spp., such as *Ixodes minor* and *Ixodes affinis,* have been implicated in enzootic transmission cycles of *B. burgdorferi*, but these species rarely bite humans (Oliver et al. 2003).

The distribution of *I. pacificus,* the vector for *B. burgdorferi* found on the West Coast, correlates with reports of Lyme borreliosis in areas where dusky-footed wood rats (*Neotoma fuscipes*), western grey squirrels (*Sciurus griseus*), deer mice (*Peromyscus maniculatus*), and California kangaroo rats (*Dipodomys californicus*) serve as reservoir hosts (Burkot et al. 1999; Dennis et al. 1998; Eisen et al. 2006). Rodents and the principally nidiculous *I. spinipalpis* (Burkot et al. 1999) are the main components in the natural infection cycle (Dennis et al. 1998; Oliver et al. 2003), and infection is transmitted to the bridge vector *I. pacificus* when this tick co-feeds with *I. spinipalpis* on dusky-footed wood rats and deer mice (Burkot et al. 1999).

Ixodes pacificus immature ticks preferably feed on lizards, such as the western fence lizard (*Sceloporus occidentalis*) and the southern alligator lizard (*Elgaria multicarinata*) (Lane and Loye 1989; Wright et al. 1998). The role of the lizard as a barrier host has been proposed to be the main reason for the low infection rate of nymphal *I. pacificus* (Tälleklint-Eisen and Eisen 1999). These two lizard species have been shown to not only lack reservoir competence for *B. burgdorferi*, but also their blood has been show to have immune-mediated borreliacidal activity (Kuo et al. 2000).

4. DIAGNOSIS

Four different techniques can be used to diagnose *B. burgdorferi* in clinical laboratories: 1) culture with Barbour-Stoenner-Kelly II (BSK-II) medium (Kelly 1971), 2) detection of *Borrelia burgdorferi*-specific antibodies (towards the C6 protein encoded for by the VlsE), 3) detection of *Borrelia burgdorferi*-specific antibodies via Western Blot, or 4) targeted PCR (Aguero-Rosenfeld et al. 2005). In endemic areas of the USA, presence of physician-identified EM following the bite of an *Ixodes scapularis* (black-legged tick) is considered pathognomonic for acute Lyme borreliosis even in the absence of laboratory confirmation (CDC 1997; Nichol et al. 1998). No chemical or hematological change is characteristic for infection with *B. burgdorferi*. Due to an inflammatory reaction abnormalities in protein concentration or white blood cell count may be observed in body fluids like cerebrospinal fluid (CSF), urine or synovial fluid, but molecular analysis or culture is necessary to attribute these findings to infection with a specific pathogen.

5. ANIMAL MODELS

To reproduce clinical signs associated with the different stages of human Lyme disease, several animal models have been established, each reflecting certain aspects of the disease, but none covering the full spectrum. Non-human primates (Philipp et al. 1993), dogs (Magnarelli et al. 1987; Appel et al. 1993), New Zealand White rabbits (Burgdorfer et al.1984), and mice (Schaible et al.1989) are amongst the most commonly used species. C3H/HeJ mice are predominantly established for the study of carditis and arthritis following infection with *B. burgdorferi* (Barthold et al. 1990; Barthold et al. 1991).

Erythema migrans following *B. burgdorferi* infection has been described in non-human primates, specifically rhesus monkeys (*Macaca mulatta*), and baboons (*Papio* spp.), and New Zealand white rabbits (Burgdorfer et al. 1984; Kornblatt et al. 1984a; Kornblatt et al. 1984b; Philipp et al. 1993; Foley et al. 1995; Roberts et al. 1998; Hefty et al. 2002). Lesions appear several days after intradermal inoculation of cultured spirochetes or attachment and feeding of infected ticks and expand gradually; secondary EMs are described (Kornblatt et al. 1984; Foley et

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al. 1995; Hefty et al. 2002). For the study of erythema migrans, the New Zealand white rabbit (NZW) is the animal model of choice based on size, reproducibility, and ease of maintenance. Skin lesions in rabbits macroscopically resemble human EM lesions, show histological resemblance to human EMs, and *B. burgdorferi* is readily cultured from rabbit skin biopsies and other tissues (Foley et al. 1995).

III. SOUTHERN TICK-ASSOCIATED RASH ILLNESS OF UNKNOWN ETIOLOGY

1. INVESTIGATED / PROPOSED ETIOLOGIC AGENTS

a. *Borrelia burgdorferi*

Borrelia burgdorferi is known to cycle in nature in the southern United States, where a competent vector of the spirochete, *Ixodes scapularis*, is also present (Luckhart et al. 1991; Kardatzke et al. 1992; Luckhart et al 1992, Mukolwe et al. 1992b; Oliver et al. 1995; Sanders and Oliver 1995). Although *Ixodes scapularis* inhibiting the southern United States are rarely infected with *B. burgdorferi* (Magnarelli et al. 1986; Piesman and Sinsky 1988; Luckhart et al. 1991; Kardatzke et al. 1992), these ticks are able to acquire and transmit southeastern (Sanders and Oliver 1995) and northeastern isolates (Piesman and Sinsky 1988; Jacobs et al. 2003) of this pathogen. Moreover, infection in a patient in the southern US has not been laboratory confirmed, leaving the existence of autochthonous Lyme borreliosis in this region questionable (Wormser et al. 2006). To date, *B. burgdorferi* has not been recovered from STARI patients (Wormser et al. 2005 a; Wormser et al. 2005b).

STARI is consistently associated with *A. americanum*. However, laboratory studies have shown that the LST is an inefficient vector for *B. burgdorferi* (Piesman and Sinsky 1988; Mather and Mather 1990; Mukolwe et al. 1992a; Oliver et al. 1993; Sanders and Oliver 1995; Piesman and Happ 1997). One reason for this inefficiency might be that the number of *B. burgdorferi* exposed to saliva of *A. americanum* has experimentally been shown to have a borreliacidal effect leading to a decrease by 87% within 48 hours as determined by fluorescent microscopy after

staining (Ledin et al. 2005). However, reports of infection of LST with *B. burgdorferi* in southern states (Texas, Alabama, Missouri) as determined by culture, indirect immunofluorescence (IFA), direct immunofluorescence, and/or PCR, exist in the literature (Levine et al. 1991; Luckhart et al. 1991; Teltow et al. 1991; Luckhart et al. 1992; Feir et al. 1994; Strohmdahl et al. 2001; Clark 2004; Taft et al. 2005).

b. *Borrelia lonestari*

First discovered in LST in the 1980s, spirochetes identified by dark-field microscopy and direct immunofluorescence in LST collected in a Lyme endemic area were believed to be *Borrelia burgdorferi* (Schulze et al. 1984). The same conclusion was drawn by investigators examining LST collected from white-tailed deer from North Carolina via direct FA staining in 1986 (Magnarelli et al. 1986). It was not until 1996 that phylogenetic analysis revealed the presence of a spirochete distinct from *B. burgdorferi* in LST, which was named *Borrelia lonestari* (Barbour et al. 1996). Later work showed that *B. lonestari* overlaps in its geographic distribution with LST, its only known vector (Moore et al. 2003, Murdock et al. 2009), and is present in 1 to 10% of ticks examined (Paddock and Yabsley 2007).

Borrelia lonestari became of great interest in association with the etiology of STARI. In 2001 this spirochete was amplified from the skin biopsy of a patient who presented with an erythema migrans at the site of a lone star tick bite (James et al. 2001); an observation that made *B. lonestari* the most probable putative agent of STARI. The organism was considered to be uncultivable (Barbour et al. 1996) until 2004, when it was successfully cultured in embryonic tick cell lines (ISE6) (Varela et al. 2004). The availability of culture material improved the ability to study the organism and its potential role in the etiology of STARI in detail (Varela et al. 2004). However, subsequent evaluation of 30 STARI patients failed to detect *B. lonestari*, leaving the 2001 report as the first and only one to date (James et al. 2001; Wormser et al. 2005a).

c. *Rickettsia amblyommii*

New *Rickettsia* species are continuously described (La Scola et al. 2009). To date, six members of the spotted fever group (SFG) of clinical interest have been reported from the United States: *R. massiliae*, *R. parkeri*, *R. honei, R. rickettsii*, *R. amblyommii, and R. akari* (Renvoisé et al. 2009). All six species are tick-borne with the exception of *R. akari* (Renvoisé et al. 2009). Common clinical findings associated with infection with members of this group range from mild to severe and include fever, maculopapular rash (often spreading from the extremities inwards, sparing the face), and eschar at the tick bite site (Parola et al. 2009; Renvoisé et al. 2009; Cragun et a. 2010). *Amblyomma* spp. have been implicated in the transmission of *R. parkeri*, *R. rickettsii*, *R. africae*, *R. amblyommii,* and *R. tamurae,* all of which have been associated with febrile illness (Guedes et al. 2005; Fournier et al. 2006; Renvoisé et al. 2009).

Rickettsia amblyommii was associated with STARI after being detected in a lone star tick which was removed from a patient who subsequently developed a rash (Billeter et al. 2007). The average prevalence of *R. amblyommii* in LST populations has been reported to be 41.2% with a range of 0 to 84.0% (Mixon et al. 2006), and a high prevalence of LST collected from human patients is infected with the pathogen (Jiang et al. 2010). Cross-reactivity of *R. amblyommii* and *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), are thought to account for a high seroprevalence for the spotted fever group *Rickettsia* within the geographical range of the LST (Apperson et al. 2008; Strohmdahl et al. 2008). However, evaluation of paired serum samples from STARI patients via IFAs did not show seroconversion to *R. amblyommii*. Therefore, this data does not support *R. amblyommii* as an etiologic agent of this condition (Nicholson et al. 2009).

2. *AMBLYOMMA AMERICANUM*

Amblyomma americanum has been shown to be an excellent vector for disease agents. All three stages (larvae, nymphs and adults) of this aggressive 3-host-tick feed on humans and a variety of other animals (Childs and Paddock 2003). Historically, the LST was restricted to the southeastern and south-central US, where it is the tick most commonly reported from humans. During the last decade there have been reports of this tick as far north as Maine (Keirans and Lacombe 1998; Ijdo et al. 2000), indicating an expansion in distribution (Paddock and Yabsley 2007). While white-tailed deer (*Odocoileus virginianus*) are the main host for all stages of LST, all three stages are found to feed on other wildlife as well as domestic animals, including companion animals and humans (Paddock and Yabsley 2007).

Amblyomma americanum is well established as the primary vector of multiple *Ehrlichia* spp., including *E. chaffeensis,* which causes human monocytotropic ehrlichiosis (HME) (Anderson et al. 1991; Anderson et al. 1993). HME is a potentially life-threatening disease that is predominantly reported from people in the southeastern US, but has also been reported from other parts of the United States as well as other countries (Miura and Rikihisa 2007). A minority of patients infected with the agent also develop a skin rash, which is more diffuse and more similar to rickettsial rashes than single distinct lesions expanding from the tick bite site (Dummler and Bakken 1995; Paddock and Childs 2003) The fatality rate of HME without treatment is estimated to be 2 to 3% (Olano et al. 2003). White-tailed deer are the primary reservoir host for *E. chaffeensis* (Dawson et al. 1994; Lockhart et al. 1997). A second agent, *E. ewingii*, which was originally suspected to be only a canine pathogen, infects neutrophils resulting in a clinical disease in people that is indistinguishable from that caused by *E. chaffeensis* (Buller et al. 1999). While infections with both agents are nationally notifiable conditions in the United States, *E. ewingii* has not yet been cultured limiting the diagnostic confirmation of infection to amplification by PCR. Therefore, data on the prevalence of human *E. ewingii* infection is not fully elucidated (Doudier et al. 2010). A third, newly recognized LST transmitted agent is the "Panola Mountain *Ehrlichia*" (PME), which is closely related *to Ehrlichia ruminantium* (Loftis et al. 2008) and was consecutively detected in a blood sample from a patient suffering from persistent neck pain

following a LST bite (Reeves et al. 2008). White-tailed deer have been identified as a reservoir host for PME (Yabsley et al. 2008) and it has been shown to be present throughout the range of the LST (Loftis et al. 2008).

