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The Role Of Cattle And Gulf Coast Ticks (Amblyomma Maculatum) In The Epidemiology Of Rickettsia Parkeri Infection

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THE ROLE OF CATTLE AND GULF COAST TICKS (*AMBLYOMMA MACULATUM)* IN THE EPIDEMIOLOGY OF *RICKETTSIA PARKERI* INFECTION

By

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I hypothesized cattle and Gulf Coast ticks (GCT), *Amblyomma maculatum,* may be involved in epidemiology of *Rickettsia parkeri* infection. I demonstrated transient rickettsemia by polymerase chain reaction (PCR) for the *17 kilodalton (kDa)* gene in 33% (2/6) of calves experimentally exposed to *R. parkeri* either by direct inoculation or by placement of *R. parkeri*-infected GCT on calves' ears. Calves (4/4) receiving GCT developed lesions at attachment sites consistent with a pathologic condition known as "gotch" ear whether or not GCT were infected with *R. parkeri,* suggesting the condition is related to GCT attachment and not to rickettsial infection. In calves exposed to *R. parkeri*, biopsy of injection sites and attachment sites revealed rickettsial organism by immunohistochemistry. Unexposed calves were seronegative, whereas, exposed calves seroconverted (produced antibodies).

In a cross-section of Mississippi sale barn cattle, I did not demonstrate rickettsemia, although 7.1% (13/183) were GCT-infested and 49.5% (91/183) were immunofluorescent antibody (IFA) positive for SFG rickettsiae (1:32 dilution). In addition, 21.7% (5/23) and 4.3% (1/23) of GCT from cattle were PCR positive for the *17 kDa* gene and *rompA* gene, respectively.

I sequenced a *rompA* amplicon from one GCT and found it was 100% identical to a *R. parkeri* sequence in GenBank (U43802).

I compared the distribution of SFG rickettsiae by IFA in salivary glands, midgut, ovaries, and Malpighian tubules from laboratory-reared and field-collected GCT and documented my approach to successful and consistent dissection of tick tissues.

Since *R. parkeri* is transmitted by GCT, these ticks are significant cattle pests, and at least some GCT from cattle are naturally-infected with *R. parkeri*, cattle with *R. parkeri*-infected ticks may increase exposure of people and wildlife to the organism, and the ticks themselves may serve as invertebrate reservoirs of the pathogen.

This study is the first to my knowledge to outline a clear case definition of "gotch" ear, and document experimentally the role of GCT in its pathology. Also, I demonstrated rickettsemia in calves experimentally exposed to *R. parkeri*, presence of GCT on Mississippi sale barn cattle, natural *R. parkeri* infection in GCT from cattle, and distribution of SFG rickettsiae in GCT.

DEDICATION

I dedicate this dissertation to my very favorite (and only) sister, Linda, who has always been my inspiration and who never failed to rush to my side in time of need, lead the way, and assure me. On July 29, 2009 she lost her battle with brain cancer.

ACKNOWLEDGEMENTS

In 2005 I took my first course in Medical Entomology. This was at Iowa State University at the insistence of my friend, Dr. Radford Davis, a classmate from vet school. He said it was a great course and the professor, Dr. Wayne Rowley, would be teaching the course one last time before retiring. During this time I was pursuing my master of public health (MPH) degree from the University of Iowa. I was reared by two microbiologists and really enjoyed all the coursework in public health. However, that medical entomology course I took as an elective was my absolute favorite. It seemed to bring everything together as far as what I'd studied or been interested in. My mathematics was pure problem-solving, the public health was what I had grown up on, and the epidemiology held everything together with the veterinary medicine. However, when I finished my MPH, my husband, Philip, and I decided to return to Mississippi so we could live close to my mother. We prayed about what to do next with my enthusiasm for my now well-rounded education in veterinary medicine and public health. Philip and I had an idea and talked to our friend from church, Dr. Fred Musser. It had occurred to me that one course in medical entomology was not enough and learning more about medical and veterinary entomology would provide the missing link in my educational pursuits. Fred spoke with the head of the department of entomology and plant pathology, Dr. Clarence Collison, on my behalf.

At this point, there was no doubt in my mind that the Lord was in the plan because I never could have crafted the plan that emerged on my own. If Fred had not talked to Dr. Collison, or Dr. Collison had not given me a chance, my pursuit of entomology would have

ended. Not only that, but Dr. Collison suggested I work with Dr. Jerome Goddard, the medical entomologist for the Mississippi Department of Health. Jerome was a perfect choice and became my major professor. Jerome has boundless ideas and we designed a wonderful course of study. It included some far-flung ideas that would never have come to fruition if it weren't for the arrival of Dr. Andrea Varela-Stokes. Dr. Stokes provided the laboratory space, expertise in molecular techniques, and the friendship and mentoring that made the program possible.

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I thank Drs. Jerome Goddard and Andrea Varela-Stokes, my co-major professors for their vast knowledge and expertise but especially for their guidance and kindness in my research efforts. Andrea helped me during the entire calf experiment and also at the sale barns when she was pregnant with Marco. Jerome has been salt and light every step of the way. I also thank my committee members, Drs. Clarence Collison, Blake Layton, and Carla Huston for ensuring my time in the graduate program was a true learning experience. I thank Dr. Christopher D. Paddock for sharing his vast expertise, his uniquely critical, but kind eye, for providing cell

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CHAPTER I

INTRODUCTION

The overall objective of this study was to elucidate the natural history of the emerging tick-borne pathogen, *Rickettsia parkeri,* in its United States tick vector, *Amblyomma maculatum* Koch, specifically with respect to cattle. *Rickettsia parker*i was thought to be nonpathogenic until 2004 when Dr. Christopher Paddock, Centers for Disease Control and Prevention, Atlanta, Georgia, documented a case of rickettsiosis due to *R. parkeri* in a man from Virginia (Paddock et al. 2004). Since then, at least 12 total cases have been reported in the United States, four of which were from Mississippi, underscoring the presence of this rickettsia in the state (Paddock et al. 2008). The name American Boutonneuse Fever (ABF) has been proposed to describe disease caused by *R. parkeri* because its clinical presentation is similar to Mediterranean Boutonneuse Fever, including the presence of eschars at tick attachment sites (Goddard 2004).

Extensive cross-reactivity exists among spotted fever group (SFG) rickettsiae (Raoult and Roux 1997). In fact, serologic assays, such as the immunofluorescence assay, which may use only a single antigen from the organism known to be pathogenic in that geographic location, may have hampered correct identification of several novel SFG rickettsioses, including *R. parkeri* (Raoult and Roux 1997, Paddock et al. 2004, Parola et al. 2005b). Thus, some cases diagnosed as mild Rocky Mountain spotted fever (RMSF) may have actually been *R. parkeri* infection (Raoult and Paddock 2005).

Unraveling the natural history of this emergent pathogen depends on recognizing its uniqueness among the SFG rickettsiae, which begins with the ecology of its primary US vector, *A. maculatum,* the Gulf Coast tick (GCT).

Our central hypothesis is that cattle may be involved in the ecology and transmission of *R. parkeri* rickettsiosis by the Gulf Coast tick, and may have the capacity to serve as a reservoir for human infection with *R. parkeri*. Our rationale is based on the fact that cattle are natural hosts for *A. maculatum* ticks (Parker et al. 1939), and *R. parkeri* is known to infect *A. maculatum* ticks (Parker et al. 1939, Parker 1940, Goddard 2004, Paddock et al. 2004, Goddard and Paddock 2005). Also, *R. parkeri* is pathogenic to humans (Paddock et al. 2004) and *A. maculatum* ticks are known to infest cattle (Bishopp and Hixon 1936, Bishopp and Trembley 1945, Williams et al. 1978, Byford et al. 1992, Mock 2000, Barker et al. 2004, Broce and Dryden 2005, Ketchum et al. 2005, Highfill 2006, Wright et al. 2007). Since *A. maculatum* ticks will also attack humans and may be a competent vector of pathogens, it is possible that cattle play a role in the natural history of *R. parkeri* infection and provide a significant ecological component to human disease. However, there are no data to describe vertebrates as reservoirs or amplifiers in the natural transmission of *R. parkeri,* and little is known of the life cycle of *R. parkeri* in ticks.

This manuscript includes the following: 1) a review of *R. parkeri,* the Gulf Coast tick, (*A. maculatum*), and a description of the potential role of this rickettsial organism and this tick in ABF rickettsiosis ; 2) results of research with calves experimentally infected with *R. parkeri* both by needle injection and by placement of *R. parkeri*-infected *A. maculatum* ticks on them; 3) analysis of a sample of Mississippi cattle, and ticks removed from those cattle, for evidence of infection and exposure to SFG rickettsiae; 4) description of an approach to the dissection of Ixodid ticks; and 5) report on the distribution of *R. parkeri* in selected tissues of experimentally-

infected Gulf Coast ticks (*Amblyomma maculatum*) compared with hemolymph-positive, field-collected ticks.

CHAPTER II

HISTORICAL REVIEW

Rickettsia parkeri is one of the most recently recognized tick-borne rickettsial agents in the United States associated with human disease. In 1937, R. R. Parker and coworkers identified a unique spotted fever group (SFG) rickettsia in Gulf Coast ticks (*Amblyomma maculatum*) removed from cattle in Texas (Parker et al. 1939). This rickettsia was eventually named *R. parkeri* in his honor (Lackman et al. 1949, Lackman et al. 1965). Subsequent infection of guinea pigs with *R. parkeri* caused a self-limiting, febrile illness similar to, but milder than, Rocky Mountain spotted fever (RMSF), which Parker termed 'maculatum infection' (Parker 1940). Parker noted a similarity between *R. parkeri* and *R. conorii,* the agent of Boutonneuse fever (Mediterranean spotted fever) in the clinical and serologic characteristics of infections in guinea pigs inoculated with this agent. Paddock (Paddock et al. 2004) and Walker (Walker and Fishbein 1991) noted a similarity to *R. africae*, the agent of African tick-bite fever. Eventually, these three species were found to be closely related genetically (Fournier et al. 1998). When first recognized, Parker and others speculated that *R. parkeri* might also cause a RMSF-like disease in humans. However, it was still considered by most to be a non-pathogen. In 2002, the first confirmed human infection with *R. parkeri* was identified in a Virginia patient who presented with an eschar-associated febrile illness (Paddock et al. 2004). Other patients from Mississippi and Virginia were reported in 2005 (Finley et al. 2006) and 2007 (Whitman et al. 2007), respectively. Since that report, the total number of probable and confirmed cases has risen to twelve (Paddock et al. 2008). Goddard (Goddard 2004) has proposed the name "American

Boutonneuse Fever" (ABF) for the new disease based on clinical similarities between this infection and Boutonneuse fever caused by *R. conorii*. Although the agent has occasionally been found in other *Amblyomma* ticks (for example, it was identified in *A. americanum* from Mississippi and Kentucky in the mid-1980s) (Goddard and Norment 1986), *R. parkeri* seems to be generally associated with the tick species, *A. maculatum,* in the U.S. (Goddard and Norment 1986, Sumner et al. 2007). *Rickettsia parkeri* has been identified in *A. maculatum* ticks collected in many states within its range, including Mississippi, suggesting that this pathogenic rickettsia is endemic throughout a relatively large expanse of the United States (Sumner et al. 2007). It is conceivable that many cases of spotted fever reported in this area were actually caused by *R. parkeri* (Raoult and Paddock 2005). Because the primary vector for *R. parkeri* in the United States is *A. maculatum* (Sumner et al. 2007), determining the vertebrate hosts involved in the maintenance of the pathogen in nature depends in part on understanding the ecology of this tick.

The tick vector

There are two major families of ticks, Ixodidae (known as hard ticks because of their hard sclerotized dorsal plate) and Argasidae (known as soft ticks, because of the lack of a dorsal plate) (Campbell and Barker 1998, Estrada-Pena et al. 2005). A third family, Nuttalliellidae contains only a single species found in southern Africa (Horak et al. 2002, Estrada-Pena et al. 2005). Ixodid ticks spend more than 90% of their lives unattached from their hosts (Needham and Teel 1991). Most of them are exophilic and live in open environments such as the edges of meadows and forests. They are highly responsive to stimuli that indicate the presence of hosts, such as chemical stimuli, humidity, airborne vibrations and body temperatures associated with warm-blooded animals (Sonenshine 1991). There are two major host-seeking behaviors

exhibited by exophilic ticks. One is an ambush strategy where ticks climb vegetation and wait for passing hosts with their front legs held out (questing). Another is the hunter strategy. Ticks leave their habitats and run toward their hosts to attack them. Some species exhibit both strategies (Needham and Teel 1991, Sonenshine 1991). There is also evidence of a male pheromone attractive to unfed females. Specifically, it has been documented that unfed female *A. maculatum* ticks readily attach to their hosts in the presence of feeding *A. maculatum* males. Conversely, unfed females are reluctant to attach when fed males are absent (Gladney 1971, Obenchain and Galun 1982).