Amblyomma americanum is also recognized as a vector for the causative agent of tularemia, *Francisella tularensis* (Hopla 1953; Hopla 1955; Hopla 1960). A broad spectrum of animals has been shown to be affected by this organism, whereas in the United States hares (*Lepus* spp.) and cottontail rabbits (*Sylvilagus* spp.) are key hosts (Farlow et al. 2005). The clinical presentation of infection with *F. tularensis* in human patients depends on the route of infection. Vector-borne infection generally results in the ulceroglandular or glandular form which is characterized by fever, ulcer at the site of bacterial exposure and enlargement of regional lymph nodes (Tärnvik 2007). Further, LSTs transmit *Theileria cervi,* an apicomplexan protozoa, which causes theileriosis in white-tailed deer fawns. When combined with heavy tick infestation and poor nutritional status infection with this parasite can contribute to severe clinical disease (Goddard and Varela-Stokes 2009).

3. DIAGNOSIS AND ANIMAL MODELS

Until an etiologic agent of STARI is identified, diagnostic testing of patients is restricted to negative established tests for known pathogens (Campbell et al 1995; Wormser et al. 2005a) and recognizing the clinical presentation in combination with a history of tick exposure (Masters et al. 2008). Prior to the work reported in the present dissertation, no animal models for STARI had been described.

IV. IMPORTANCE OF UNDERSTANDING THE ETIOLOGY OF ERYTHEMA MIGRANS FOLLOWING A TICK BITE

1. DISTRIBUTION OF LYME DISEASE

a. Human risk assessment

Within the United States, autochthonous transmission of *B. burgdorferi*, and the occurrence of Lyme disease is restricted to endemic areas. Based on established case definitions, human cases of Lyme disease are reported through a national surveillance system by county of residence. Therefore, reports from states outside the areas of endemicity are not considered acquired infections in those states. In fact, Lyme borreliosis is rarely, if ever, reported to be acquired from states south of Virginia and Maryland (Dennis 2005; Wormser et al. 2006). In 2009, the five states with the highest reported incidences (confirmed cases per 100,000 persons) were Delaware (111.2), Connecticut (78.2), New Hampshire (75.2), Massachusetts (61.0), and Maine (60.0) (CDC 2010). Over a ten year period (1999 to 2008), less than 4% of cases were reported from outside established endemic areas (CDC 2010). The total number of reported cases of Lyme disease increased in the US from 16,273 in 1999 to 29,959 in 2009 (CDC 2010).

b. Canine seroprevalence

Dogs are companion animals that typically live in close association with their owners but often have a lifestyle that leads to greater exposure to ticks in their usual habitats (Bowman et al. 2009). Indeed, dogs are estimated to be about six times more likely to be exposed to infected ticks than humans, underscoring their usefulness as sentinels (Johnson et al. 2004). Testing of canine patients without significant travel histories can give an insight into the presence of a pathogen in that area, although not necessarily the abundance or severity of risk for humans living in that area (Beall et al. 2008). Geographic mapping of exposure to *B. burgdorferi* in dogs shows great similarities with that of human case reports for Lyme borreliosis (Duncan et al. 2005; Bowman et al. 2009). However, as changes in climate and habitat might influence the distribution of reservoir hosts and tick vectors, endemic areas are subject to modification (Ogden 2006;

Bacon et al. 2008; Bowman et al. 2009). Monitoring of exposure-specific antibody responses in canines may aid in recognizing changes in human exposure risk (Little et al. 2010)

2. DISTRIBUTION OF STARI

The true distribution of STARI cases is unknown but is thought to follow that for *A. americanum,* which is present throughout most of the eastern US (Keirans and Lacombe 1998; Ijdo et al. 2000; Paddock and Yabsley 2007; Masters et al. 2008). Without an etiologic agent or specific diagnostic testing, identification of this condition is based on clinical presentation (Masters and Donnell 1995) and lack of evidence of infection with other pathogens (Wormser et al. 2005a). Within areas where Lyme disease is endemic, an EM measuring at least 5 cm in diameter is considered to be a confirmed positive case of Lyme borreliosis for surveillance purposes; no additional diagnostic evidence is considered necessary (CDC 1997; Bacon et al. 2008). This approach allows STARI cases to be reported as Lyme disease cases (CDC 1997; Paddock and Yabsley 2007). Furthermore, the lack of a surveillance system for STARI cases makes spatial analysis or any conclusions regarding distribution or prevalence difficult. The distribution of STARI cannot be characterized further until an etiology is identified.

V. SUMMARY

STARI and Lyme borreliosis have a similar clinical presentation, with an expanding erythematous skin rash developing at the site of a tick bite. However, knowledge about the degree of similarity between these two conditions is lacking. Without detection of the causative agent for STARI and a national surveillance system, the current understanding of the spectrum of signs and symptoms associated with this condition are based on case reports. To date, no long-term study enrolling STARI patients has been done leading to a lack of knowledge about possible sequelae (Masters et al. 2008). This information is of great interest because Lyme borreliosis is associated with potentially severe chronic disease affecting the nervous system, the heart, and joints (Wormser et al. 2006). With the expanding distribution of the LST into areas where Lyme disease is endemic, the distinction based on geographic distribution becomes invalid which further emphasizes the need for diagnostic approaches to differentiate the two conditions.

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CHAPTER III

BACTERIAL DIVERSITY IN *AMBLYOMMA AMERICANUM* WITH A FOCUS ON MEMBERS OF

THE GENUS *RICKETTSIA*[1]

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ABSTRACT

The lone star tick, *Amblyomma americanum*, is commonly reported from people and animals throughout the eastern U.S. and is associated with transmission of a number of emerging diseases. To better define the microbial communities within lone star ticks, 16S rRNA gene based analysis using bacteria-wide primers, followed by sequencing of individual clones (n=449) was used to identify the most common bacterial operational taxonomic units (OTUs) present within colony-reared and wild *A. americanum*. The colony-reared ticks contained primarily sequences affiliated with members of the genus *Coxiella* (89%; 81/91), common endosymbionts of ticks, and *Brevibacterium* (11%; 10/91). Similarly, analysis of clones from unfed wild lone star ticks revealed that 96.7% (89/92) of all the OTUs identified were affiliated with *Coxiella*-like endosymbionts, as compared to only 5.1%-11.7% (5/98 – 9/77) of those identified from wild lone star ticks after feeding. In contrast, the proportion of OTUs identified as *Rickettsia* sp. in wild-caught ticks increased from 2.2% (2/92) before feeding to as high as 46.8% (36/77) after feeding, and all *Rickettsia* spp. sequences recovered were most similar to those described from the spotted fever group *Rickettsia*, specifically *R. amblyommii* and *R. massiliae*. Additional characterization of the Rickettsiales tick community by PCR, cloning, and sequencing of 17 kDa and GltA genes confirmed these initial findings and suggested that novel *Rickettsia* spp. are likely present in these ticks. These data provide insight into the overall, as well as the rickettsial community of wild lone star ticks and may ultimately aid in identification of novel pathogens transmitted by *A. americanum*.

INTRODUCTION

Amblyomma americanum, the lone star tick, is an important arthropod pest and disease vector that has dramatically increased in both number and geographic distribution in recent years largely due to expansion of white-tailed deer populations (Childs and Paddock 2003; Paddock and Yabsley 2007). Although deer serve as the keystone host for both immature and adult stages of this aggressive tick, lone star ticks also readily bite humans and a variety of other animals; indeed, *A. americanum* is the tick most commonly reported from people in the southern United States, accounting for up to 83% of human tick bites in some surveys (Felz et al. 1996). Historically restricted to relatively isolated pockets primarily in the southeastern and south-central United States, lone star ticks have expanded regionally and nationally in recent years, with populations now established as far north as Maine (Keirans and Lacombe 1998; Ginsberg et al. 2002; Childs and Paddock 2003; Paddock and Yabsley 2007). At the same time, a number of novel *A. americanum*-associated pathogens and diseases have been described in the last two decades (Ewing et al. 1971; Goddard and Norment 1986; Anderson et al. 1991; Kirkland et al. 1997; Reeves et al. 2008). This expanding geographic range together with our increasing awareness of the pathogens and diseases associated with *A. americanum* lends a sense of urgency to efforts to understand the bacterial diversity present in this arthropod in natural systems.

Amblyomma americanum has been associated with several human diseases, including ehrlichioses, spotted fever caused by rickettsial agents, and Southern Tick Associated Rash Illness (STARI). Lone star ticks are well established as the primary vector of at least three distinct ehrlichial pathogens: *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME) (Anderson et al. 1991), *E. ewingii*, the cause of human granulocytic ehrlichiosis (Buller et al. 1999), and an *E. ruminantium*-like organism referred to as the Panola Mountain *Ehrlichia* (PME) (Reeves et al. 2008). Human ehrlichiosis, particularly when caused by *E. chaffeensis*, is potentially life-threatening and is considered the most common tick-borne disease of people in the southern US (McQuiston et al. 2003; Olano et al. 2003; Manangan et al. 2007; Miura and Rikihisa 2007; Wimberly et al. 2008). The historical literature also links Rocky

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Mountain spotted fever caused by *R. rickettsii* with *A. americanum* (McDade and Newhouse 1986), but more recent data suggest another rickettsial agent, such as *R. amblyommii*, may be associated with a spotted fever like illness in people (Apperson et al. 2008).

Perhaps the most perplexing of the lone star tick-associated diseases is STARI, a Lyme borreliosis-like condition in which people develop an erythema migrans rash following the bite of a lone star tick which cannot be attributed to infection with *B. burgdorferi*, the only known cause of Lyme borreliosis in North America (Wormser et al. 2005a; Wormser et al. 2005b; Philipp et al. 2006). In endemic areas, erythema migrans following a tick bite is considered pathognomonic for Lyme borreliosis and a physician-diagnosed erythema migrans is considered sufficient evidence to warrant report as a confirmed case for surveillance purposes (CDC 1997). However, in areas where Lyme borreliosis is not known to be endemic, such as the southern US, the etiology of *A. americanum*-associated erythema migrans remains elusive (Blanton et al. 2008; Masters et al. 2008). Attempts to attribute STARI to infection with *B. lonestari*, a relapsing fever-like spirochete present in wild lone star ticks and associated with a single case of STARI, or with *R. amblyommii*, have not been confirmed with clinical microbiological studies to date (James et al. 2001; Wormser et al. 2005a; Wormser et al. 2005b; Billeter et al. 2007; Nicholson et al. 2009).

A full characterization of the spectrum of the bacterial community in *Amblyomma americanum* is an important first step to establish a basis for further investigations of this tick as a vector of known and yet to be discovered pathogens. Recently, multiple studies have convincingly demonstrated the ubiquitous presence of a *Coxiella*-endosymbiont in wild and colony-reared lone star ticks (Jasinskas et al. 2007; Klyachko et al. 2007; Zhong et al. 2007; Clay et al. 2008). However, attempts to characterize complete microbial communities in wild lone star ticks have not included colony-reared *A. americanum* as controls or evaluated changes in diversity of microbes induced by tick feeding (Clay et al. 2008). For the study reported here, our goal was to document differences in the degree of microbial diversity present in colony-reared ticks and wild caught ticks before and after feeding. The population of vector-borne pathogens increases within arthropod vectors in response to blood feeding (de Silva and Fikrig 1995; Azad and Beard 1998), and so we used blood feeding as a means to increase the proportion of

bacteria which were perhaps more likely to be transmitted to hosts. Our results show that microbial diversity is severely limited in colony-reared ticks, and somewhat restricted in unfed, wild caught ticks, but that feeding greatly increases the microbial diversity detected, particularly for the members of the Rickettsiales, in wild caught ticks which presumably fed on infected vertebrates in their immature stages.

MATERIALS AND METHODS

Samples / Source of Ticks

Colony-reared adult *A. americanum* were obtained from the Oklahoma State University Tick Laboratory (Stillwater, OK USA) which has maintained the same line of lone star ticks since 1976 with the last introduction of new colony material two years prior to this study. (L. Coburn, personal communication). Wild adult lone star ticks were collected from Lake Carl Blackwell Recreational Area, OK (Site 1) and Panola Mountain State Park, GA (Site 2) in May of 2008 using dry ice traps and drags as previously described (Lockhart et al. 1997). A subset of 100 adult lone star ticks from Site 1 were fed in two groups of 50 (25 males and 25 females) on each of two pathogen-free Class A beagle dogs that had been shown to be free of infection with all pathogens of interest prior to tick feeding as determined by specific PCR for *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and *Borrelia* spp. on whole blood as described below. In addition, dogs were evaluated for evidence of antibodies to *Ehrlichia, Anaplasma,* and *B. burgdorferi* via a commercial ELISA (SNAP 4Dx, IDEXX Laboratories, Inc., Westbrook, ME) according to manufacturer's instructions, and by indirect fluorescent antibody assay for *Rickettsia rickettsii* using a commercial laboratory service (Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK). A subset of 100 colony-reared lone star ticks were fed in two groups of 50 (25 males, 25 females) of each of two specific-pathogen free New Zealand white rabbits. Ticks were allowed to feed until females were replete and then collected for PCR.

Nucleic acid extraction

From each of the pools of unfed adults, 100 adults (50 males and 50 females) were dissected and all internal tissues transferred into a sterile 1.5 ml tube containing 0.8 ml 1X PBS and total DNA was extracted using a commercially available kit (FastDNA® SPIN Kit for Soil, MP Biomedicals, Solon, OH) according to the manufacturer's instructions. From the two pools of fed ticks (Site 1, colony-reared), ticks were dissected and all internal tissues and contents from feeding transferred into a sterile vial containing 1X PBS. Due to large differences in body mass post feeding, fed males and fed females were pooled and evaluated separately. The volume of PBS used was adjusted according to body weight of the ticks dissected to reach the same ratio (body weight:PBS). Total DNA was extracted from fed ticks via a modified lysis bead-beating protocol as previously described (Dojka et al.1998). Total nucleic acid was extracted from 100 micro liters of whole blood using the GFX genomic blood DNA purification kit (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's directions.

PCR amplification, cloning, and sequencing.

Multiple DNA targets were utilized to identify organisms in these samples, including bacteria-wide 16S rRNA gene, *Rickettsia*-specific 17 kDa fragment, and rickettsial citrate synthase gene (gltA). All pools were also tested for individual organisms using PCR protocols established for *Borrelia* spp., *E. chaffeensis*, *E. ewingii*, *E. ruminantium*, *A. platys*, and *A. phagocytophilum*. All primers, amplification protocols, and references are listed in Table1.
A 16S rRNA gene fragment (~800bp) was amplified as previously described (Elshahed et al. 2003) from the bulk community DNA of each *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia ruminantium*, *Anaplasma platys* and *Anaplasma phagocytophilum*. All primers, amplification protocols, and references are listed in Table 1.

A 16S rRNA tick pool using primers 8F and 805R (Table 1) in 50µl reactions containing the following (final concentrations): 26.7µl pure water, 10µl of 5X buffer (Promega), 5µl $MgCl_2$, 2µl

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deoxynucleoside triphosphate mixture (10µM), 2 µl of the 8F and 805R primers (Invitrogen Corp., Carlsbad, CA), and 1µl (1.5 units) of GoTaq Flexi DNA polymerase (Promega, Madison, WI), and 2 µl DNA. PCR amplification was carried out on a Gene Amp PCR system 9700 thermocycler. The 16S rRNA gene amplification used a protocol involving initial denaturation for 5 min at 94℃ and 39 cycles of 92℃ for 0.5 min, 50℃ for 1 min, and 72℃ for 2 min, followed by a final extension at 72℃ for 20 min.   PCR products obtaine d were visualized on a 2% agarose electrophoresis gel.

Amplification of the 17 kDa fragment (434bp) of *Rickettsia sp.* was achieved using a nested PCR protocol using the external primers 17K-5 and 17k-3 (Table 1) in a 25 µl reaction that contained 13.5 µl molecular biology-grade water, 2.5 µl 25µM MgCl2 (Roche Applied Biosystems, USA), 2.5 µl 10X PCR Gold Buffer (Roche), 0.25 µl 10µM dNTPs (Promega), 0.5 µl of each primer (10 µM), 0.25 µl AmpliTaq Gold (Roche), and 5 µl of sample DNA.   Cycling conditions in the primary reaction were 95°C for 3 min followed by 40 cycles of 95 °C for 15 sec, 48°C for 30 sec, 70°C for 30 sec, followed by 72°C for 7 min. For the secondary reaction, 5 µl of primary product was used as template in a 25 µl reaction containing the same PCR components with the exception of the primers 17kD1 and 17kD2 (Table 1) and cycling conditions in the secondary reaction were 95°C for 3 min followed by 40 cycles of 95 °C for 15 sec, 50°C for 30 sec, 70°C for 30 sec, followed by 72°C for 7 min.

To detect the citrate synthase (gltA) fragment (380bp) of *Rickettsia* sp., PCR primers RpCS.877 and RpCS.1258 (Table 1) were used in a 25 µl reaction that contained 17.5 µl molecular biology-grade water, 2.5 µl 25µM MgCl2 (Roche Applied Biosystems, USA), 2.5 µl 10X PCR Gold Buffer (Roche), 0.25 µl 10µM dNTPs (Promega), 0.5 µl of each primer (10 µl), 0.25 µl AmpliTaq Gold (Roche), and 1 µl of sample DNA.   Cycling conditions were 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, followed by 72°C for 5 min.

The amplicons produced in all reactions were directly cloned using a TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmids from a total of 96 clones from each amplification protocol and each pool were submitted for sequencing

(1,152 total sequence requests); sequencing and assembly procedures were performed at the Advanced Center for Genome Technology at the University of Oklahoma as previously described in detail (Elshahed et al. 2003).

Phylogenetic analysis

For each of the 3 gene targets (16S, gltA, 17 kDa), sequences were initially compared to entries in GenBank database using BLASTnr (Altschul et al. 1997) for rough determination of phylogenetic affiliation. Sequences were also aligned in Greengenes NAST aligner to a 7,862-character global alignment (DeSantis et al. 2006b), and ran through Greengenes classifier (DeSantis et al. 2006a). In addition to Greengenes classifier output, the NAST-aligned sequences were imported to Greengenes May 2007 ARB database and added to the ARB universal dendogram using ARB parsimony function to determine their position in the global phylogenetic tree (Ludwig et al. 2004).

For OTU assignment and phylogenetic tree construction, sequences were aligned using ClustalX (Thompson et al. 1997), and the alignments were exported to PAUP (Version 4.01b10; Sinauer associates, Sunderland, Mass). A pair-wise distance matrix generated in PAUP was exported to DOTUR (Schloss and Handelsman 2005), and used for assignment of operational taxonomic units (OTUs) at the standard 97% sequence similarity cutoff for analysis of the 16S sequences, 98% for *Rickettsia*-specific 17 kDa gene, and 99% for the gltA *Rickettsia* gene. The cutoff for 16S rRNA gene sequences was based on widely accepted species cutoff values for such gene (DeSantis et al. 2006b). The cutoffs for 17 kDa and gltA rickettsial genes was based on typical average sequence divergence between gltA and 17 kDa genes identified in various rickettsial genomes and PCR based analysis of pure cultures of rickettsial species. Basic diversity measurements e.g. Shannon index, average nucleotide diversity, and Good's coverage were calculated as previously described (Good 1953; Martin 2002; Magurran 2004). Phylogenetic trees were constructed using OTUs from this study as well as representatives of closely related reference sequences. Distance neighbor joining trees with no corrections, F-84

corrections, and Jukes-Cantor corrections were constructed using PAUP, and all gave similar tree topologies.

Nucleotide sequence accession numbers

Sequences generated in this study were deposited in GenBank database under the accession numbers GQ302888-GQ302959.

RESULTS

Bacterial diversity in unfed colony-reared and wild-caught *Amblyomma americanum*

In the clone library from colony-reared lone stars, the majority of 16S rRNA gene sequences obtained (80/91 clones, 20 OTUs, 87.9%) were members of the genus *Coxiella*, class γ- *Proteobacteria* (Figure 1). These clones were most closely related (94 – 98 % sequence similarity) to *Coxiella* endosymbionts of *A. americanum* (Figure 2) reported in previous studies examining bacterial diversity in lone star ticks (Clay et al. 2008; Jasinskas et al. 2007; Klyachko et al. 2007; Zhong et al. 2007). In addition to *Coxiella*-affiliated clones, a few 16S rRNA gene sequences (10/91 clones, 3 OTUs) belonged to the family *Brevibacteriaceae*, within the phylum *Actinomycetes,* with their closest relatives being members of the aerobic, heterotrophic genus *Brevibacterium* (97% sequence similarity to *Brevibacterium avium* strain 3055, isolated from diseased poultry (Y17962) (Pascual and Collins 1999) (Figure 1, Figure 2).

Both populations of wild lone star ticks evaluated from two distant geographical regions within the US (Oklahoma, Georgia) had similar bacterial communities, with 16S rRNA gene sequences of *Coxiella* endosymbionts again being the most abundant (94.4% Georgia and 96.7% Oklahoma) (Figure 1). While the most abundant bacterial group within both colony-reared and wild ticks from two distinct locations were similar, the less abundant members were completely different. No *Brevibacteriaceae*-affiliated sequences were identified in the wild caught-ticks.

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Instead, sequences affiliated with the genus *Acinetobacter* (Order *Pseudomonales*, *γ-Proteobacteria)*, as well as spotted fever group *Rickettsia* spp. (closely affiliated to *R. amblyommii,* and *R. massiliae*) were identified as the minor components of the bacterial communities (Figure 1, Figure 2). No significant differences in diversity estimates were observed between wild and colony reared ticks (Table 2).

Change in microbial population distribution upon feeding adult ticks

Analysis of sequence of 175 clones (98 clones from pooled male ticks and 77 clones from pooled female ticks) from fed, wild caught ticks (Site 1) showed that blood feeding led to significantly higher diversity, when compared to unfed controls. In addition to observing previously undetected phylogenetic groups upon feeding (Figure 1b), diversity estimates clearly indicate that feeding results in an increase in the community diversity within lone star ticks, as evident by increase in diversity indices (Shannon, ACE), as well as the decrease in the library coverage (Table 2).

In both male and female wild ticks, feeding resulted in a significantly lower proportion of *Coxiella* (Figure 1), and hence a more even bacterial distribution resulting in a higher ACE estimate. In addition, feeding resulted in the identification of various OTUs not detected in clone libraries from unfed ticks, including members of the genus *Pseudomonas* (Order *Pseudomonales, γ-Proteobacteria*), members of the genus *Methylobacterium* (class *α-Proteobacteria)*, as well as sequences affiliated with the genus *Stenotrophomonas*, family *Enterobacteriaceaea*, and families *Micrococcaceaea,* and *Bacillaceaea* within the Phylum *Firmicutes*. Most importantly, the proportion of clones affiliated with the genus *Rickettsia*, order *Rickettsiales,* within the *α-Proteobacteria* significantly increased, particularly in the fed females (2.2% to 46.8%). Rickettsial clones belonged to the spotted fever group, with *R. amblyommii*, *R. massiliae* and *R. rickettsii str. Iowa* the closest relatives to our sequences (Figure 1, Figure 3). Although *Coxiella* (20%) and *Bacillaceaea* (23.6%), and a diverse array of other bacteria were present in the colony raised ticks after feeding, no sequences associated with members of the genus *Rickettsia,*

Pseudomonas, Methylobacterium, Stenotrophomonas, Enterobacteriaceaea, or *Micrococcaceaea* were found in the colony-raised fed ticks (data not shown).

Characterization of rickettsial populations in wild *A. americanum* before and after feeding using 17 kDa and gltA as group specific targets

Since members of the *Rickettsia* gp., a lineage with multiple pathogenic members, was stimulated by feeding, we decided to further characterize the rickettsial population in ticks using two Rickettsiales-specific phylogenetic markers. To this end, we cloned and sequenced PCR products of genes encoded outer membrane protein (17 kDa) and citrate synthase (gltA) in DNA pools from wild ticks (Site 1) before and after feeding.