The Gulf Coast tick

The Gulf Coast tick, *A. maculatum* [Acari: Ixodidae], is a Nearctic and Neotropical three-host tick (Sonenshine 1991, 1993, Estrada-Pena et al. 2005). *Amblyomma maculatum* has a very ornate, sclerotized, dorsal plate (Figure 1). Typical of other ticks in the family Ixodidae, *A. maculatum* is exophilic, readily bites humans, and feeds on a number of livestock and wildlife hosts. The distribution of *A. maculatum* has expanded from its original distribution along the Gulf coast to extend up to 100-150 miles from the Gulf coast, along the Mississippi River and in parts of the Atlantic states (Cooley and Kohls 1944, Ketchum et al. 2006). It also includes a discontiguous population in Oklahoma and Kansas (Cooley and Kohls 1944, Ketchum et al. 2006). Its presence has been documented in several states including Mississippi (Goddard and Paddock 2005), Florida (Cilek and Olson 2000), Georgia (Goldberg et al. 2002), South Carolina (Felz et al. 1996), Oklahoma (Barker et al. 2004), Kansas (Goddard and Norment 1983), (Ketchum et al. 2006), North Carolina (personal communication, Wes Watson, North Carolina State University), and Texas (Ketchum et al. 2006). It is also found in regions of several Central and South American countries that border the Gulf of Mexico and Caribbean Sea, including

Mexico, Guatemala, Belize, Nicaragua, Honduras, Costa Rica, Colombia, Venezuela, and some parts of Ecuador and Peru (Estrada-Pena et al. 2005). Recent research on *A. maculatum* demonstrates that developmental diapause does not appear to be induced by the photoperiod in fed, laboratory nymphs which may explain why its range is limited to tropical regions (Loymeyer et al. 2009).

Parasitism of humans by *A. maculatum* has been documented in at least eight southern and southeastern states (Merten and Durden 2000). Bites by these ticks accounted for 12% of all human tick bites described in a survey of Mississippi human tick biting (Goddard 2002). Thus, these ticks may act as a suitable route of infection for tick-borne agents to humans.

Larvae, nymphs and adults of the GCT have relatively limited host groups (Hoogstraal and Aeschlimann 1982). Adults attack a variety of vertebrates including dogs, cattle, horses, sheep, deer, coyotes, rabbits, and humans (Hixson 1940, Bishopp and Trembley 1945). Cattle and some other ruminants appear to suffer some specific ear pathology associated with adult GCT bites, as is discussed in detail below. However, GCT nymphs appear to have a predilection for the withers, midline, and tail-head of cattle (Ketchum et al. 2005). As larvae and nymphs, these ticks are also common pests of ground-inhabiting birds and small rodents (Bishopp and Trembley 1945, Keirans and Litwak 1989, Estrada-Pena et al. 2005). Host specificity in *A. maculatum* has been described as moderate stage-stage host specificity (Marrelli et al. 2007). Although some *Amblyomma* ticks share the same group of primary hosts in nature, the phylogenetic trees based on their second internal transcribed spacer (ITS2) sequences suggest no host association patterns (Marrelli et al. 2007).

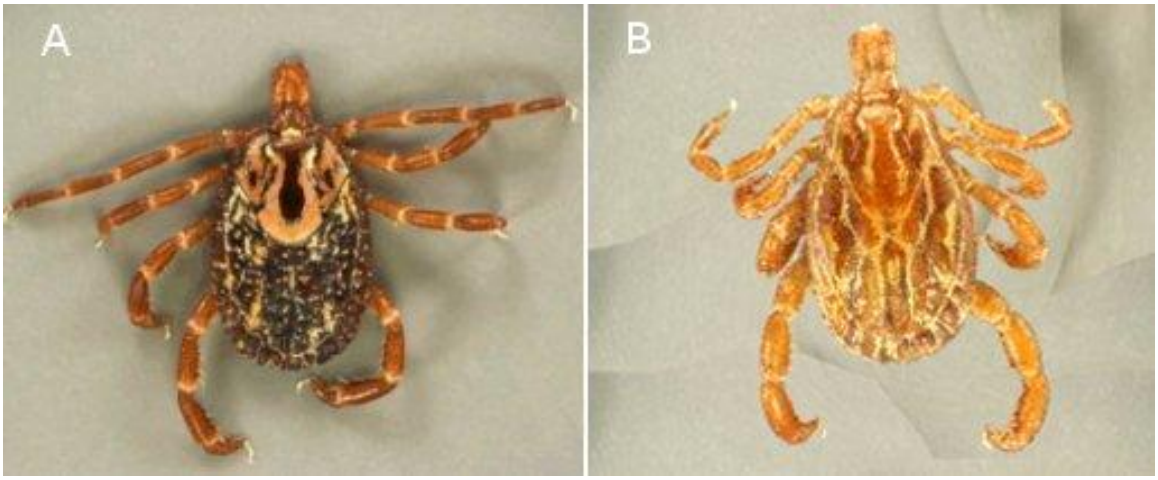


Figure 1. Adult Amblyomma maculatum (Gulf Coast tick) female (A) and male (B). Photos courtesy of Dr. Blake Layton, Department of Entomology and Plant Pathology, Mississippi State University, 2006.

The vertebrate host

Since the first reported case of infection with *R. parkeri* in *A. maculatum* ticks from cattle in Texas in 1937 by R. R. Parker, it has been suspected that cattle play a role in the epidemiology of *R. parkeri* infection (Parker et al. 1939). Early studies focused on the implications of a large Gulf Coast tick burden as a predisposing factor for screwworm infestation (Bishopp and Hixon 1936, Gladney 1976). More recent studies conducted on cattle infested with *A. maculatum* ticks have documented decreased weight gain in heavily infested animals and noteworthy alterations in blood composition (Williams et al. 1978). Total leukocyte counts were significantly decreased in heavily infested animals. Also, substantial increases occurred in total serum protein, total serum globulin; alpha, beta, and gamma globulin fractions and plasma fibrinogen. Also, significant decreases occurred in the albumin/globulin ratio (Williams et al. 1978). These changes in blood parameters are indicative of a metabolic reaction to the tick infestation and the resultant initiation of an immune response. Total energy balance may be altered when an animal is exposed to ectoparasite infestations, resulting in decreased

productivity (Byford et al. 1992). The greatest decrease in average daily gain (ADG) due to ectoparasite infestation in Hereford cattle was attributed to the Gulf Coast tick (Byford et al. 1992). No loss in ADG was apparent in Brahman cattle with similar tick infestations. One estimate attributed the loss in the United States specifically due to tick infestation to be nearly $400 million annually (Williams et al. 1977, Stacey et al. 1978, Williams et al. 1978, Byford et al. 1992). In addition to the nuisance effect of ticks to cattle and the blood loss from heavy infestations, research on cattle infested with *A. maculatum* has shown growth performance reduced by as much as 20% (Broce and Dryden 2005). The presence of rickettsemia in cattle associated with Gulf Coast tick infestation and possible implications on cattle productivity have not been investigated.

Amblyomma ticks and cattle

Although there is a wide host range for GCT, cattle appear to be the preferred hosts for the adult stage (Barker et al. 2004). With some infestations of adult *A. maculatum*, the ears may become thickened and curled, causing a condition known as “gotch" ear (Bishopp and Hixon 1936, Highfill 2006). It has been reported that this deformity is evident when there are more than 10 ticks per ear (Highfill 2006). Further, the condition is apparently due to damage to muscle and cartilage in the ear (Mock 2000, Highfill 2006, Wright and Barker 2007, Wright et al. 2007). In very young calves, up to a third of the ear may be lost after becoming necrotic (Mock 2000). However, little is known about the pathogenesis or epidemiology of “gotch” ear in cattle except that it generally involves *A. maculatum* ticks. Whether these ticks are infected with *R. parkeri*, and whether this affects the pathology of “gotch” ear has not been investigated.

Rickettsiae

Rickettsiae are short, Gram-negative pleomorphic bacteria that retain fuschin when stained as described by Gimenez (Gimenez 1964). In the past they were regarded as microorganisms positioned somewhere between viruses and true bacteria. Rickettsiae are thought to be the closest living relatives to the bacteria that are considered the theoretical origin of the mitochondria organelle existing in most eukaryotic cells. Rickettsiae are associated with arthropods that may act as vectors, reservoirs, or amplifiers of the bacteria (Raoult and Roux 1997, Parola and Raoult 2001). Rickettsial diseases are zoonoses caused by bacteria in the order Rickettsiales (Parola et al. 2005a). Members in the genus *Rickettsia* belong to the family Rickettsiaciae. Another important family in the order Rickettsiales, Anaplasmataceae, includes the genera *Ehrlichia*, *Anaplasma*, and *Wolbachia*. Rickettsial pathogens are highly specialized for obligate intracellular survival in both the vertebrate and invertebrate hosts (Azad and Beard 1998). Rickettsial organisms are deposited directly into the bloodstream of vertebrate hosts by the bite of a tick vector. The endothelial cells of the host react to organisms by engulfing them and carrying them into the cell's cytoplasm within a vacuole. Ultimately, the organisms escape the vacuole into the cytoplasm of the cell where rickettsiae multiply and cause cell injury and death. Damage to the endothelial cells and the vascular system in general may be widespread and include damage to skin, muscle, heart, and lungs of the vertebrate host (Walker and Fishbein 1991, Forbes et al. 2002).

Rickettsia species are carried as micro-parasites by many ticks, fleas, and lice, and cause diseases such as typhus, rickettsialpox, Brill-Zinsser disease, Boutonneuse fever, Rocky Mountain Spotted Fever (RMSF), and endemic typhus (Heymann 2004).

Spotted fever group rickettsiae

Members of the genus *Rickettsia* may be classified into 3 major groups: spotted fever group (SFG), typhus group (TG), and scrub typhus group (STG) (La Scola and Raoult 1997, Andersson et al. 1999, Raoult et al. 2005) based on vector, host, and antigenic cross-reactivity (Stothard and Fuerst 1995) although their classification is continually being modified as new data become available (Parola et al. 2005a). Rickettsiae infect and multiply in almost all organs of their invertebrate hosts in the life cycle of the typical SFG member. Rickettsiae may be transmitted transovarially to at least some offspring when the ovaries and oocytes of an adult female tick are infected (Azad and Beard 1998). Once an oocyte is infected, all subsequent life stages resulting from that infected oocyte will also be infected. Ticks can transmit rickettsiae to their vertebrate hosts while feeding whenever these microorganisms infect ticks' salivary glands (Parola and Raoult 2001). Since larvae, nymphs and adults may infect susceptible vertebrate hosts, ticks are the main reservoir host of rickettsiae (Raoult and Roux 1997, Parola and Raoult 2001). At least 10 SFG rickettsiae have been identified in Ixodid ticks in the Western Hemisphere (Parola et al. 2005a).

Ecology of tick vectors

Ticks have been recognized as parasites of humans and animals for thousands of years (Sonenshine 1991). They were first demonstrated as capable of transmitting infectious diseases in 1893 when Smith and Kilbourne identified the protozoal agent of Texas cattle fever in a tick (Assadian and Stanek 2002). Their account was also the first to document a zoonotic disease and served as the foundation of all later work on the animal host and the arthropod vector (Assadian and Stanek 2002).