In general, both phylogenetic trees from both gene sequences gave similar topologies, with rickettsial populations identified that belong to two main lineages. The first lineage is comprised of close relatives of the *R. amblyommii* and *R. japonica* group, and the other is a relatively minor component which may represent a novel rickettsial species. Analysis of results using both targets revealed a higher proportion of clones within the *R. amblyommii* group. In unfed ticks, 56.3% (17 kDa) – 80% (gltA) were *R. amblyommii* affiliated; upon feeding, this proportion increased to 95.2% (17 kDa) - 95.6% (gltA).

Other pathogenic microbes described from lone star ticks

All tick pools (before and after feeding) were tested for specific pathogens using established PCR protocols and were found to be PCR negative for *Ehrlichia chaffeensis*, *E. ewingii*, *E. ruminantium*, *Anaplasma* spp*., B. burgdorferi*, and *B. lonestari.* In addition, both dogs failed to seroconvert to *Ehrlichia*, *Anaplasma*, *B. burgdorferi*, or *Rickettsia.*

DISCUSSION

In this study, we characterized the spectrum of bacteria present in *Amblyomma americanum* from an established laboratory colony and compared it to that found in wild lone star ticks before and after feeding in order to identify both common and less frequent components of the natural tick micro fauna. As expected from previous reports (Jasinskas et al. 2007; Klyachko et al. 2007), *Coxiella* was very commonly identified in both colony-reared and wild caught ticks although the proportion decreased upon tick feeding due to increase in other organisms, most notably *Rickettsia* spp. (Figure 1). The near-ubiquitous presence of *Coxiella spp.* in multiple-organ systems suggests that these might be obligate endosymbionts for lone star ticks. A reduced genome compared to the pathogen *C. burnetii,* as well as reduced reproductive success of lone star ticks without *Coxiella,* support this hypothesis (Jasinskas et al. 2007; Klyachko et al. 2007; Zhong et al. 2007). Colony ticks, but not wild ticks, also harbored *Brevibacterium*, a gram-positive soil organism not previously described from lone star ticks (Clay et al. 2008). Because it was absent from wild ticks, we suspect this organism may be present or maintained as a result of the colony rearing process.

Wild lone star ticks, which presumably feed on a variety of wild vertebrates as larvae and nymphs and thus acquire a complex micro fauna, harbored bacteria not found in the colony ticks, namely spotted fever group *Rickettsia* spp. and *Acinetobacter* sp.. Another recent study investigated the bacterial community of unfed adult lone star tick and identified four bacterial components: the *Coxiella* endosymbiont, *R. amblyommii*, *Pantoea agglomerans*, and an *Arsenophonus* endosymbiont (Clay et al. 2008). However, numbers of clones evaluated, relative proportion of the population comprised by each bacteria, and overall diversity estimates were not reported (Clay et al. 2008), precluding direct comparison to our results. *Acinetobacter,* a gram-negative soil organism occasionally associated with nosocomial infections, was previously reported from larval but not adult lone star ticks (Clay et al. 2008).

Upon blood feeding, the microbial diversity in the wild *A. americanum* increased, allowing detection of organisms not identified in the ticks prior to feeding, including those in the genera *Pseudomonas, Stenotrophomonas,* and *Methylobacterium.* Members of the closely related

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genera *Pseudomonas* and *Stenotrophomonas* are widespread gram-negative bacteria that can induce clinically relevant opportunistic infections (Toleman et al. 2007; Crossman et al. 2008; Looney et al. 2009); both genera have been previously reported from Ixodid ticks (Benson et al. 2004; Moreno et al. 2006). To our knowledge, this is the second report of *Pseudomonas* spp. (Clay et al. 2008) and the first of *Stenotrophomonas* from lone star ticks. *Methylobacterium* spp. are commonly found in soil and on the surface of plants; infection of immunocompromised individuals has been reported but this genus has not been previously identified in ticks although another Rhizobiales (*Ochrobactrum* sp.) was reported from larval lone star ticks (Clay et al. 2008). Fed adult *A. americanum* in the present study also harbored bacteria in the families Bacillaceaea, Enterobacteriaceaea, and Micrococcaceaea. Two other members of the Enterobacteriaceaea, *Enterobacter agglomerans* and an *Arsenophonus* endosymbiont were reported from unfed lone star ticks previously (Clay et al. 2008) but the sequences from our *A. americanum* were distinct.

Male and female ticks differed, in some instances dramatically, in both type and the relative proportion of bacteria present. Overall, fed male lone star ticks had a comparatively smaller proportion of *Rickettsia* spp. and more bacteria that are thought to be environmental or commensals. For example, *Stenotrophomonas* was much more common in male (81.6% of sequences) than female (2.6% of sequences) lone star ticks, suggesting surface contamination may have been responsible for its presence. In contrast, *Rickettsia* spp. and the *Coxiella* endosymbiont comprised a larger share of the sequences found in fed female ticks, an observation consistent with the fact that these organisms are transovarially transmitted and increase in number with tick feeding (de Silva and Fikrig 1995; Azad and Beard 1998; Macaluso et al. 2001; Stromdahl et al. 2008; Zanettii et al. 2008)

Analysis of *Rickettsia* spp. by 3 different genes (16S, 17 kDa, gltA) showed at least three distinct spotted fever group *Rickettsia* were present in these ticks. Known pathogens in this group share a common clinical presentation, including fever, headache and dermal rash, which can be combined with an eschar at the site of the tick bite (Parola et al. 2009). Sequences from our ticks were most closely related to *R. amblyommii*, *R. massiliae,* and *R. rickettsii*. Additionally,

our analysis revealed a higher proportion of clones within the *R. amblyommii* group in the fed

ticks, suggesting that this group is selectively stimulated by feeding or that it has a higher relative

growth rate than other *Rickettsia* species. Further, it appear that feeding selects for a single or

very few OTUs within this group, as evident by the prevalence of a single OTU in fed libraries of

both genes, as opposed to a relatively more diverse, even distribution within unfed libraries of

both genes. Thus feeding appears to stimulate only specific strains with the rickettsial community

of *A. americanum*.

Rickettsia amblyommii is widely distributed in *A. americanum* populations, and is

suspected to account for a high rate of seropositivity to the spotted fever group due to cross-

reactivity (Apperson et al. 2008, Strohmdahl et al. 2008). This agent has been associated with a

rash in a human patient following a tick bite (Billeter et al. 2007) although its role as a human

pathogen has not yet been fully established (Parola et al. 2009). To date, *R. massiliae* has only

been associated with *Rhipicephalus sanguineus, R. turanicus* and *Ixodes ricinus* (Eremeeva et al.

2006; Fernandez-Soto et al. 2006; Ogawa et al. 2006; Blanc et al. 2007). To our knowledge, this

is the first report of *R. massiliae* in lone star ticks.

In North America, *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted

fever (RMSF), is vectored by *Dermacentor* spp. and, occasionally, *R. sanguineus* (Demma et al.

2006). However, *Amblyomma* spp. ticks infected with *R. rickettsii* have been reported from South

America (Guedes et al. 2005). Historic reports of lone star ticks as a vector of RMSF in North

America are thought largely due to the presence of related spotted fever group *Rickettsia* spp. in

A. americanum, such as *R. amblyommii* (Burgdorfer 1969). Our finding in this study of

sequences that most closely resemble, yet are distinct from, *R. rickettsii* (96%) suggests novel

spotted fever group *Rickettsia* species may be present in lone star ticks although additional

specific data are required to confirm that assertion. Novel spotted fever group *Rickettsia* continue

to be reported (Fournier et al. 2006, Fujita et al. 2006, Mediannikov et al. 2008), underscoring the

importance of continuing to pursue the role of these organisms as human and veterinary

pathogens.

Specific PCRs optimized for detection of *Ehrlichia* spp., *Anaplasma* spp., and *Borrelia* spp. did not identify these individual pathogens in these lone star ticks. These results were somewhat surprising as *E. chaffeensis*, *E. ewingii*, and *B. lonestari* are known to be present in white-tailed deer and lone star ticks from this area (Mixson et al. 2006). Pooling of the ticks prior to DNA extraction may have suppressed detection of infrequent organisms. As a basic rule, the greater the diversity within sequences isolated from a distinct microbial population, the more likely minor populations are masked (Schloss and Handelsman 2006). This concept is supported in the present study by the statistics shown in Table 2. Pooling ticks prior to analysis has been previously demonstrated to decrease prevalence estimates of *E. chaffeensis* infection in wild lone star ticks (Lockhart et al. 1997). Moreover, tick populations with the same geographical background show shifts in their pathogen load over time (Varela et al. 2004; Bacon et al. 2005), and thus at the time of collection these ticks may not have contained the pathogens.

Direct comparison of colony-raised, unfed and fed wild-caught adult *A. americanum* reflect the dynamics within the bacterial community associated with this tick. Environmental influences appear to have a less significant influence on the proportion inhibited by the different groups of bacteria compared to the feeding process. While some bacteria are acquired from the skin of the host, which is incorporated in the feeding lesion, feeding causes pathogens, especially *Rickettsia* spp. to multiply. The findings of this study establish a basis for further investigations on the background of various diseases in human and veterinary medicine associated with *A. americanum.*

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FIGURES AND TABLES.

Figure 1a. Bacterial community structure in colony reared and wild *A. americanum* from two different locations.
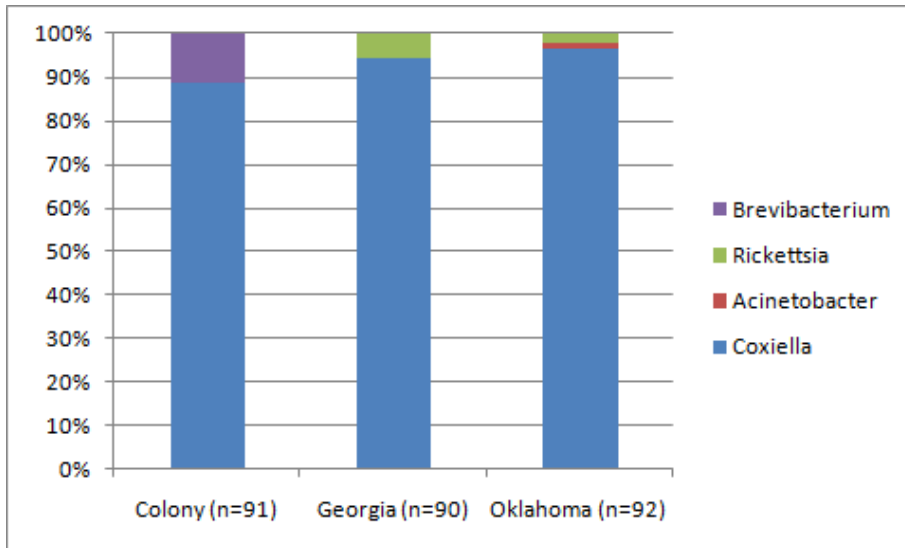
Figure 1b. Effect of feeding on bacterial community structure within male and female wild *A. americanum* collected from Lake Carl Blackwell, Oklahoma (Site 1).
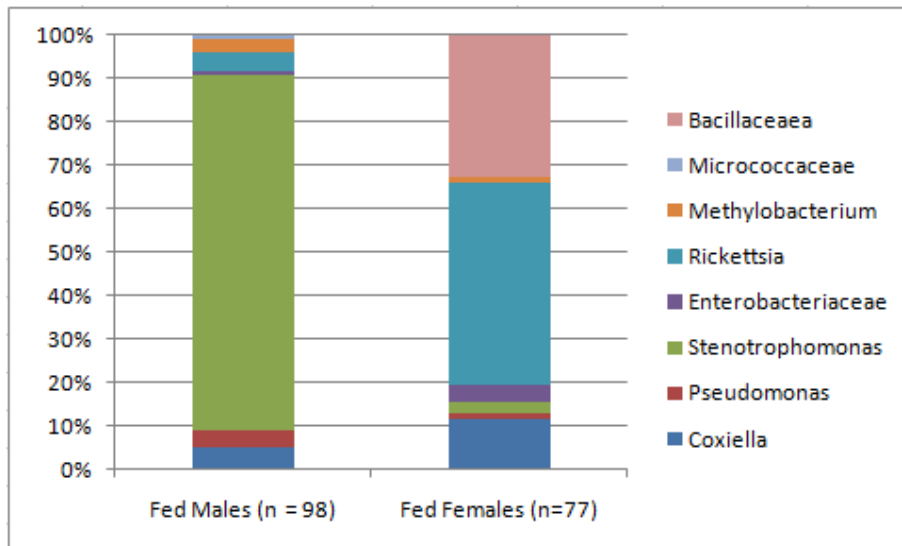
Figure 2. Distance neighbor-joining tree based on the 16S rRNA gene sequences of bacterial operational taxonomic units (OTUs) in colony reared and wild-caught lone star ticks (Site 1) before and after feeding encountered in clone libraries constructed in this study (excluding members of the order Rickettsiales). Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1000 replicates. The frequency of occurrence of each OTU is reported in parentheses.