Ecologic aspects of tick vectors are important in the epidemiology and clinical appearance of tick-borne diseases (Parola and Raoult 2001). There are three main routes of infection for tick vectors: feeding on bacteremic animals, transstadial infection, and transovarial infection. Whenever ticks become infected with bacteria by feeding on bacteremic animals, subsequent infection may occur transstadially (from stage to stage; for example from larva to nymph and adult) and transovarially (from one generation to the next via the female ovaries) (Parola and Raoult 2001). Transstadial passage of bacteria is necessary for the competence of ticks as vectors (Parola et al. 2005b). Spotted fever group rickettsiae may be transmitted by all three routes (Raoult and Roux 1997). In addition, some Ixodid ticks produce a painless bite which enhances their potential as vectors of pathogens. They may remain unnoticed, attached to the host, and feed for long periods of time (Sonenshine 1991). Each stage of the tick generally feeds only once and hosts may include a variety of vertebrates in diverse environments.

As mentioned, ticks not only act as vectors of disease agents, but sometimes as reservoirs of tick-transmitted agents (Parola and Raoult 2001). Since each stage of the Ixodid tick generally feeds only once, bacteria acquired by them during feeding typically are only transmitted to another host when a tick capable of transstadial transmission has molted to its next stage of development. Because ticks may serve as reservoirs of pathogens, the distribution of the particular disease caused by rickettsiae may be identical to that of its tick vector (Parola and Raoult 2001). Therefore, examination of the distribution of the tick vector may yield valuable information in the epidemiology of rickettsioses.

Rickettsia parkeri

In 1937, Ralph Robinson Parker (1888-1949) isolated a rickettsia-like organism which differed significantly from *R. rickettsii*, the etiologic agent of RMSF. He isolated this organism from *A. maculatum* ticks collected from cattle in a Texas county at a time when all confirmed cases of tick-borne spotted fever were attributed to *R. rickettsii* (Raoult 2004). Subsequently, in 1965 the agent isolated by Parker was confirmed as a unique SFG rickettsia and named *R. parkeri* in his honor (Lackman et al. 1965, Raoult and Roux 1997). Although researchers speculated that *R. parkeri* might be a potential cause of human illness, it remained an obscure rickettsia for decades and was generally referred to as a non-pathogen (Walker and Fishbein 1991, Raoult and Roux 1997, Paddock et al. 2004, Raoult 2004, Hechemy et al. 2005).

Extensive cross-reactivity exists among SFG rickettsiae, especially *R. rickettsii, R. conorii, R. africae,* and *R. parkeri.* Even with immunofluorescence assay, the standard reference method in rickettsial serology, there are wide antigenic cross-reactions among SFG rickettsiae (Raoult and Roux 1997). Historically, the only antigen used in serologic testing for rickettsial disease was one known to be pathogenic to humans in that geographic location. In fact, conclusions drawn from these nonspecific serologic assays may have hampered the correct identification of several novel SFG rickettioses (Parola et al. 2005b). Consequently, since *R. rickettsii* is often the only antigen used in serologic analysis for routine diagnosis of RMSF, misdiagnosis of other SFG diseases is likely (Paddock et al. 2004). An obfuscating factor is that multiple and distinct pathogenic rickettsiae may circulate in several species of tick vectors whereas other rickettsiae may be associated with only one tick species (Parola et al. 2005b). Another complex situation, demonstrated by Burgdorfer in the 1980s, is the fact that ticks infected with one species of rickettsiae may be refractory to infection with another (Burgdorfer et al. 1981). Historically, pathogenicity has been demonstrated in animal models. However, such models have unreliable

predictive value for human disease since the animal being used may have mild disease, whereas the human patient may experience severe illness (Parola et al. 2005b). Many aspects of rickettsial pathogenesis remain unknown.

Pathogenicity of *Rickettsia parkeri*

The role of *Amblyomma* ticks as vectors of pathogens and the pathogenicity of *R. parkeri* have recently earned increased attention. Specifically, the medical importance of the Gulf Coast tick, *A. maculatum* Koch, has been highlighted with the discovery of the pathogenicity of *R. parkeri* associated with this tick (Goddard and Paddock 2005). In 2004, Paddock et al. described the first recognized case of infection in a patient with *R. parkeri* (Paddock et al. 2004). In that case, the patient had antibodies to both *R. rickettsii* and *R. akari* as well as the formation of an eschar. The term eschar, also called a tache noire, refers to a black, necrotic region which may occur at the tick attachment site. Biopsy of the patient's eschar in the index case, yielded rickettsiae, which were characterized by molecular biology as *R. parkeri* (Paddock et al. 2004).

Novel rickettsioses caused by distinct SFG rickettsiae have been documented in Europe, Africa, Australia and Asia (Raoult and Roux 1997). Similarities among SFG rickettsioses have been identified by comparing clinical and serologic characteristics of these infections in guinea pigs (Lackman et al. 1965). Phylogenetic analyses evaluating genotypic relationships among SFG rickettsiae show *R. parkeri* most closely related to Old World pathogens including *R. conorii* and *R. africae* (Stothard and Fuerst 1995, Fournier et al. 1998). It has been suggested that the disease caused by *R. parkeri* be named "American Boutonneuse Fever" because of the similarity of the clinical presentation of the disease with those documented in Africa and in Europe referred to as Boutonneuse fever (Goddard 2004). Interestingly, *R. conorii* and *R. africae* may

also produce eschars, 72% and 100%, respectively, in infected patients, whereas other rickettsiae, e.g., *R. rickettsii*, do not (Raoult and Roux 1997).

Other *Amblyomma* tick vectors of *Rickettsia parkeri*

The occurrence of *R. parkeri* in *A. maculatum* ticks as well as in other *Amblyomma* ticks has been documented (Parker 1940, Philip and White 1955). For example, *R. parkeri* has been reported in *A. triste* ticks from Uruguay (Venzal et al. 2004) and Brazil (Silveira et al. 2007). In addition, *R. parkeri* was experimentally inoculated into *A. americanum* ticks and remained viable for two generations (Goddard 2003). *Rickettsia parkeri* was reported for the first time from *A. americanum* in 1986 in ticks collected in Mississippi and Kentucky (Goddard and Norment 1986). More recently *R. parkeri* was reported in *A. americanum* ticks collected in Georgia and Tennessee (Cohen et al. 2009). However, the range of *R. parkeri* and the frequency of infection, has not been fully documented (Sumner et al. 2007).

In a 2004 study, *R. parkeri* was the only SFG rickettsia detected in *A. triste* ticks from Uruguay. The data in this study suggested that *A. triste* is a host of SFG rickettsia in Uruguay, and that *R. parkeri* could be the causative agent of human cases of rickettsioses in Uruguay (Venzal et al. 2004). A 2005 study in Brazil demonstrated the capability of *A. cajennense* ticks to support development of *R. parkeri* when experimentally infected (Sangioni et al. 2005). In a 2008 study, the occurrence of *R. parkeri* in *A. triste* ticks was reported in ticks collected in close proximity to locations of several eschar-associated spotted fever cases in Argentina (Nava et al. 2008). With the widespread use of molecular techniques to differentiate rickettsial organisms, other *Amblyomma* ticks may yet be identified as vectors of *R. parkeri*. However, there are few data currently available which describe the vertebrates involved as reservoirs or amplifiers in the

natural transmission of *R. parkeri* or that describe the life cycle of *R. parkeri* in their tick vectors (Paddock 2005).

Significance of these studies

We hypothesize that cattle may be involved in the biology and ecology of *R. parkeri* and the Gulf Coast tick, *A. maculatum* Koch (Ixodidae). The rationale for our hypothesis is that *R. parkeri* is transmitted by *A. maculatum* ticks, and these ticks are a significant pest to cattle, often inducing "gotch" ear and other pathology; thus, cattle may play a role in the maintenance of this pathogen in nature. In addition, since rickettsiae are generally transmitted both transstadially and transovarially in their tick vectors (Azad and Beard 1998), *A. maculatum* may serve as a reservoir for *R. parkeri* rickettsial disease (i.e. ticks themselves may be reservoirs). Results from this research have supplied several missing components to the natural history of *R. parkeri*. Elucidating these missing elements is critical for understanding the risk factors for infection and ultimately for developing an approach to the control and prevention of this, and other, vector-associated zoonotic diseases. That is, if cattle become rickettsemic after exposure to *R. parkeri*-infected ticks, cattle may be sufficient to serve as reservoirs for tick transmission to humans. In fact, even if cattle serve only as a blood source and transport mechanism for *A. maculatum* ticks, cattle may be an important ecologic component for these ticks to serve as vectors of pathogens.

CHAPTER III

CATTLE AS HOSTS FOR *RICKETTSIA PARKERI* INFECTION

Abstract

Calves exposed to *Rickettsia parkeri* by needle-exposure to inoculum or by *R. parkeri*-infected *Amblyomma maculatum* ticks seroconverted and 2 out of 6 were transiently rickettsemic. Calves remained clinically normal, except for the development of lesions in all tick-infested calves. These lesions were consistent with "*gotch*" ear, suggesting that *R. parkeri* is not implicated in the condition.

Introduction

Rickettsia parkeri is one of the most recently recognized US tick-borne rickettsial agents associated with human disease. It causes a disease similar to, but milder than, Rocky Mountain Spotted Fever (RMSF), sometimes referred to as "American Boutonneuse Fever" (Goddard 2004). In 1937, R. R. Parker identified a unique spotted fever group (SFG) rickettsia, eventually named *R. parkeri* (Lackman et al. 1965), in Gulf Coast ticks (*Amblyomma maculatum*) removed from Texas cattle. Subsequent *R. parkeri* infection of guinea pigs caused a self-limiting, febrile illness Parker termed 'maculatum infection' (Parker 1940). Parker noted a similarity in the clinical and serologic characteristics of infections in guinea pigs inoculated with either *R. parkeri* or *R. conorii,* the agent of Boutonneuse fever (Mediterranean spotted fever) while Paddock (Paddock et al. 2004) and Walker (Walker and Fishbein 1991) noted a similarity to *R. africae*, the agent of African tick-bite fever. Eventually, these three species were found to be closely related

genetically (Fournier et al. 1998). Parker speculated early on that *R. parkeri* might cause a RMSF-like disease but it was not until 2002, that the first confirmed infection with *R. parkeri* was identified in a Virginia patient who presented with an eschar-associated febrile illness (Paddock et al. 2004). Patients from Mississippi and Virginia were reported in 2005 (Finley et al. 2006) and 2007 (Whitman et al. 2007), respectively. Since then, the total number of cases has risen to twelve (Paddock et al. 2008). Four of the twelve cases documented so far have been in Mississippi residents. Also, Paddock (personal communication) found *R. parkeri* infection rate as high as 40% in *A. maculatum* ticks tested in Mississippi. In fact, based on the current information, Mississippi may be an epicenter of *R. parkeri* infection.

Adult *A. maculatum* ticks prefer feeding on cattle ears, sometimes causing a condition called *"gotch"* ear (Bishopp and Hixon 1936, Highfill 2006, Wright and Barker 2007, Wright et al. 2007). The ears become thickened and furled, especially in young animals. If the tick burden is severe, the cartilage of the ear may be undermined, the ear may become necrotic, and the tip may slough entirely. However, a concise case definition of "gotch" ear is lacking as well as a description of the underlying etiology, distribution, susceptibility, risk factors, and prevention strategies. In the past, tick-bite lesions on cattle ears caused by these ticks attracted screwworm flies and provided a substrate for oviposition and subsequent burrowing and feeding (Bishopp and Hixon 1936, Gladney 1976), thereby predisposing cattle with GCT-laden ears to screwworm infestation.

We hypothesized that *"gotch"* ear might be attributable to *A. maculatum*-transmitted *R. parkeri* infection since *R. parkeri* produces eschars in humans. Furthermore, because *R. parkeri* appears to have high prevalence in ticks compared with other SFG rickettsiae (Sumner et al. 2007), and cattle exposure is likely in certain locations (Bishopp and Hixon 1936, Bishopp and Trembley 1945, Williams et al. 1978, Byford et al. 1992, Mock 2000, Barker et al. 2004, Broce

and Dryden 2005, Ketchum et al. 2005, Highfill 2006, Wright et al. 2007), cattle may be a potential *R. parkeri* reservoir. Alternatively, since rickettsiae are generally transmitted both transstadially and transovarially in their tick vectors (Azad and Beard 1998), *A. maculatum* itself may serve as a *R. parkeri* reservoir.

Materials and Methods

We defined "gotch" ear as follows: a condition of the ear associated with tick infestation, predominantly GCT, which includes variable degrees of edema, lesions at tick-attachment sites on the outer or inner pinnae, including crusting, alopecia, erythema, and excoriation; with or without curling of the tip of the pinnae and a loss of a portion of the ear.