Ornithodoros moubata symbiont (AB001521)
Haemaphysalis concinnae symbiont (AF521888)
Coxiella *Rhipicephalus sanguineus* symbiont (D84559)
Coxiella burnetii (M21291)
Haemaphysalis longicornis symbiont (AY342035)
UnfedColony19 (2)
UnfedWild3 **(5)**
UnfedColony1 **(50)**
FedWild25 **(1)**
FedWild19 **(6)**
UnfedWild1 **(54)**
Coxiella endosymbiont of *Amblyomma americanum* (AY939824)
FedWild16 **(4)**
Endosymbiont *Ifremeria nautilei* (Gastropoda) (AB238964)
Rickettsiella sp. GSU (AF383621)
Rickettsiella grylli (U97547)
Legionella anisa (AY744776)
Legionella pneumophila (AF122885)
Francisella tularensis (Z21931)
Pseudomonas aeruginosa (AY162139)
Pseudomonas plecoglossicida (EF544606)
FedWild33 **(1)**
FedWild3 **(4)**
Pseudomonas sp. CYN01B (AB175661)
Microbiota *Ctenocephalides felis* (Siphonaptera) (EF121344)
UnfedWild12 **(1)**
Acinetobacter junii (FM164636)
Acinetobacter calcoaceticus (AY800383)
Arsenophonus endosymbiont *Dermacentor variabilis* (AY265347)
Enterobactera glomerans (AF130887)
Klebsiella oxytoca (AB004754)
Escherichia coli (AJ567617)
Collimonas sp. CTO 113 (AY281151)
Burkholderia sp. NAH1 (AF247815)
Comamonas sp. D1 (AF532869)
FedWild2 **(63)**
FedWild29 **(1)**
Bacterium human skin clone nbt215e02 (EU536530)
Saperda vestita (Coleoptera) gut clone SV69AB2-1(AY770425)
UnfedWild7 **(2)**
FedWild14 **(1)**
Rickettsia amblyommii (U11012)
FedWild37 **(2)**
FedWild6 **(1)**
Rickettsia rickettsii (U11021)
Rickettsia parkeri (L36673)
Rickettsia conorii (U12460)
Anaplasma phagocytophilum (AY055469)
Ehrlichia chaffeensis (AF147752)
Neorickettsia risticii (M21290)
Acyrthosiphon pisum symbiont (Hemiptera) clone APe2_3 (AB074616)
Methylobacterium sp. G296-5 (AF395035)
FedWild17 **(1)**
FedWild4 **(2)**
FedWild27 **(1)**
Methylobacterium aquaticum (AJ635303)
Symbiont *Acromyrmex octospinosus* (Hymenoptera) (AF459799.1)
Bacterium *Ixodes scapularis* clone W2-7 (AY394615)
Brevundimonas bullata (AB023428)
Ochrobactrum sp. (AY331580)
Brevibacterium avium (Y17962)
Bacterium *Pachnoda ephippiata* (Coleoptera) gut clone PeH13 (AJ576331)
UnfedColony2 **(7)**
UnfedColony20 **(1)**
UnfedColony11 **(1)**
UnfedColony3 **(1)**
Bacterium ocean crust clone EPR3967-O2-Bc31 (EU491780)
FedWild10 **(1)**
Corynebacterium pseudogenitalium (X81872)
Corynebacterium renale (D37806)
FedWild23 **(2)**
FedWild31 **(1)**
Geobacillus pallidus (EU935594)
FedWild35 **(2)**
FedWild40 **(1)**
FedWild34 **(17)**
FedWild36 **(1)**
FedWild38 **(1)**
Bacillus sp. SD43 (DQ462180)
Bacillus sp. BAcB2 (AF497248)
Bacillus cereus (EF032682)
FedWild42 **(2)**
FedWild7 **(1)**
FedWild39 **(1)**
Borrelia lonestari (AY682920)
Spirochaeta africana (X93928)
Leptospira interrogans (EF536994)
Methanospirillum hungatei (M60880)

Coxiellaceae

Pseudomonales

Enterobacteriaceae

Burkholderiales

Rickettsiales

Methylobacteria

Actinomycetates

Bacillaceae

Proteobacteria

Spirochetes

0.05 substitutions/site

Figure 3a. Distance neighbor-joining tree based on the 16S rRNA gene sequences of operational taxonomic units (OTUs) affiliated with members of the order Rickettsiales encountered in clone libraries constructed in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1000 replicates. The frequency of occurrence of each OTU is reported in parentheses.
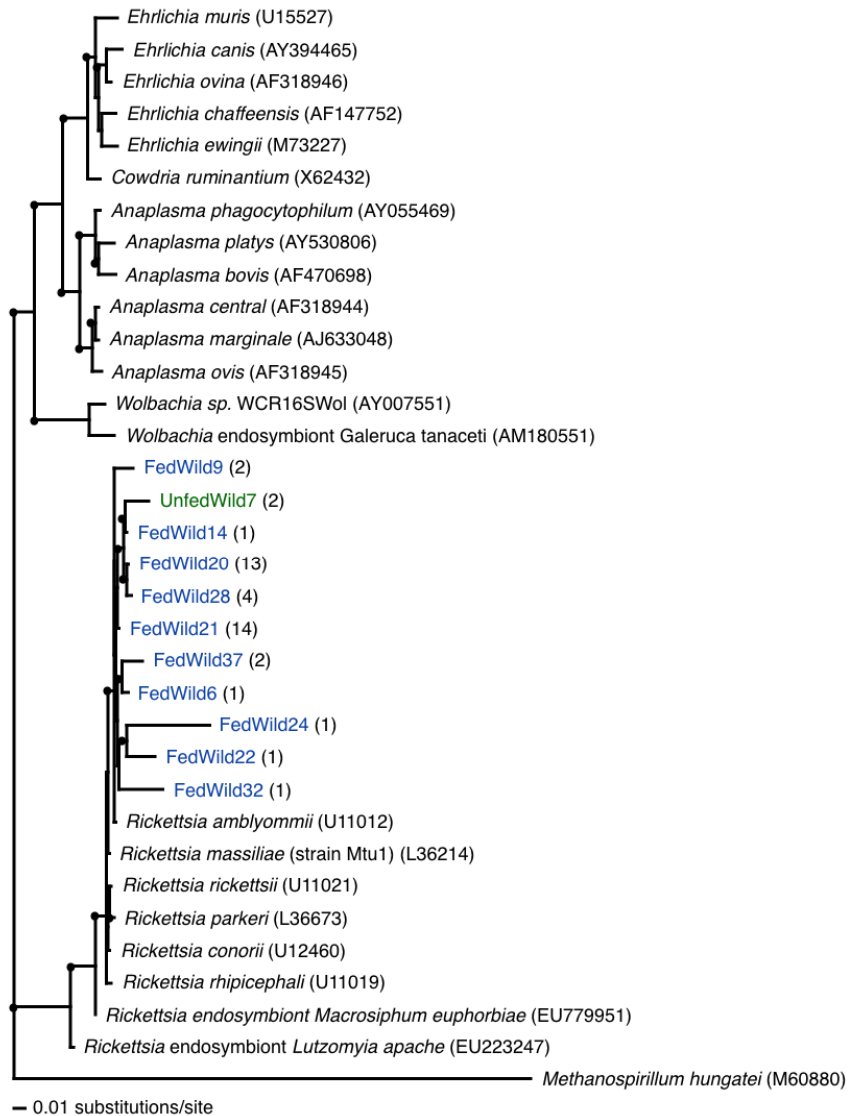
Figure 3b. Distance neighbor-joining tree based on the 17 kDa gene sequences obtained in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1000 replicates. The frequency of occurrence of each OTU is reported in parentheses.

Figure 3c. Distance neighbor-joining tree based on the gltA gene sequences obtained in this study. Bold circles indicate a bootstrap value of >50%. Bootstrap values were based on 1000 replicates. The frequency of occurrence of each OTU is reported in parentheses.

Table 1. Gene targets, primer sequences, and references for PCR protocols used in this study

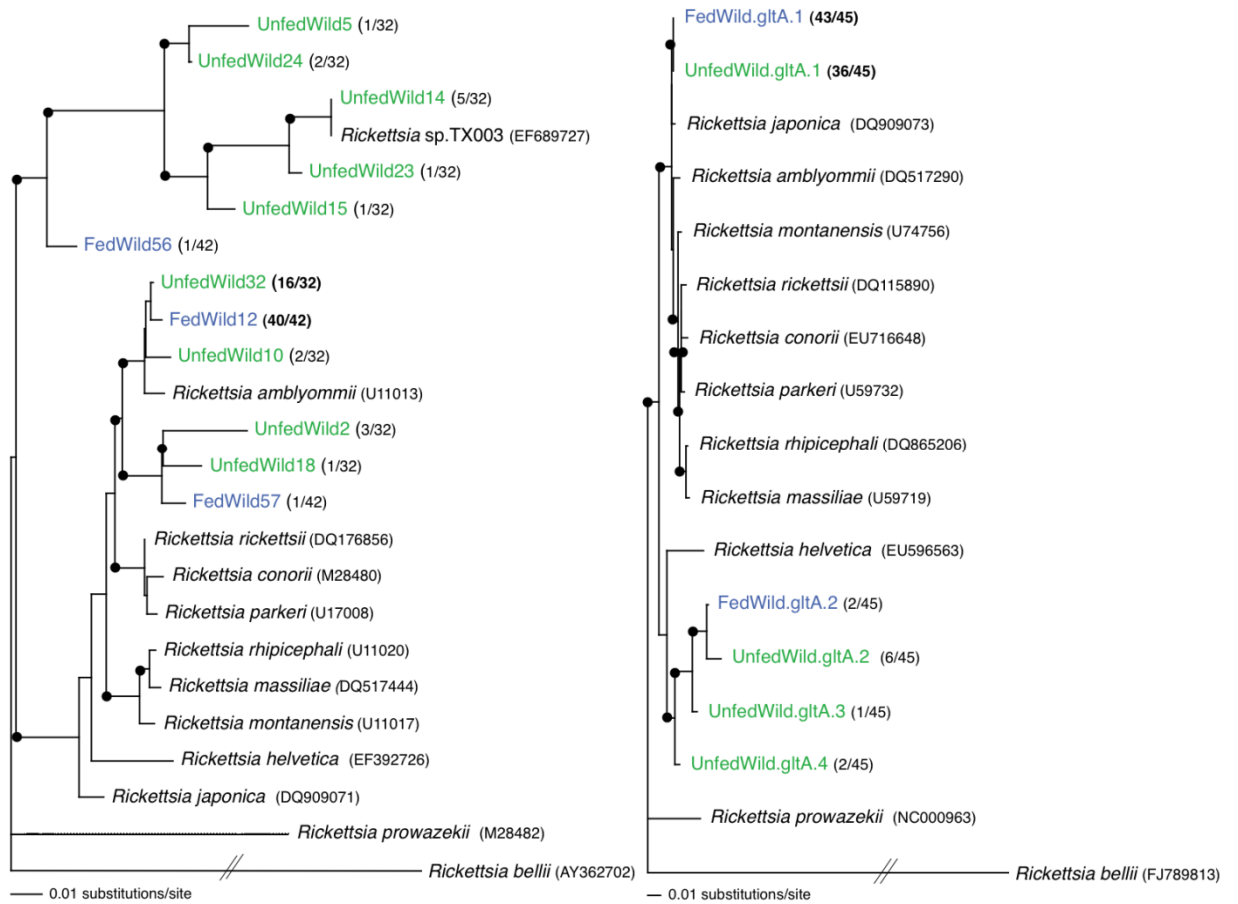| Targeted gene	Primer		Sequence	Reference
Bacteria-wide 16S rDNA	8F		5'-AGAGTTTGATCCTGGCTCAG-3'	Elshahed et al. 2003
	805R		5'-GACTACCAGGGTATCTAATCC-3'	
Borrelia flagellin (flaB)	FLALL	1°	5'-ACATATTCAGATGCAGACAGAGGT-3'	Barbour et al. 1996
	FLARL		5'-GCAATCATAGCCATTGCAGATTGT-3'	
	FLALS	2°	5'-AACAGCTGAAGAGCTTGGAATG-3'	
	FLARS		5'-CTTTGATCACTTATCATTCTAATAGC-3'	
Ehrlichia & _Anaplasma_ 16S rDNA	ECB	1°	5'-CGTATTACCGCGGCTGCTGGCA-3'	Dawson et al. 1996, Anderson et al. 1992a, Anderson et al. 1992b
	ECC		5'- AGAACGAACGCTGGCGGCAAGCC-3'	
E. chaffeensis 16S rDNA	HE3	2°	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	
	HE1		5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3'	
E. ewingii 16S rDNA	HE3	2°	5'-TATAGGTACCGTCATTATCTTCCCTAT-3'	
	EE72		5´-CAATTCCTAAATAGTCTCTGACTATT-3´	
A. phagocytophilum 16S rDNA	GE9f	2°	5'-AACGGATTATTCTTTATAGCTTGCT-3'	
	GA1UR		5'-GAGTTTGCCGGGACTTCTTCT-3'	
A. platys 16S rDNA	DGA	2°	5'-TTATCTCTGTAGCTTGCTACG-3'	
	GA1UR		5'-GAGTTTGCCGGGACTTCTTCT-3'	
Ehrlichia ruminantium citrate synthase	Ehr3CS-185F	1°	5'-GCCACCGCAGATAGTTAGGGA-3'	Yabsley et al. 2008
	Ehr3CS-777R		5'-TTCGTGCTCGTGGATCATAGTTTT-3'	
	Ehr3CS-214F	2°	5'-TGTCATTTCCACAGCATTCTCATC-3'	
	Ehr3CS-619R		5'-TGAGCTGGTCCCCACAAAGTT-3'	
Rickettsia 17 kDa	17k-5	1°	5'-GCTTTACAAAATTCTAAAAACCATATA-3'	Stothard 1995
	17K-3		5'-TGTCTATCAATTCACAACTTGCC-3'	
	17KD1	2°	5'-GCTCTTGCAACTTCTATGTT-3'	
	17KD2		5'-CATTGTTCGTCAGGTTGGCG-3'	
Rickettsia citrate synthase (gltA)	RpCS.877		5'-GGGGGCCTGCTCACGGCGG-3'	Regnery et al. 1991
	RpCS.1258		5'-ATTGCAAAAAGTACAGTGAACA-3'	

Table 2. Diversity estimates obtained from bacterial 16S rRNA clone libraries generated in this study

	clones (n)	Shannon	ACE average	Goods Coverage
colony-reared	91	2.01	48.2	87.9
Georgia	90	1.73	19.7	92.2
Oklahoma	92	1.44	24.8	93.5
fed ticks (OK)	175	2.59	68.9	64.3

CHAPTER IV

BACTERIAL COMMUNITY IN STARI ASSOCIATED *AMBLYOMMA AMERICANUM*,

OKLAHOMA[1]

_____

[1] Heise, S. R., M. S. Elshahed, T. C. Holbrook, and S. E. Little.
Submitted to Vector-Borne and Zoonotic Diseases.

ABSTRACT

Specific and bacteria-wide PCR was used to define the bacterial community in *Amblyomma americanum* nymphs collected at the residence of a STARI patient. Organisms most frequently identified were within the order Rickettsiales (*Rickettsia amblyommii*, *Rickettsia* spp., *Ehrlichia chaffeensis*) followed by Orders Rhizobiales and Acinetobacter. The etiology of STARI remains unknown.

INTRODUCTION

Amblyomma americanum, the lone star tick (LST), is the most common tick found on people in the southeastern and south-central United States and is known to transmit a number of pathogens (Childs and Paddock 2003). The LST is well established as the primary vector of both *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, causative agents of human ehrlichiosis, and has also been shown to transmit certain members of the spotted fever group *Rickettsia* (Childs and Paddock 2003). In addition, *A. americanum* feeding has been associated with development of erythema migrans (EM), a condition of unidentified etiology referred to as southern tick-associated rash illness (STARI) or Masters' disease (Masters et al. 2008). Putative agents of STARI based on individual case reports include *Borrelia lonestari* and *Rickettsia amblyommii* (James et al. 2001; Billeter et al. 2007).

Distinction between the clinical presentation of STARI and Lyme borreliosis (LB) is difficult and generally based on geographic correlations. In areas of North America where LB is endemic, lack of laboratory evidence of infection as seen in approximately 15% of patients with EM may be due to prompt treatment, precluding detection, or infection with another agent. In recent decades, the geographic range of LSTs has expanded into areas where LB is endemic (Murdock et al. 2009), suggesting some patients diagnosed with LB on the basis of EM alone likely have STARI instead. Without an etiologic distinctiveness, distinguishing between LB and STARI remains difficult.

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MATERIALS AND METHODS

In August of 2008, a 44-year-old man presented with a circular erythematous rash with distinct central clearing on the right scapular region (Figure 1). A LST nymph had been removed from the center of the affected area 3 days prior. The rash measured 8.25 cm by 5.75 cm, was mildly pruritic, and borders appeared slightly raised. Headache and mild fatigue were reported. After treatment with doxycycline (100 mg/day x 10days) the rash resolved without complications. The individual resides in an area with high tick populations (Lake Carl Blackwell, Oklahoma, 36N 8' 6.23"; 97W 13' 10.01"), spends the majority of his day outdoors, and lacked recent travel history. He estimates receiving several hundred tick bites annually without experiencing any complications before or since. The area immediately surrounding the home includes several acres of uncultivated land inhabited by companion animals, horses and wildlife, including white-tailed deer (*Odocoileus virginianus*).

Within the week of rash recognition, approximately 6,000 LST nymphs were collected within 2 hours from the immediate vicinity of the residence using dry ice traps. A subset of nymphs (n = 400) was fed on a pathogen-free class A dog. The nymphs were then surface sterilized by successive washing in 3% hydrogen peroxide, 95% alcohol, 0.1% sodium hypochlorite, and PBS. Fed nymphs (n=50) were tested individually by PCR for *Rickettsia* spp. (17 kDa gene), *Borrelia* spp. (flagellin B), and *Ehrlichia* spp. (16S rDNA, citrate synthase), and for bacteria-wide 16S rDNA-based screening as previously described (Heise et al. 2010). Two pools of 100 ticks each of unfed and fed nymphs, surface sterilized as described above, were used in 16S rDNA-based assays with subsequent phylogenetic analysis to characterize the bacterial community as previously described (Heise et al. 2010) and sequences generated deposited in GenBank (accession numbers HM010771 – HM010915).

RESULTS

In the bacterial clone library from unfed nymphs, the greatest proportion of 16S rDNA sequences (22/70 clones, 31.4%) were members of the genus *Rickettsia.* The majority of rickettsial clones from unfed nymphs (18/22) were most closely related (97-100% similarity) to *Rickettsia amblyommii.* The remaining 4 sequences were most closely associated with *Rickettsia* sp. described from other ixodid tick species. The second largest percentage of clones in unfed ticks (15/70 clones, 21.4%) belonged to the order Rhizobiales, followed by members of the genus *Acinetobacter* (9/70 clones, 12.9%).

Within the clone library constructed from fed nymphs, the percentage of clones affiliated with the genus *Rickettsia* increased from approximately a third to more than half (40/78 clones, 51.3%); all clones identified were affiliated with *Rickettsia amblyommii* (94-100% similarity). Members of the order Rhizobiales were still abundant (11/78 clones, 14.1%), and the percentage of the family *Methylobacteriaceae* increased from 4.3% in the unfed to 19.2% (15/78 clones). Out of 50 individually tested fed nymphs, one tick had *Ehrlichia chaffeensis* (2.0%) and 6 ticks had *Rickettsia amblyommii* (12%). No other specific agents were identified (*Borrelia* spp., *E. ewingii*, *E. ruminantium*). The dog on which the ticks were fed did not test positive for any of the agents considered and did not show seroconversion to *Rickettsia* spp. or *Ehrlichia* spp. (data not shown).

DISCUSSION

Despite efforts to identify the causative agent, STARI remains a perplexing clinical presentation without clear etiology (Masters et al. 2008). We identified molecular evidence of *Ehrlichia chaffeensis*, *Rickettsia amblyommii*, and other *Rickettsia* spp. in nymphs collected from the home of an individual with a presentation consistent with STARI. A minority of patients infected with *E. chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME), also develop a dermal rash, but the rash of HME is more diffuse and variable and not expanding from the tick attachment site (Paddock and Childs 2003).

Rickettsia amblyommii is widely distributed in *A. americanum* populations (Mixon et al. 2006; Heise et al., 2010) and serologic cross-reactions with *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, have been proposed to lead to a high rate of seropositivity to spotted fever group *Rickettsia* in patients with a history of LST exposure (Apperson et al. 2008). Furthermore, *R. amblyommii* has been associated with a STARI rash in a human patient (Billeter et al. 2007) although serologic testing of 8 STARI patients did not support etiologic correlation (Nicholson et al. 2009).

Other *Rickettsia* spp., similar but not identical in sequence to those described from ticks, were also identified in these ticks, suggesting novel *Rickettsia* spp. are present in LSTs. Novel *Rickettsia* spp. have been reported from LSTs and other *Amblyomma* spp. (*10*, FJ793521, EU826507, FJ269037, GU169050, DQ269439, AF483202, EF451001) and are sometimes associated with focal rashes with central vesicular eruptions or eschars. In contrast, STARI rashes resemble a target lesion with central clearing; eschars are not reported (Masters et al. 2008). In light of the frequent occurrence of diverse *Rickettsia* spp. in LST and some reports that STARI patients may respond to doxycycline (Masters et al. 2008), additional consideration should be given to the potential role of novel *Rickettsia* spp. in STARI. Although the clinical presentation of STARI has been recognized for more than 20 years (Masters et al. 2008), the causative agent remains unknown.

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FIGURES.

Figure 1. Circular rash (8.25 cm x 5.75 cm) in the right scapular region that developed around

the attachment site of a nymphal *Amblyomma americanum*.

CHAPTER V

DEVELOPMENT OF AN ANIMAL MODEL FOR

SOUTHERN TICK-ASSOCIATED RASH ILLNESS (STARI)[1]

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ABSTRACT

Southern tick-associated rash illness (STARI) is a Lyme disease-like illness of unknown etiology associated with the lone star tick (LST). To develop an animal model for STARI, wild-caught adult LSTs from an area where STARI is known to occur were allowed to feed on New Zealand white rabbits; colony-raised ticks were fed on additional rabbits as negative controls. Skin was evaluated daily for evidence of erythema migrans, and serum, whole blood, and skin biopsy samples were collected at 0, 7, 14, and 21 days post tick infestation (DPTI) and evaluated for evidence of infection via PCR using specific primers targeting known tick-borne pathogens as well as 16S rDNA bacteria-wide PCR followed by sequencing. A total of 4 distinct, circular expanding erythematous lesions 3.0-3.5 cm in diameter developed on rabbits infested with wild caught LSTs; lesions did not develop on rabbits that received colony-raised ticks. PCR revealed that wild-caught ticks contained both *Borrelia lonestari* and *Rickettsia amblyommii*, putative agents of STARI, but no specific pathogens were identified from the skin biopsy samples, a finding consistent with reports from human STARI patients.