Eight healthy Holstein bull-calves from the Mississippi State University dairy, varying in age from 3 to 6 months, with no detectable antibodies to *R. parkeri* by immunofluorescent assay (IFA) (Appendix C) or detectable circulating rickettsiae by polymerase chain reaction (PCR) (Appendix D) were maintained in a tick-free environment[1]. Calves were divided into two groups: injected animals and animals exposed to injected ticks. Each of three calves chosen at random for the injection group was inoculated by three different routes with 0.3 ml of *R. parkeri*-infected Vero cell suspension[2] at each route: intradermally over the triceps muscle of the left shoulder, intravenously in the left jugular vein, and subcutaneously near the other injection sites. As a negative control, 0.3 ml of an uninfected Vero cell suspension was injected at the same locations in the control calf.

[1] All studies were approved by the Institutional Animal Care and Use Committee at Mississippi State University.

[2] *Rickettsia parkeri* infected Vero cells were obtained from Dr. Christopher D. Paddock, CDC, Atlanta, GA. Growth parameters for the rickettsiae in Vero cells were as follows: inocula for all experiments were from 2 to 6 passage infected Vero cell suspensions with an approximate dose of 1.8×10^6 Vero cells in 1 milliliter for the negative control and approximately 2.63×10^6 Vero cells infected with *R. parkeri* in 1 milliliter (75-80% infected).

For tick-exposed calves, two hundred eighty-five engorged, nymphal ticks were obtained from an Oklahoma State University colony and injected percutaneously with either *R. parkeri*-infected cell-suspensions (210 ticks) or sterile PBS (75 ticks) using a 30-gauge needle as previously described (Goddard 2003). The ticks were hemolymph-tested (Appendix B) (Gimenez 1964) (Appendix A) and IFA-tested (Appendix C) by an investigator blinded to the nature of the injection. The ticks were also examined by nested PCR for the *rompA* gene (Appendix D). After molting to the adult stage, a minimum of 15/18 (83.3%) *R. parkeri*-injected ticks were positive when tested by nested PCR for the *rompA* gene, while all PBS-injected ticks were negative for this gene. Although the IFA results were equivocal, the hemolymph test was 86% (16/18) positive for the *R. parkeri*-injected ticks.

Fifteen to twenty *R. parkeri*-injected adult *A. maculatum* (approximately 50% male) ticks were placed on the right ear of each of three calves. Similarly, fifteen to twenty PBS-injected ticks were placed on the right ear of the control calf. We covered the tick-infested ear of each calf with a sock adhered to the ear base. We removed ticks from the calves' ears on the seventh day after placement and biopsied injection and tick-attachment sites under local anesthesia.

Blood was collected from all calves once prior to the experiment and three times weekly thereafter by jugular venipuncture for hematologic, molecular, and serologic tests. The calves were given a physical exam including monitoring of temperature, respiration, heart-rate, appetite, and attitude on each sampling day for 30 days. The calves' tick attachment-sites were also visually inspected for signs of "gotch" ear as described above. We screened calf sera in both groups for antibodies using *R. parkeri*-coated slides for IFA at a 1:32 dilution. We used fluorescein-isothiocyanate-labeled goat anti-bovine immunoglobulin-G (Kirkegaard & Perry Laboratories) to determine serologic response to infection. We titrated sera showing titers ≥ 32 by 2-fold dilutions to their endpoints (Appendix D).

DNA was extracted using an Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, Piscataway, NJ) for all sampled days post-infection (DPI) for each calf and evaluated the extracts using a nested PCR assay designed to amplify a segment of the *rompA* gene based on a published protocol (Sumner et al. 2007) (Appendix D). We also used a nested PCR assay designed to amplify a segment of the *17 kilodalton (KDa)* gene based on a published protocol (Paddock et al. 2004) (Appendix D).

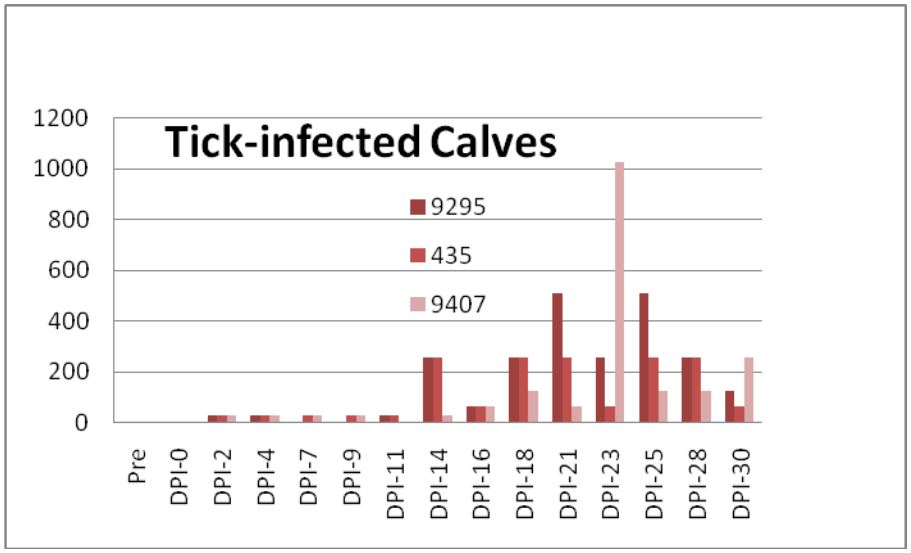
Results

Calves exposed to *R. parkeri* did not develop signs of systemic disease during the course of the study. They remained bright and alert, non-febrile, and maintained healthy appetites. Both negative control calves were negative by PCR techniques. All calves (negative control calves and *R. parkeri*-exposed calves) were negative for *R. parkeri* by nested PCR of the *rompA gene.* However, two of six calves exposed to *R. parkeri* were transiently positive to SFG rickettsiae by PCR of the *17KDa* gene (one tick-infected calf on DPI-23 and one inoculum-infected calf on DPI-11 and DPI-14). All *R. parkeri*-exposed calves seroconverted whereas both negative-control calves remained seronegative (Figure 2). Lesions at attachment sites from all four calves receiving ticks were documented including edema of the ear, crusting and erythematous lesions on the outer pinna, and curling of the tip of the ear (Figure 3A), and, in some cases, severe excoriation (Figure 3B). These lesions were present in the right ear of all tick-infested calves, regardless of whether ticks were *R. parkeri*-infected. Indurated swellings ranging from approximately 2 to 6 cm developed at injection sites in all *R. parkeri*-injected calves. Immunohistochemistry of tissue samples revealed rickettsial organisms (Christopher D. Paddock, CDC) (Figure 4)

Discussion

During the course of this study, all calves remained clinically normal except for lesions consistent with "gotch" ear in the right ears of all tick-infested calves. None of the calves exposed to *R. parkeri* by injection of inoculum developed ear lesions, although they did develop lesions at injection sites consistent with a local inflammatory response. These results suggest *R. parkeri* infection may not be a factor contributing to *"gotch"* ear. In fact, this study implicates the GCT themselves as the major contributors to the pathology of "gotch" ear, since all calves infested with ticks, whether or not the ticks were exposed to *R. parkeri,* developed signs consistent with "gotch" ear.

The ticks fed successfully to repletion on the calves. In keeping with other studies on the roles of rickettsiae, ticks and vertebrate hosts, the ticks appear to act as vectors and reservoirs, while the cattle provide a blood source for the ticks thereby promoting the spread of *R. parkeri* indirectly. Pathology to the calves in this study was limited to the ears of the tick-infected calves, and the injection sites in the inoculum-injected calves.



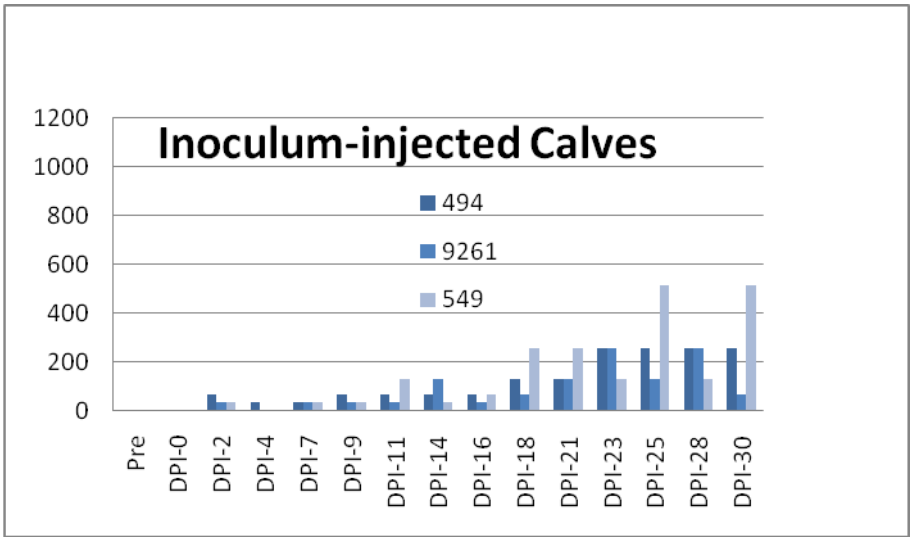


Figure 2. Tick-infected calves and *Rickettsia parkeri*-injected calves seroconverted at a 1:32 dilution on DPI-2; most were transiently infected and then remained positive for the duration of the study.

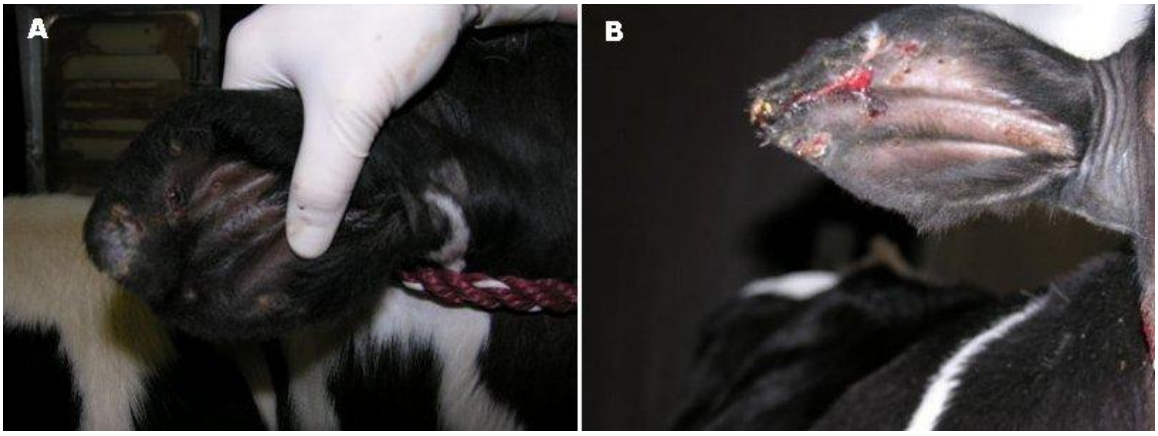


Figure 3. "Gotch" ear in calf infested with *Rickettsia parkeri*-infected *Amblyomma maculatum* ticks (A) and "Gotch" ear of calf infested with *Amblyomma maculatum* ticks without organism (B).

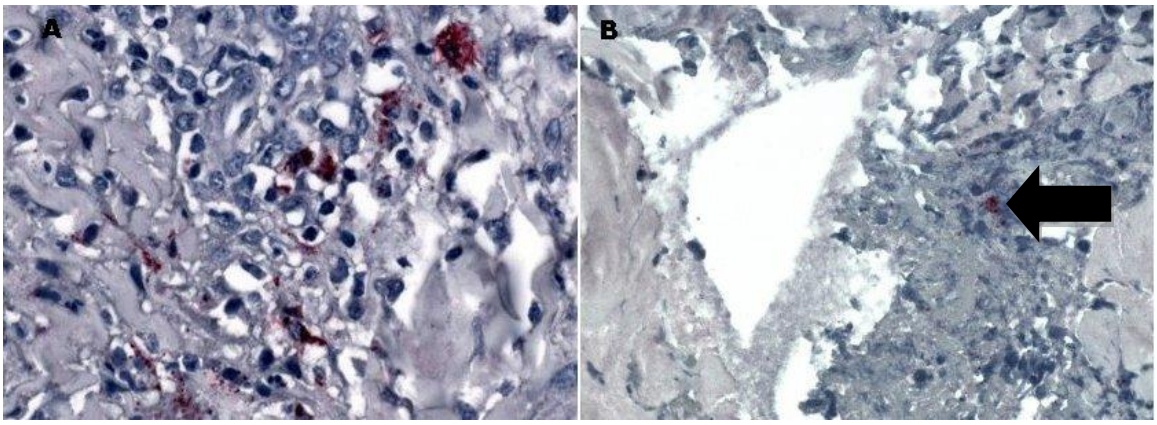


Figure 4. Immunohistochemistry (Christopher D. Paddock, CDC) of biopsied ear in calf infested with *Rickettsia parkeri*-infected *Amblyomma maculatum* ticks revealing rickettsial organisms (A) and immunohistochemistry (Christopher D. Paddock, CDC) of biopsied injection site in calf exposed to *Rickettsia parkeri* by needle injection revealing rickettsial organisms (arrow) (B).

CHAPTER IV

RICKETTSIA PARKERI IN GULF COAST TICKS (*AMBLYOMMA MACULATUM*)

INFESTING MISSISSIPPI CATTLE

Abstract

Amblyomma maculatum (Gulf Coast ticks) removed from cattle, as well as their cattle hosts, were tested for *Rickettsia parkeri,* a recently recognized human pathogen. Cattle were not rickettsemic by polymerase chain reaction (PCR) but 49.7% were seropositive (1:32) for spotted fever group (SFG) rickettsiae. Ticks removed from cattle were 11.8% hemolymph positive and 8.7% IFA positive for SFG rickettsiae. Approximately 22% (5/23) and 4% (1/23) of harvested ticks were SFG positive by PCR of the *17kDa* gene and the *rompA* gene, respectively. An amplicon for the *rompA* gene from one tick was successfully sequenced and showed 100% similarity with the homologous sequence of *R. parkeri.* Thus, *A. maculatum* from cattle may harbor *R. parkeri* and produce antibodies to SFG rickettsiae.

Introduction

Cattle have been known hosts for Gulf Coast ticks (GCT), *Amblyomma maculatum,* since 1937 when R. R. Parker and coworkers removed these ticks from cattle in Texas (Parker et al. 1939). At that time, Parker identified a rickettsial organism in GCT, similar to, but distinct from, *Rickettsia rickettsii,* the etiologic agent of Rocky Mountain Spotted Fever (RMSF). Parker speculated this organism might cause a RMSF-like illness which he referred to as “maculatum” infection (Parker 1940). Eventually, “maculatum agent” was named *R. parkeri* (Lackman et al.

1965), and a human case of *R. parkeri* infection was confirmed in 2002 in a Virginia man (Paddock et al. 2004). Since that report, the total number of cases has risen to twelve (Paddock et al. 2008) with four occurring in Mississippi residents.

The specific role of cattle in *R. parkeri* maintenance in GCT is unknown. Adult GCT prefer feeding on cattle ears and when infestations involve sufficient numbers, the ears may become thickened and curled, causing a condition referred to as *"gotch*" ear (Wright and Barker 2007). Little is known about the pathogenesis or epidemiology of *"gotch"* ear except that it usually involves GCT. Preliminary studies demonstrated transient rickettsemia in calves experimentally exposed to *R. parkeri* as well as evidence of *"gotch"* ear in calves experimentally infested with *R.* parkeri-infected and uninfected GCT (Edwards et al, unpublished data) (Chapter III).

The distribution of GCT in Mississippi counties was documented in 2005 (Goddard and Paddock 2005) (Figure 5). There are few data to describe vertebrates as reservoirs or amplifiers in the natural transmission of *R. parkeri*, and little information exists on the description of the natural history of *R. parkeri*. However, we believe the importance of cattle as hosts for these ticks implicates them in the ecology and transmission of *R. parkeri* rickettsiosis by GCT.

Materials and Methods

Cattle study

Cattle sale auctions are distributed throughout Mississippi (Figure 6). From July through October 2008, which encompasses peak GCT activity on cattle in Mississippi (Goddard and Paddock 2005), we collected GCT and blood samples from cattle (Table 1) at cattle auctions in

six Mississippi counties[3] (Figure 7). Cattle were sampled as they were held in a chute without regard to age, gender, or breed. Blood was obtained regardless of the presence of ticks. Ticks collected from cattle were deposited in labeled vials for transport to the lab where they were held in a humidity chamber until processing. Using an Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, Piscataway, NJ), nucleic acid was extracted from whole blood samples and the extracts were evaluated using a nested Polymerase Chain Reaction (PCR) assay designed to amplify a segment of the *17 kilodalton (KDa)* gene and a nested PCR designed to amplify a segment of the *rompA* gene based on published protocols (Paddock et al. 2004, Sumner et al. 2007) (Appendix D). Amplified product was separated by electrophoresis in 2% agarose gels containing ethidium bromide.

An indirect fluorescent antibody test (IFA) with *R. parkeri* (Tate's Hell isolate, CDP) antigen-coated slides, was performed on cattle sera to detect immunoglobulin (Ig)-G antibodies reactive with SFG rickettsiae. Each serum sample was screened at both a 1:32 and a 1:64 dilution to identify which animals were seropositive. Reactivity was determined using fluorescein isothiocyanate–conjugated goat anti–bovine IgG (Kirkegaard and Perry Laboratories) at a 1:100 dilution in phosphate-buffered saline (PBS; pH 7.4) (Appendix C).

Tick study

All information obtained for each animal was recorded on a numbered chart (Figure 8). Data were subsequently entered into a spreadsheet for further analyses. Ticks removed from cattle were hemolymph tested (Figure 9, Appendix A) (Gimenez 1964) (Appendix B), IFA tested (Appendix C), and assayed for SFG rickettsiae by nested PCR (Appendix D). For PCR testing, the

[3] Accompanying the attending veterinarian designated for these sales and in accordance with the Mississippi Department of Agriculture guidelines outlined for this purpose per our request.

ticks were minced using a scalpel blade and DNA was extracted using an Illustra Tissue and Cells Genomic Prep Mini Spin kit (GE Healthcare, Piscataway, NJ). A segment of the *17KDa* gene was amplified as described above. A segment of the *rompA* gene was also amplified based on a published protocol (Paddock et al. 2004). Positive PCR products were sequenced through MWG Biotech (Huntsville, Alabama) and sequences were analyzed using ClustalX2 and the BLAST program (version 2.0, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/blast.

Results

We collected blood samples from 183 cattle and examined their ears for ticks. We removed 39 GCT from 13 of 183 (7.1%) cattle at five of the six sale barns visited which represents 25% (6/24) of Mississippi sale barns (Table 1, Figure 7). Cattle comprised a variety of beef breeds and ranged in age from 1 to 14 (mean = 5) years with 96% (176/183) female. All cattle were negative for *R. parkeri* by PCR of both the *17KDa* gene and the *rompA* gene. Nearly 50% (91/183) of the cattle were positive when tested for SFG rickettsiae by IFA at a 1:32 dilution (Appendix C). However, they were negative at a 1:64 dilution.

Spotted fever group rickettsiae from 11.8% (4/34) of GCT were detected by the hemolymph test (Figure 9, Figure 10, Appendix B) and 8.7% (2/23) by IFA assay (Figure 11, Appendix D), including ticks from one sale barn in Walthall County where *A. maculatum* had not previously been reported (Goddard and Paddock 2005). Approximately 22% (5/23) and 4% (1/23) of ticks harvested from the cattle were SFG positive by nested PCRs of the *17kDa* gene and the *rompA* gene, respectively. The SFG-positive ticks were taken from cattle in Noxubee and Lauderdale county sale barns. We were able to sequence a *rompA* amplicon from one tick;

this showed 100% similarity with the homologous sequence of *R. parkeri* (GenBank accession no. U43802).

Discussion

Sale barn cattle are not typically representative of the general cattle population and therefore conclusions drawn about them cannot be extrapolated to the general population. Studies have demonstrated presence of GCT in Mississippi counties (Goddard and Paddock 2005) and the occurrence of human *R. parkeri* rickettsiosis in Mississippi has recently been documented (Finley et al. 2006). To our knowledge, the current study is the first documenting GCT on cattle in Mississippi and the first demonstrating evidence of *R. parkeri* in ticks harvested from those cattle. Our data show that cattle appear to serve as indirect amplifiers rather than reservoirs for *R. parkeri* based on absence of rickettsemia and development of antibodies to SFG rickettsiae. That is, they may play a role in the maintenance of the organism, possibly serving as blood meals for GCT and increasing distribution of ticks and indirectly, organism.

Sale barn cattle were chosen because they represent a livestock population from various geographic locations and diverse management systems. Since we detected *R. parkeri* in GCT removed from cattle and cattle produced antibodies to SFG rickettsiae without evidence of infection, we suspect GCT themselves may play a role as a rickettsial reservoir. However, further studies are required to thoroughly test this hypothesis.

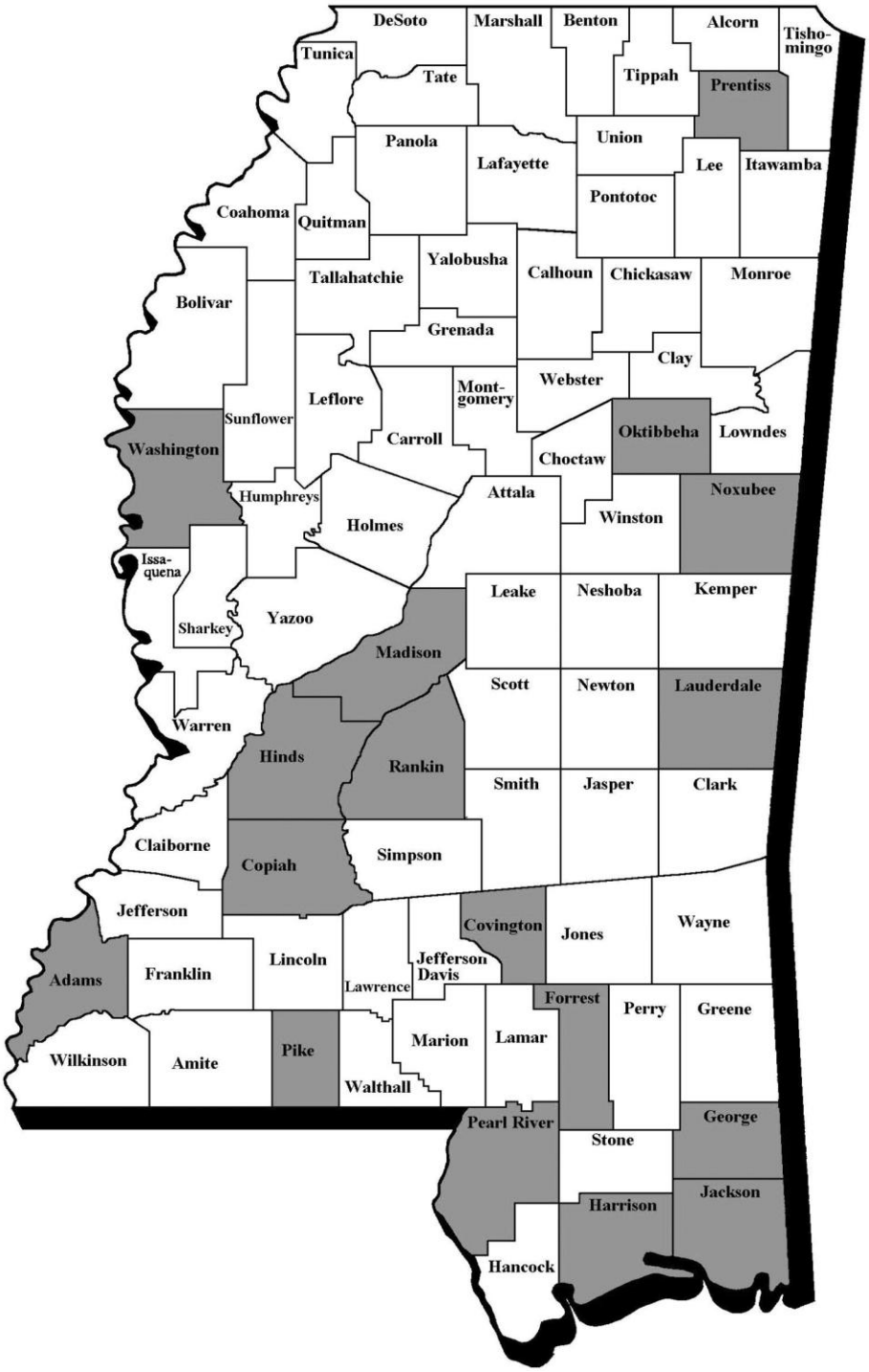


Figure 5. Geographic distribution of *Amblyomma maculatum* collections in Mississippi by county, 2005 (Goddard and Paddock 2005).