INTRODUCTION

Southern tick-associated rash illness (STARI) refers to a condition in which people develop erythema migrans (EM) and mild constitutional signs following attachment and feeding of an *Amblyomma americanum* tick (Masters et al. 2008). The condition, also referred to as Masters' Disease, is widely recognized in patients from the southern U.S., where *A. americanum* is the most common tick found on people and Lyme borreliosis (LB) is rare or absent (Wormser et al. 2005, Wormser et al. 2006). Clinical diagnosis of STARI is based on development of EM following the bite of *Amblyomma americanum*, the lone star tick (LST) in an area where LB is not known to occur (Wormser et al. 2005). To date, patients with EM from outside LB endemic areas lack serologic, PCR, or culture evidence of *B. burgdorferi* infection (Philipp et al. 2006, Wormser et al. 2005).

At present, the etiology of STARI has not been identified. Putative agents based on individual case reports include *Borrelia lonestari* and *Rickettsia amblyommii* (James et al. 2001, Billeter et al. 2007). However, a comprehensive evaluation of 30 STARI patients from Missouri failed to identify evidence of *B. lonestari* infection (Masters et al. 2005), and paired serum samples from STARI patients tested negative for seroconversion to *R. amblyommii* (Nicholson et al. 2009). The situation is further complicated by an overlap in the occurrence of STARI and LB; in recent decades, populations of *A. americanum* have increased in areas where LB is endemic (Childs and Paddock 2003), suggesting some patients diagnosed with LB on the basis of EM alone likely have STARI instead. Without an etiology, distinguishing between LB and STARI remains difficult.

Erythema migrans following *B. burgdorferi* infection has been described in non-human primates and New Zealand white rabbits (Burgdorfer et al. 1984; Kornblatt et al. 1984; Foley et al. 1995; Roberts et al. 1998; Philipp et al. 1993; Hefty et al. 2002). The circular, erythematous rashes appear several days after either intradermal inoculation of cultured spirochetes or attachment and feeding of infected ticks. The rashes progressively expand around the site of inoculation; secondary EMs are also described (Kornblatt et al. 1984; Foley et al. 1995; Hefty et al. 2002). Although EM is not considered of major pathologic significance in LB, this lesion is the only objective evidence of STARI and the basis for confusion regarding the two conditions. In the present paper we describe macroscopic and microscopic lesions that developed around feeding sites of wild-caught, but not colony-reared, *A. americanum* ticks on New Zealand white rabbits and propose that this model for STARI be used in future work to investigate the etiology of this enigmatic condition.

MATERIALS AND METHODS

Source of ticks

In the spring of 2009, *Amblyomma americanum* adults were collected from a property of an individual presenting with erythema migrans associated with the bite of a LST near Lake Carl Blackwell, Stillwater, Oklahoma in the previous fall, using dry ice (CO_2) traps (Lockhart 1997). Adult ticks were freed from the tape and placed in cardboard cups in groups of 100. Until processing, the ticks were held in humidity chambers. Colony-reared adult *A. americanum* (n=200) were obtained from the Oklahoma State University Tick Laboratory (Stillwater, OK USA) and used as controls.

Ixodes scapularis nymphs (n=30) were obtained from the Oklahoma State University Tick Laboratory (Stillwater, OK USA). An 8-week-old female C3H/He mouse (Charles River Laboratories) was inoculated subcutaneously with 100 µl of culture containing 1 x 10^4 *B. burgdorferi*. At 7 days post tick infestation (DPTI), the mouse was infested with 30 *I. scapularis* nymphs as previously described (Almazán et al. 2003). Replete nymphs that had detached were collected twice a day and placed in a humidity chamber where they were allowed to molt. Ticks were held for 8 weeks after molting. Systemic infection of *Borrelia burgdorferi* in the mouse was confirmed by culture of heart, stifle joint and skin and PCR of heart, spleen, skin, and bladder collected on DPTI 15 in BSK-II media (Kelly 1971).

Infestation and monitoring of rabbits

Wild-caught or colony-reared adult *A. americanum* were fed on New Zealand white rabbits. Each rabbit was infested with 50 wild-caught adult ticks (n=2 rabbits) or 50 colony-reared ticks (n=2 rabbits) by placing the ticks under soft pads adhered to the shaved skin of the lateral thorax; 25 ticks were placed on each side of each rabbit. The pads were then covered by a 100-mm wide stockinet secured by elastic tape. As a positive control for erythema migrans caused by

B. burgdorferi, an additional rabbit was infested with adult *I. scapularis* (n=9) infected by feeding as nymphs on a mouse as described above.

Lesions associated with tick feeding were evaluated by visual inspection and documented by photographing (Canon PowerShot SD450 Digital ELPH, 3x lens, 5.0 mega pixels, Macro setting); the diameter of each observed EM was measured to the nearest 0.1 cm and recorded. Blood was collected into 7ml EDTA tubes (BD Vacutainer® K3 EDTA 12mg, Becton Dickinson and Company, Franklin Lakes NJ, USA) and 10ml serum tubes (BD Vacutainer® Serum, Becton Dickinson and Company, Franklin Lakes NJ, USA) from the ear vein of each rabbit using 23Gx 3/4" winged infusion sets (Surflo® winged infusion set, Terumo Corporation, Tokyo, Japan) prior to the study and once a week for the duration of the study. Sterile 6 mm punch biopsy samples were collected using sterile biopsy punches (Biopsy Punch 6mm, Miltex, Inc., York, PA, USA), forceps (Sterile disposable forceps, TWD TradeWinds, Inc., Pleasant Prairie, WI, USA) and surgical blades (Rib-Back® Carbon steel surgical blades number 11, Becton Dickinson and Company, Franklin Lakes, NJ, USA). On DPTI 0, 7, 14, and 21 biopsy samples were collected from each rabbit infested with *A. americanum* and DPTI 9, 13, and 21 from the rabbit infested with *I. scapularis*. On DPTI 0 biopsy samples were taken from the lateral area of the hind leg. Following tick infestation biopsy samples were taken from the margin of the lesions if present or from within the area of tick attachment when lesions were absent. On DPTI 42, rabbits were euthanatized and tissues (skin from the tick feeding site, ear, popliteal lymph node, stifle joint synovia, heart, lung, bladder, kidney, liver, spleen, spinal cord, brain) collected.

Assaying ticks and tissues for evidence of infection

PCR

Adult ticks were dissected using sterile surgical blades (Rib-Back® Carbon steel surgical blades number 20, Becton Dickinson and Company, Franklin Lakes, NJ, USA) and needles under a dissecting microscope. DNA was extracted via phenol-chloroform extraction using a previously published protocol. Unfed wild-caught ticks (n=100), fed wild-caught ticks (n=100), unfed colony-

raised ticks (n=100), and fed *Ixodes scapularis* (n=3) were dissected and DNA was extracted individually. Fed colony-raised ticks (n=87) were dissected and pooled for DNA extraction and PCR. Extracted nucleic acid from each LST or tick pool was analyzed using a nested PCR approach with primer sets specific for *Borrelia* spp. (flagellin B gene), *Rickettsia* spp. (17 kDa fragment of an outer membrane protein), *Ehrlichia chaffeensis* (16S rDNA) and *Ehrlichia ewingii* (16S rDNA). Positive PCR results were confirmed by sequencing as previously summarized. *Ixodes scapularis* were analyzed using *Borrelia* spp. specific primers (Heise et al 2010).

Culture

Skin biopsy samples from each rabbit infested with *A. americanum* were inoculated into DH82 cells and BSK-II medium for culture of *Ehrlichia* spp. and *Borrelia burgdorferi*, respectively. Skin biopsy samples from the rabbit infested with *I. scapularis* were inoculated into BSK-II medium. BSK-II medium (prepared by the College of Veterinary Medicine, University of Georgia, GA, USA) was supplemented with 6% heat-inactivated rabbit serum (Gibco/Invitrogen, Grand Island, N.Y., USA) and 1% antibiotics (Antibiotic Mixture for Borrelia (100X), Sigma®, St. Louis, MO, USA) as previously described (Kelly 1971). Samples (approximately 0.5 cm^3) of ear, stifle joint, lymph node, bladder, heart and spleen collected from each rabbit at necropsy were inoculated into 10ml BSK-II media and incubated at 33°C. For each culture a 10µl aliquot was examined for presence of motile spirochetes using dark field microscopy every 7 days until spirochetes were seen or for a total of 42 days.

Prior to inoculation onto DH82 cells the biopsy samples were rinsed in 1x phosphate buffered saline (PBS) containing 10% (?) Penicillin-Streptomycin-Fungizone (Invitrogen Corporation, Carlsbad, CA, USA; contains 10,000 units penicillin, 10,000 µg streptomycin , and 25 µg of amphotericin B/ml). The process was repeated three times. After that the samples were resuspended in minimum essential medium (MEM) with 10% fetal bovine serum and inoculated onto a monolayer of uninfected DH82 cells in a 25 cm^2 tissue culture flask. Cultures were evaluated for presence of cytopathic effect using an inverted microscope daily. Once a week cells were harvested from the cultures and spotted on glass slides using a cytocentrifuge

(Thermo Scientific Shandon cytospin catalog # 78300002). Slides were air-dried, stained with Diff-Quick (IMEB Inc. San Marcos, CA, USA) according to the manufacturer's instructions and evaluated using a light microscope at 40x.

RESULTS

Lesions

A total of 4 distinct, grossly-apparent lesions of expanding circular erythema developed on rabbits infested with wild-caught *A. americanum* adults (Figure 1). Both rabbits in this group developed one lesion at each side of the dorsal midline within the area of tick infestation. Mild reddening and thickening of the skin developed at each attachment site of both male and female ticks shortly after tick feeding commenced. On DPTI 6, multiple, isolated circular rashes exceeding the diameter of other tick bite lesions significantly, developed at the attachment site of individual ticks of each rabbit (2 rash lesions on each of 2 rabbits). The rashes, which reached a maximum diameter of 3.0-3.8 cm, increased in size from DPTI 6 to DPTI 8, with the edges becoming indistinct by DPTI 9 and resolving by DPTI 10.

Mild reddening and thickening of the skin around tick attachment sites also developed on rabbits infested with colony-raised *A. americanum* (Figure 2), but no distinct, expanding areas of circular erythema were observed. Two distinct erythema migrans rash lesions developed on the rabbit infested with *B. burgdorferi*-infected *I scapularis* adults on DPTI 10 and DPTI 12, respectively (Table 1b). Both lesions exceeded 5 cm in diameter and continued to expand until margins became indistinct.

PCR and culture results

Rickettsia spp. were identified by PCR (17 kDa target) in 8/100 unfed wild-caught *A. americanum*; sequencing revealed *R. amblyommii* (7/8 amplicons 98 to 99% similar to a published sequence, GenBank accession number U11013) and a *Rickettsia* sp. isolated from ticks in Texas (1/8 94% similar to published sequence, GenBank accession number EF689727).

Rickettsia spp. were also identified in 12/100 fed *A, americanum*; all sequences derived from fed wild-caught LST were confirmed (98 to 100% similar to published sequences, GenBank accession numbers U11013 and AY375162) to be *R. amblyommii*. *Borrelia* spp. were identified by PCR (flagellin B target) in 2/100 unfed and 1/100 fed wild-caught ticks; sequencing identified all amplicons as *Borrelia lonestari* (98% to 99% similar to published sequences, GenBank accession number AF273671 and DQ485689). All wild-caught LST (fed and unfed) were negative by PCR (16S target) for infection with *Ehrlichia chaffeensis* and *Ehrlichia ewingii*. Colony-raised adult *A. americanum* were negative by PCR for *Borrelia* spp., *Rickettsia* spp., *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* before and after feeding.

Organisms were not recovered from culture of skin biopsy samples from rabbits infested with wild-caught or colony-reared *A. americanum* after 6 weeks in BSK-II media or DH82 cells. Spirochetes were present in two out of three skin biopsy samples collected on DPTI 9 from the rabbit infested with *B. burgdorferi*-infected *Ixodes scapularis* after 7 days in culture in BSK-II media. Cultures from skin biopsy samples collected 14 and 21 days after tick infestation contained spirochetes 7 or 14 days after inoculation into BSK-II. All positive microscopic results were confirmed to be *B. burgdorferi* by PCR performed on culture material.