Figure 6. Distribution of sale barns in Mississippi, 2008.

Table 1. Gulf Coast *ticks* (*Amblyomma maculatum*) infected with spotted fever group rickettsiae, Mississippi sale barn cattle, 2008.

Sale barn	County	Date collected	Number ticks	Sex	Hemolymph positive	PCR pos *17KDa*	PCR pos *rompA*	IFA pos	Cattle breed	Age
1	Lee	9-Jul	0							
2	Clay	15-Jul	1	Male	0	0	0	0	Brahma	Unknown
3	Noxubee	28-Jul	1	Female	0	0	0	0	Hereford	3
			3	Male	0	0	0	0		
			10	Female	1	2	1§	1	Hereford	2
			1	Male	1	2	0	0		
4	Walthall	5-Aug	1	Male	0	0	0	1	Angus cross	Unknown
			1	Male	0	0	0	0	Black & White†	Unknown
			1	Female	1	0	0	0		
5	Adams	19-Aug	1	Nymph	*	*	*	*	Charolais cross	5
			1	Female	1	*	*	*	Charolais cross	5
			7	Male	0	*	*	*	Angus cross	5
			3	Female	*	*	*	*		
			2	Male	0	*	*	*	Angus cross	Unknown
			1	Female	0	*	*	*		
6	Lauderdale	6-Oct	2	Female	0	0	0	0	Angus cross	10
			1	Female	0	1	0	0	Angus	7
			1	Female	0	0	0	0	Angus	0
			1	Female	0	0	0	0	Angus	6
Total			39		4	5	1	2		13
Total tested					34	23	23	23		

† Common name for any unknown black and white cattle breed.
* Ticks lost to follow up: five died before hemolymph testing; another eleven died before PCR or IFA testing.
§ Sequence analysis for a *rompA* amplicon showed 100% similarity with the homologous sequence of *R. parkeri* (GenBank accession no. U43802).

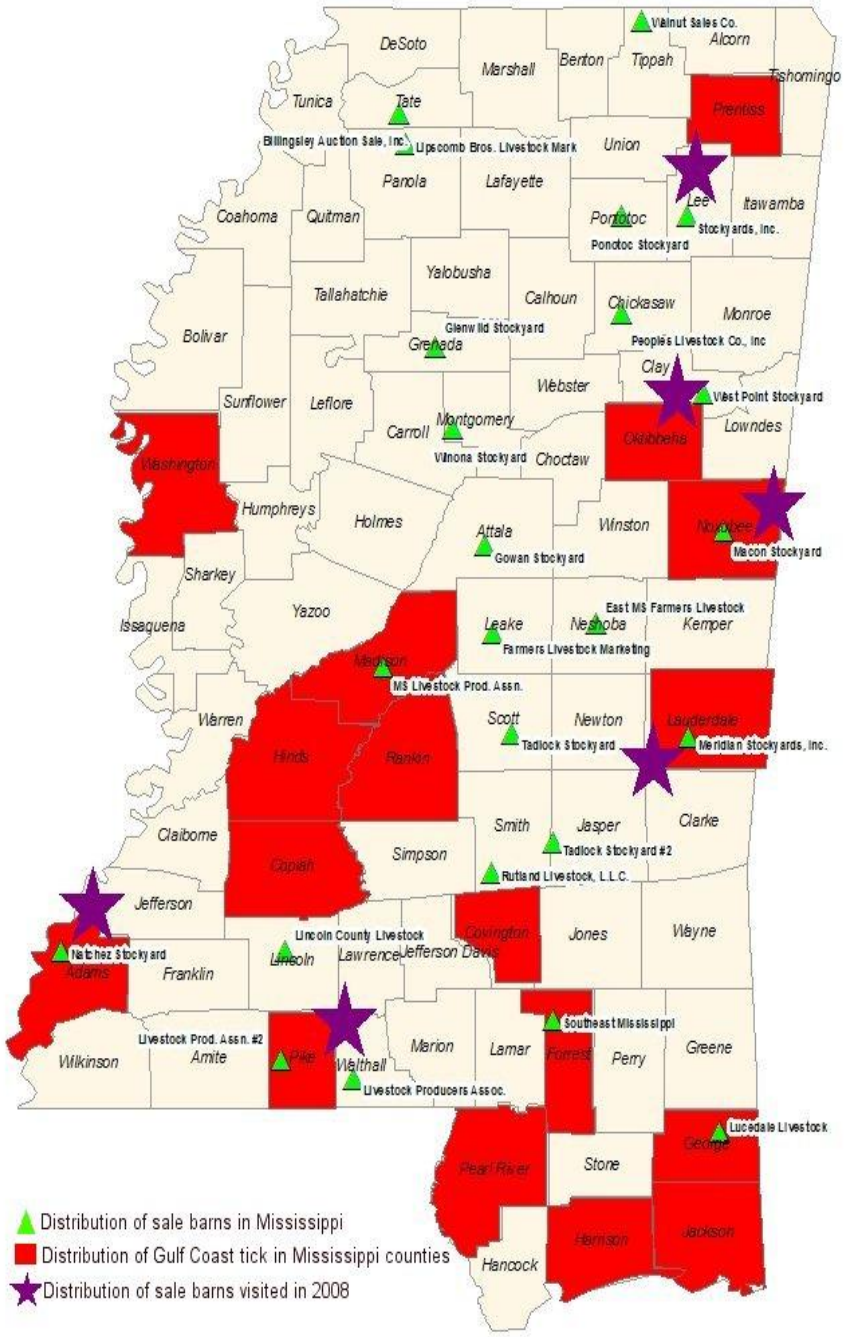
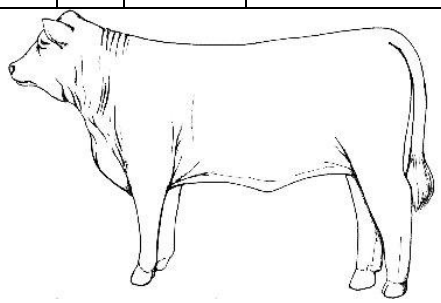
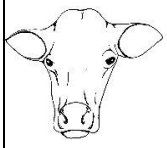
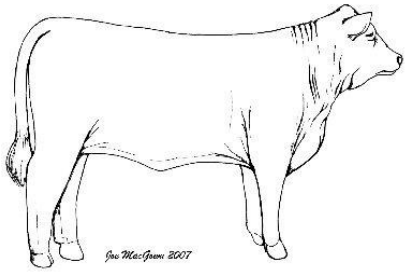


Figure 7. Geographic distribution of *Amblyomma maculatum* collections in Mississippi by county, 2005, distribution of sale barns, and distribution of sale barns visited in this study, 2008. (Map constructed by Linda Tischer)

The Role of Cattle in the Epidemiology of the Gulf Coast Tick						
CHART ID	Veterinarian's name, address, phone no.			Sale barn name, address, phone no.		
Zip				Zip		
Phone no.				Phone no.		
				County		
Lesions biopsied:					Collection tubes:	
Ears: ☐ L ☐ R ☐ Withers ☐ Back ☐ Tail head				Day: Time:	☐ Serum separator ☐ EDTA	
TUBE NO	TAG NO	COLOR	BREED	APPROX. WEIGHT	AGE	SEX: ☐ MALE ☐ FEMALE ☐ STEER
						

Date collected:	Signed:
No. of ticks collected:	Remarks:
Date tested:	
PCR results:	
Laboratory comments:	
Kristine T. Edwards Form updated: 11/27/2007	

Figure 8. Cattle chart designed in 2007 by author for data collection at sale barns.

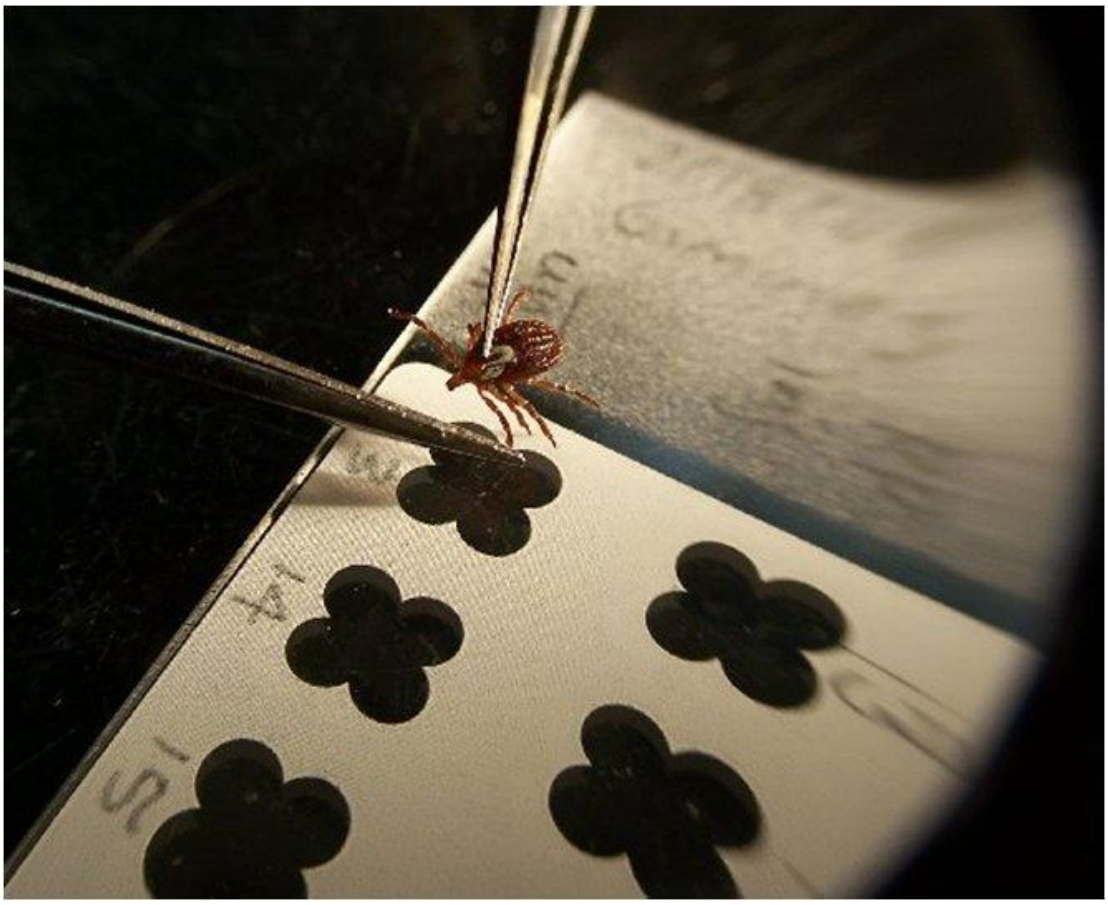


Figure 9. Hemolymph test of an *Amblyomma maculatum* female tick illustrating amputation of the pretarsus. Photo: Jerome Goddard, 2007.