DISCUSSION

Despite considerable investigation (Burkot et al. 2001; Bacon et al. 2003; Moore et al. 2003; Masters et al. 2008; Nicholson et al. 2009) the etiology of STARI remains unknown. In the current paper we report the development of an animal model for STARI. Although the etiology of this condition was not identified, distinct, erythematous rash lesions were induced in rabbit skin upon feeding of wild-caught *A. americanum* collected from an area where STARI has been documented. Rabbits infested with colony-reared ticks did not develop comparable lesions, suggesting that an infectious etiology present in wild-caught but not colony-reared ticks may be responsible. The rabbit model has been used previously in research on erythema migrans induced by *B. burgdorferi* (Burgdorfer et al. 1984; Foley et al. 1995; Foley et al. 1997; Hefty et al.

2002). The present study confirms that the New Zealand white rabbit is an excellent model for erythema migrans and documents that rabbits develop distinct EM lesions from which *B. burgdorferi* can be cultured following feeding by experimentally infected *I. scapularis*. The present work also reports for the first time that feeding by wild-caught *A. americanum* can induce distinct, circular, expanding erythematous lesions consistent with erythema migrans in the rabbit model.

Borrelia lonestari and *Rickettsia amblyommii*, putative agents of STARI, were detected in wild-caught *A. americanum* fed on the rabbits, but no specific pathogens were identified from the skin biopsy samples. The inability to detect an etiologic agent is consistent with reports from human patients with STARI (Wormser et al. 2005a; Wormser et al. 2005b; Nicholson et al 2009). Further, although a very small number of animals were used, it is interesting that the lesions observed in rabbits infested with *A. americanum* developed earlier (first documented on DPTI 6) than those caused *by B. burgdorferi* (DPTI 10 - 12). These findings are consistent with reports from human STARI patients (Wormser et al. 2005a). Case series from Missouri and North Carolina showed a general similarity of clinical presentations associated with the bite of *A. americanum* (Masters et al. 1998; Wormser et al. 2005a; Wormser et al. 2005b). Skin lesions at the site of the tick bite took on average 5 to 6 days to form, but were noticed as soon as 2 days post tick bite, which is generally faster than in LD cases, for which an average of 10 days was determined (Felz et al. 1999; Wormser et al. 2005b).

Although lesions were induced in and observations made from a very low number of animals, development of this model is a significant step towards ultimately deciphering the etiology of STARI. An animal model also provides the opportunity to evaluate putative agents experimentally and compare STARI lesions to those caused by *Borrelia burgdorferi* on both a macroscopic and microscopic level. To date, the presence of erythema migrans is the only objective evidence for STARI; this lesion is also considered pathognomonic for Lyme borreliosis, resulting in confusion of STARI and infection with *B. burgdorferi*. Using the same animal model for the study of both conditions will allow comparison of these two conditions and provides an important tool in working towards a greater understanding of the etiology of STARI.

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FIGURES AND TABLES

Figure 1a. Distinct, circular erythematous rash measuring 2.9 x 3.0 cm that developed on the right side of a rabbit 7 days after attachment of wild-caught *Amblyomma americanum* adults. One day prior to this photograph the rash measured 2.0 x 2.5 cm and an attached male LST was documented at the center of the lesion.

Figure 1b. Skin of rabbit infested with colony-raised *Amblyomma americanum* adult ticks 7 days after attachment. No rashes were observed in these rabbits during tick feeding.

Figure 1c. Two distinct, circular erythematous rashes that developed at the attachment site of two *Ixodes scapularis* adult ticks experimentally infected with *Borrelia burgdorferi* 10 and 12 days after infestation of a New Zealand white rabbit.

Table 1a. Diameter of distinct, circular erythematous lesions observed in rabbits infested with wild-caught (Rabbit 1 and 2) and colony-raised (Rabbits 3 and 4) *A. americanum* adult ticks. Each rabbit was infested with 25 LST per site (total = 50 LST per rabbit). NS = lesions not seen.

Animal ID	Source of ticks	location	Lesion diameter [cm]				
			DPTI 5	DPTI 6	DPTI 7	DPTI 8	DPTI 9
Rabbit 1	wild-caught	left	NS	indistinct margins	2.9 x 3.0	3.8 x 3.6	indistinct margins
		right	NS	indistinct margins	2.0 x 2.5	2.5 x 3.0	indistinct margins
Rabbit 2	wild-caught	left	NS	indistinct margins	indistinct margins	3.5 x 4.0	indistinct margins
		right	NS	indistinct margins	indistinct margins	2.4 x 3.3	indistinct margins
Rabbit 3	colony-raised	left	NS	NS	NS	NS	NS
		right	NS	NS	NS	NS	NS
Rabbit 4	colony-raised	left	NS	NS	NS	NS	NS
		right	NS	NS	NS	NS	NS

Table 1b. Diameter of distinct, circular erythematous lesions observed in the rabbit infested with *Ixodes scapularis* adult ticks (n=9) experimentally infected with *B. burgdorferi*. NS = lesions not seen.

	Lesion diameter [cm]					
	DPTI 9	DPTI 10	DPTI 11	DPTI 12	DPTI 13	DPTI 14
first lesion	NS	6.5 x 5.2	6.8 x 5.2	7.8 x 5.4	6.3 x 5.3	indistinct margins
second lesion	NS	NS	NS	5.2 x 4.6	5.4 x 5.0	indistinct margins

CHAPTER VI

CONCLUSIONS

The overarching objective of this research was to achieve a greater understanding of pathogens associated with *Amblyomma americanum*, the lone star tick (LST) with focus on the etiology of southern tick-associated rash illness (STARI). LST have been shown to be the tick most frequently reported to bite humans in the southern United States (Felz et al. 1996; Goddard 2002). The involvement of LSTs in STARI, a "Lyme-like illness" (Masters et al. 2008), which is characterized by the development of an expanding erythematous rash at the tick bite site poses a challenge to researchers and clinicians. While erythema migrans used to be considered to be pathognomonic for acute human Lyme borreliosis (CDC 1997; Nichol et al. 1998), it is now also known as the only objective evidence of STARI and the basis for difficulties in distinguishing the two conditions. The expansion of LSTs into areas where Lyme borreliosis is endemic (Paddock and Yabsley 2007) makes reaching a clinical diagnosis based on geographic association alone problematic. Furthermore, the increasing number of tick-borne pathogens linked to *Amblyomma* spp. (Guedes et al. 2005; Fournier et al. 2006; Loftis et al. 2008; Cohen et al 2009) emphasizes the need to further characterize potential pathogens associated with *A. americanum.*

The research presented in this dissertation contributes to a better understanding of disease associated with LSTs as summarized in the following paragraphs.

Study 1 - Chapter III

The major aim of this study was to gain insight into the overall bacterial community of wild-caught compared to colony-raised LST and the change thereof upon feeding. Blood feeding led to an increase in diversity of the microbial community, allowing detection of a wider variety of organisms and shift of predominant organisms. The most common approach in attempting to elucidate the etiology of STARI is the identification of known pathogens in samples from or directly associated with human patients (Campbell et al. 1995; Kirkland et al. 1997; James et al. 2001; Wormser et al. 2005). An insight into the bacterial community of wild-caught LST provides a valuable foundation for further research. The 16S rDNA bacteria-wide approach analysis of clones from unfed wild lone star ticks revealed that 96.7% of all the operational taxonomic units (OTUs) identified were affiliated with *Coxiella*-like endosymbionts, as compared to only 5.1 - 11.7% of those identified from wild lone star ticks after feeding. In contrast, the proportion of OTUs identified as *Rickettsia* sp. in wild-caught ticks increased from 2.2% before feeding to as high as 46.8% after feeding. More specific analysis of sequences associated with the genus *Rickettsia* targeting outer membrane protein (17 kDa), and citrate synthase (gltA) genes, supported the 16S rDNA data and revealed that sequences recovered in this study were most closely related to members of the spotted fever group, in particular *R. amblyommii,* a putative agent of STARI. Surprisingly, no *Ehrlichia* spp. or *Borrelia* spp. were identified in these ticks, and outcome that might have been influenced by the pooling process affecting detectability of minor percentages of the bacterial community. This study highlights the eminence of rickettsial species associated with LSTs and provides a foundation for identification of novel pathogens transmitted by LST.

Study 2 - Chapter IV

In August of 2008, a 44-year-old man in Oklahoma presented with a circular erythematous rash with distinct central clearing on the right scapular region following the bite of a LST, a presentation consistent with STARI. The individual resides in an area with high tick populations, and has not experienced any complications associated with tick bites before or

since. Within the week of rash recognition, LST nymphs were collected from the immediate vicinity of the residence. A subset of nymphs was fed and tested individually by PCR for *Rickettsia* spp., *Borrelia* spp., and *Ehrlichia* spp. and for bacteria-wide 16S rDNA-based screening. Pools of unfed and fed nymphs were used in 16S rDNA-based assays with subsequent phylogenetic analysis. Organisms most frequently identified were most closely associated with members of the order Rickettsiales (*Rickettsia amblyommii*, *Rickettsia* spp., and *Ehrlichia chaffeensis*) followed by the orders Rhizobiales and *Acinetobacter*. In light of the frequent occurrence of diverse *Rickettsia* spp. in LST and some reports that STARI patients may respond to doxycycline (Masters et al. 2008), additional consideration should be given to the potential role of novel *Rickettsia* spp. in STARI. Further, presence of *R. amblyommii* in LST nymphs was confirmed in this study.

Study 3 - Chapter V

Based on data collected in this study, the New Zealand white (NZW) rabbit, an established animal model for erythema migrans upon infection with the spirochete *Borrelia burgdorferi*, is proposed to be adapted as an animal model for the study of STARI. NZW rabbits were infested with wild-caught adult *Amblyomma americanum* from an area where STARI is endemic. Starting on DPTI (day post infestation) 6, each rabbit developed individual expanding circular erythematous lesions in the area of tick attachment. No comparable lesions were observed in rabbits infested with colony-raised adult LSTs, suggesting that the lesions are not induced by the feeding of the tick itself but by an agent transmitted by the LST. Another NZW rabbit infested with *Ixodes scapularis* experimentally infected with *B. burgdorferi* developed expanding circular erythematous lesions that were first recorded on DPTI 10. This rabbit was shown to be infected with the spirochete by PCR and culture. Although *Borrelia lonestari* and *Rickettsia amblyommii* were detected in wild-caught ticks fed on the rabbits, no specific pathogens were detected in samples from the rabbits infested with LSTs using culture (BSK-II media and DH82 cells), as well as PCR targeting *Borrelia* spp., *Rickettsia* spp., *Ehrlichia*

chaffeensis and *E. ewingii.* These findings are consistent with reports from STARI patients. With

the development of an animal model we gain a powerful tool for studying the etiology of STARI.

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VITA

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Amblyomma americanum, the lone star tick (LST) is the most commonly reported tick from humans in the southern United States and is associated with transmission of diseases, including STARI (southern tick-associated rash illness), a Lyme disease-like illness of people of unknown etiology, and ehrlichioses caused by *Ehrlichia chaffeensis* and *Ehrlichia ewingii*.

To better define the microbial communities within *A. americanum*, colony-raised and wild-caught ticks, including those associated with a STARI case, were analyzed before and after feeding using a 16S rDNA-wide PCR approach followed by phylogenetic analysis of sequences; ticks were further evaluated by specific PCR for *Borrelia* spp., *Rickettsia* spp., and *Ehrlichia* spp.

The data gained in these studies provides a thorough microbial characterization of the tick of interest, *A. americanum*. It provides support for previous work documenting frequent infection of LST with *Rickettsia* spp., all members of the spotted fever group, and confirms the preponderance of *R. amblyommii* in all life stages as well as the presence of *Borrelia lonestari*, another putative agent of STARI, in wild LST populations. Upon feeding, the bacterial community present in these ticks shifts significantly, with a dominant rise of the proportion of sequences associated with the genus *Rickettsia*. The development of an animal model provides a new prospective for the study of STARI. New Zealand white rabbits infested with wild-caught LSTs developed distinct circular erythematous lesions expanding from individual bite sites; similar lesions were not documented in rabbits infested with colony-raised LST suggesting that the lesions were induced by an agent transmitted by the tick rather than the tick itself. However, while *R. amblyommii* and *B. lonestari* were amplified from fed wild-caught ticks, no specific pathogens were amplified from the rabbits.

Taken together these studies work towards greater understanding of the etiology of STARI and provide valuable tools to be used in future research.

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