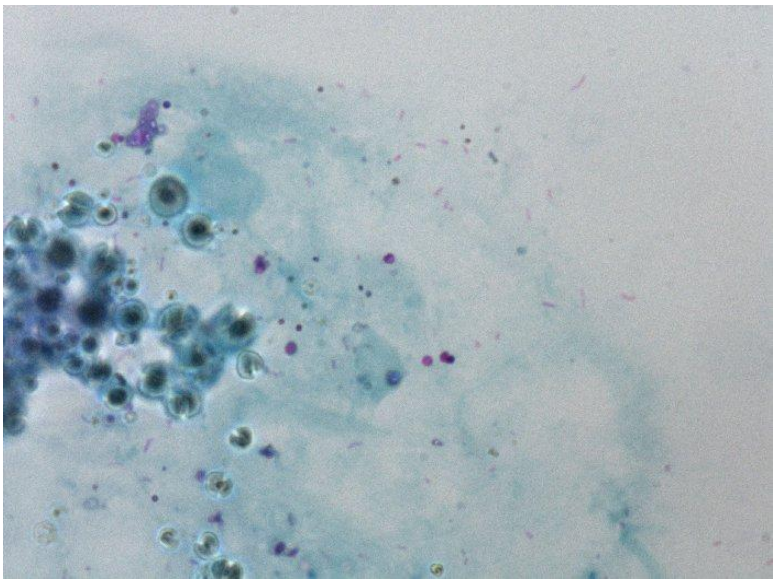


Figure 10. Gimenez stain of tick hemolymph demonstrating spotted fever group rickettsiae in a tick harvested from a cow at a sale barn in Noxubee County, Mississippi, 2008.

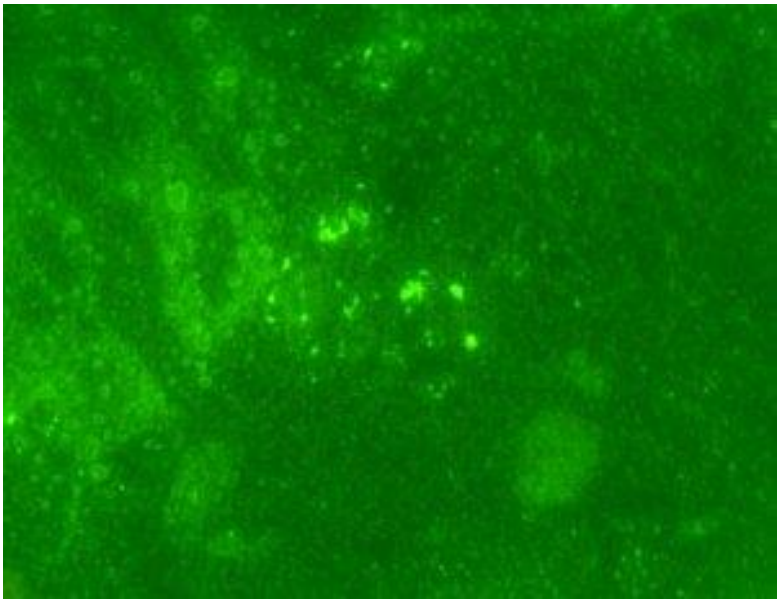


Figure 11. Fluorescence staining demonstrating spotted fever group rickettsiae in a tick harvested from a cow at a sale barn in Noxubee County, Mississippi, 2008.

CHAPTER V

EXAMINATION OF THE INTERNAL MORPHOLOGY OF THE IXODID TICK, *AMBLYOMMA MACULATUM* KOCH, (ACARI: *IXODIDAE*); A "HOW-TO" PICTORIAL DISSECTION GUIDE[4]

Introduction

There is copious scientific literature on the anatomical structure of ticks including Ixodid ticks (Acari: Ixodidae), as well as descriptions of the physiology (Obenchain and Galun 1982, Sonenshine 1991, Buczek 1999) of the digestive tract and diagrams of the internal anatomy, (Douglas 1943, Obenchain and Galun 1982). There are also stunning images of tick mouthparts and other structures produced by scanning electron microscopy which are readily available on the Internet (Balashov lu and Raikhel 1973, Pickering 2008) . However, there is inadequate documentation of how to dissect a tick, (Balashov 1968, Obenchain and Galun 1982), a process required for many tick/disease studies (Goddard and Norment 1986, Goddard 2003). The literature contains numerous line drawings (Balashov 1968, Obenchain and Galun 1982) and texts describing the process but none is adequate for the novice tick-dissector. One possible exception is found on a web page hosted by Iowa State University Department of Entomology where a limited tick dissection sequence is presented for *Ixodes scapularis* Say illustrating dissection of the midgut (VanDyk 2001). However, for the most part, unless one has the

[4] Previously published in Midsouth Entomologist, Edwards, K., J. Goddard, and A. S. Varela-Stokes. 2009. Examination of the internal morphology of the Ixodid tick, *Amblyomma maculatum* Koch, (Acari: Ixodidae); a "How-to" pictorial dissection guide. Midsouth Entomologist 2: 28-39.

guidance of an individual both knowledgeable and skilled in tick dissection, it is extremely difficult to teach oneself the complete tick dissection process from currently available materials.

Since there is little assistance in the literature on how to dissect ticks, and specifically, how to identify structures at each step of the dissection, our tutorial is intended as a stand-alone document to guide an individual new to the process through the necessary steps. We offer a "how-to" description and pictorial essay outlining our approach to tick dissection. The ticks used in this study were adult male and female Gulf Coast ticks, *Amblyomma maculatum* Koch, though the methods described herein could be applied to any species. None of the *A. maculatum* specimens we dissected adequately demonstrated ovaries so we used one female *Amblyomma americanum* Linnaeus tick, which we found to show them more clearly. The tissues we targeted are those we were analyzing for our research and are ones commonly dissected. Specifically, we included salivary glands, midgut, Malpighian tubules and ovaries in this study (Figure 12). Tracheae are also delineated because they may easily be confused with Malpighian tubules.

Materials and Methods

The following is a step-by-step pictorial description of the dissection of the Gulf Coast tick, *Amblyomma maculatum*. Note: since field caught ticks are potentially infected with a variety of disease agents, a few precautions may be necessary. For example, a white board with double-sided sticky tape around the edges may be used to sort ticks, and gloves should be used during dissection since the infectious status of ticks is often unknown. In addition, aerosolization of tick fluids is possible as tick parts are mixed with saline and should be avoided by wearing a mask during the dissection process. All ticks, parts, fluids, and supplies should be

disposed in clearly marked biohazard bags after completion of tick dissections. The dissection process is described in the following steps:

1. Prepare a small Petri dish ahead of time by pouring melted paraffin into the bottom and allowing it to cool and solidify.
2. Briefly heat the center of the small paraffin-filled Petri dish (We found a simple approach was to use the tip of a glue gun to melt the paraffin but a heated spoon may also suffice).
3. Once the paraffin is warm and starts to melt, gently grasp the tick with forceps pressing its legs and ventrum into the heated paraffin thereby immobilizing it and restraining its legs.
4. Cover the tick with a drop of phosphate buffered saline (PBS). This is an important step because it prevents desiccation of the tissues.
5. Remove the scutum with a microscalpel[5] by first cutting across the dorsal shield at the most anterior point, just distal to the basis capitulum (A regular scalpel was much too large and we ordered a special, delicate scalpel called a "microscalpel" for use only in tick dissections).
6. Continue cutting around the edge of the scutum inserting the microscalpel into the groove just inside the festoons. Lift the dorsal exoskeleton with forceps held in one hand while carefully dissecting the attached muscles and connective tissue with the microscalpel in the other hand. Cut the dorsum and remove completely.
7. At this point, connective tissue and tracheae are apparent and usually have to be removed in order to observe deeper structures.

[5] Ultra fine micro knife, catalog number #10315-12, Fine Science Tools, 373-G Vintage Park Drive, Foster City, CA 94404-1139

8. Observe anterior salivary glands – these are clear, grape-like structures at the proximal end of the tick; there are also other sets of salivary glands located near the midgut.
9. Observe gut – the gut is a dark, red, spider-shaped structure.
10. Tracheae can be seen originating from spiracular plates and must be differentiated from Malpighian tubules.
11. Observe Malpighian tubules – these tubules are clear, thin, tube-shaped structures that often have urea which appears white within the tubules. Connective tissue or tracheae may also appear white but are flat and wispy rather than tube-shaped. Further distinction between tracheae and connective tissue is often challenging. Tracheae originate from spiracular plates but connective tissue often lacks obvious organization.
12. Observe ovaries – these appear as inverted U-shaped structure distal to rectal sac.

All figures refer to *Amblyomma maculatum* Koch ticks unless otherwise stated.

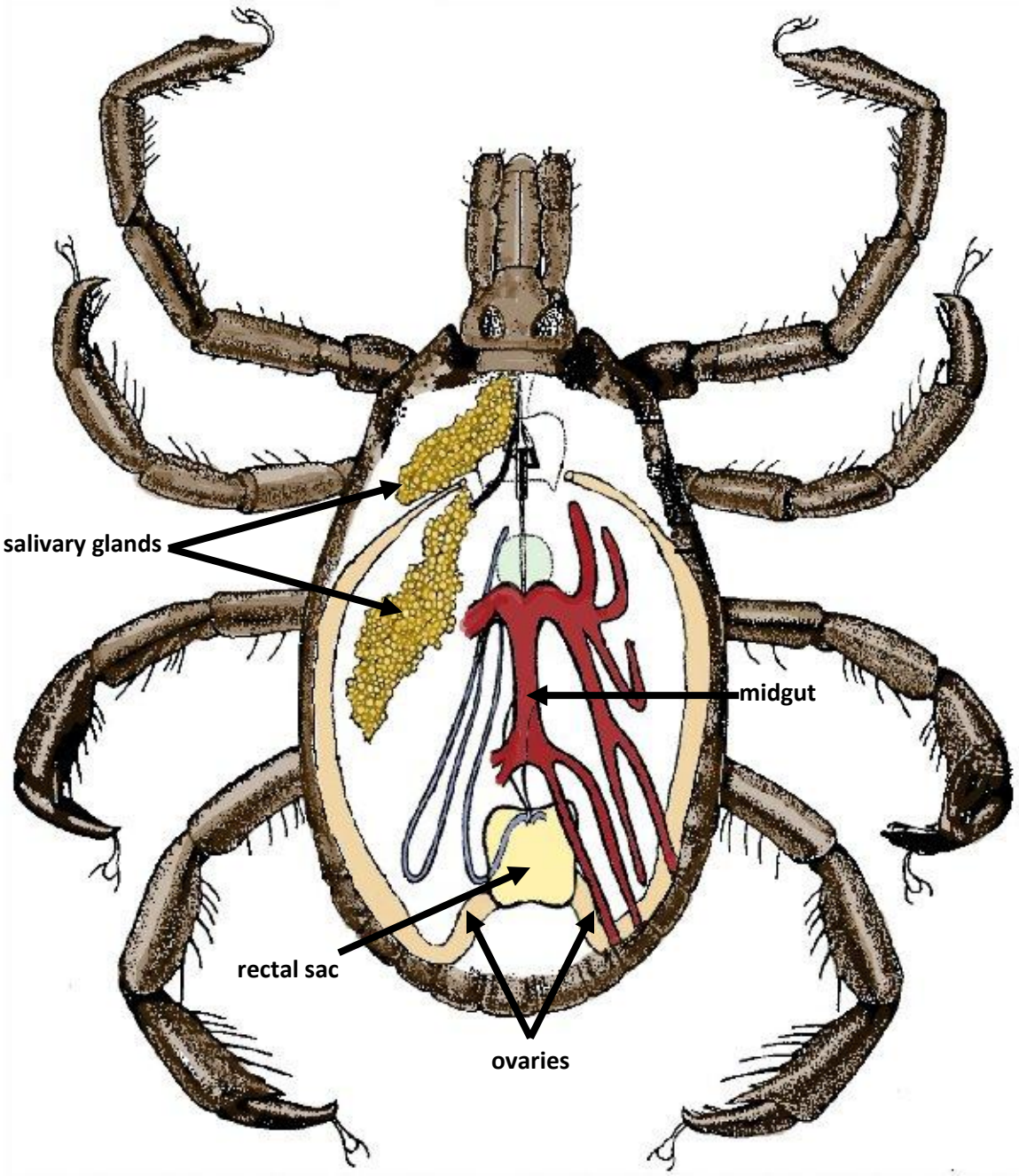


Figure 12. Line drawing of tick structures observed during dissection (original art work by Sylvia Burnett, Mississippi Department of Health; modified by KTE).

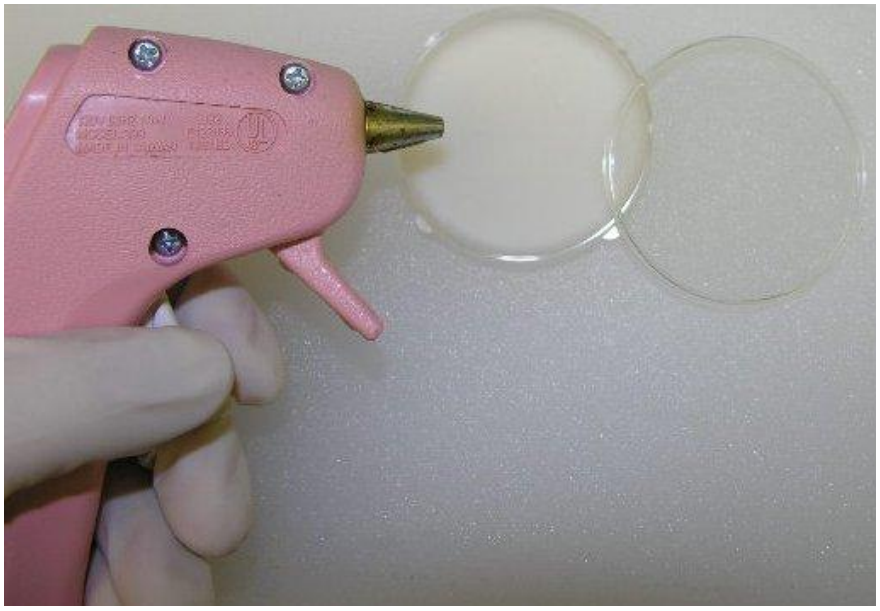


Figure 13. Steps 1 and 2 – Using glue gun to melt paraffin in Petri dish.

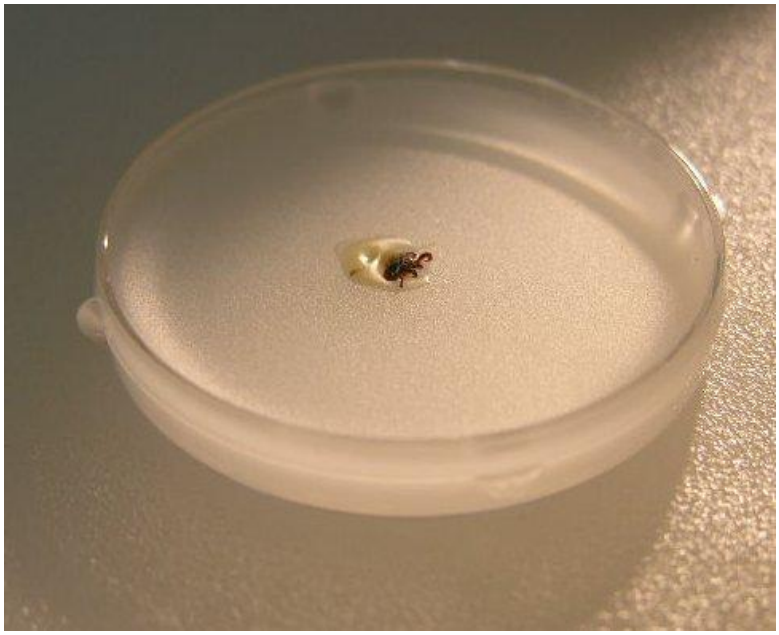


Figure 14. Step 3. Tick embedded in paraffin.

Figure 15. Step 4. Female embedded in paraffin, covered with PBS; dorsal view.

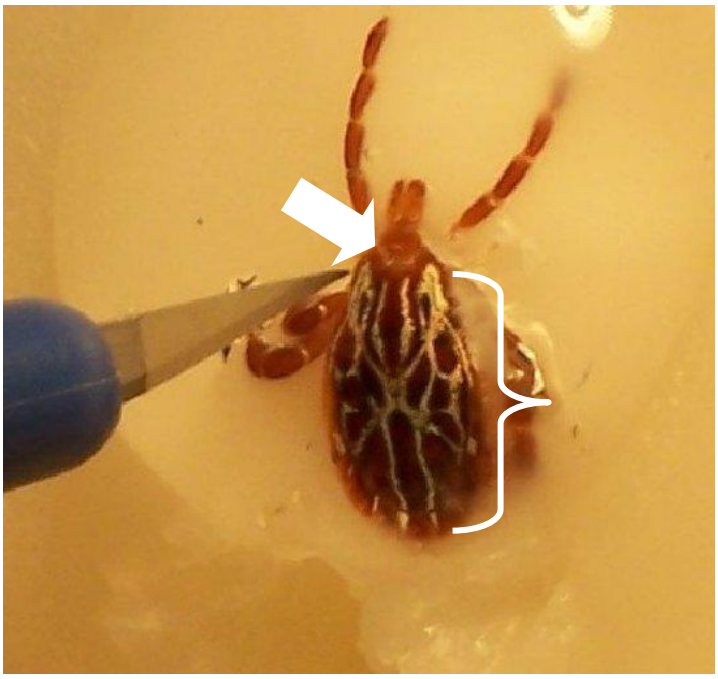


Figure 16. Step 5. Male embedded in paraffin; dorsal view. Preparing to remove scutum (bracket) with microscalpel starting at basis capitulum (arrow).

Figure 17. Step 6. Female – scutum removal.

Figure 18. Step 6 (cont.). Female – scutum removed (arrow).

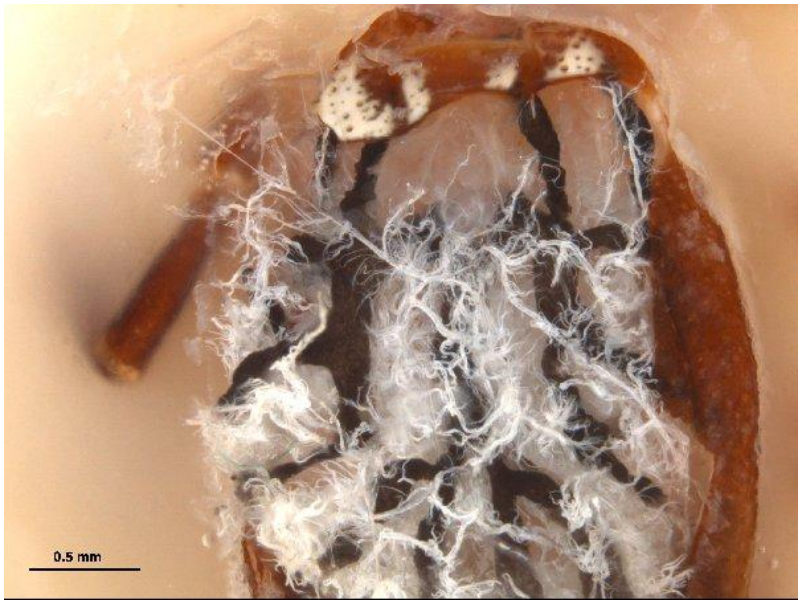


Figure 19. Step 7. Female – scutum removed. Connective tissue and tracheae are visible contrasting with surrounding gut (dark).

Figure 20. Step 8. Female – scutum removed. The complete gut is now visible as well as the anterior salivary glands (arrow).

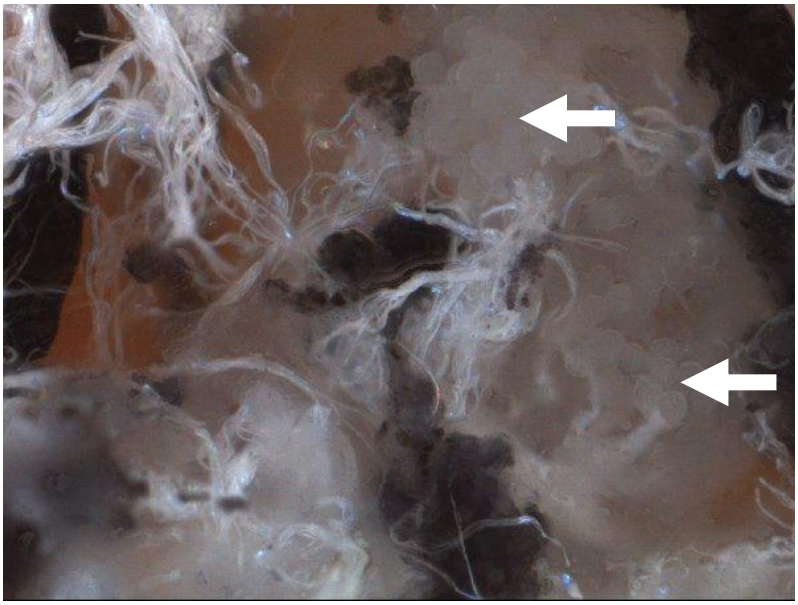


Figure 21. Step 8 (cont.). Salivary glands (arrow) – close-up.

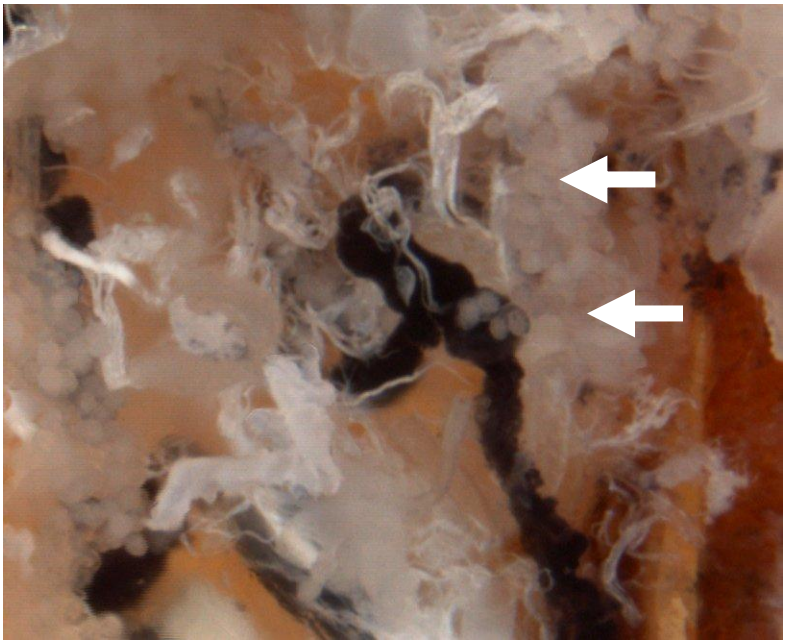


Figure 22. Step 8 (cont.). Female – close-up of salivary glands near midgut.

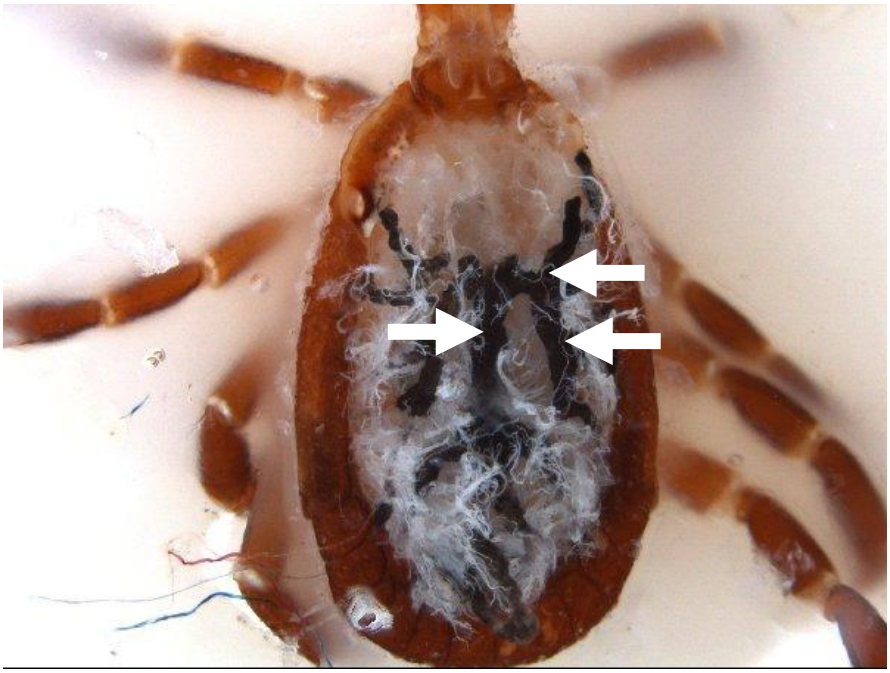


Figure 23. Step 9. Female – gut visible as dark, red, spider-shaped structure (arrows).

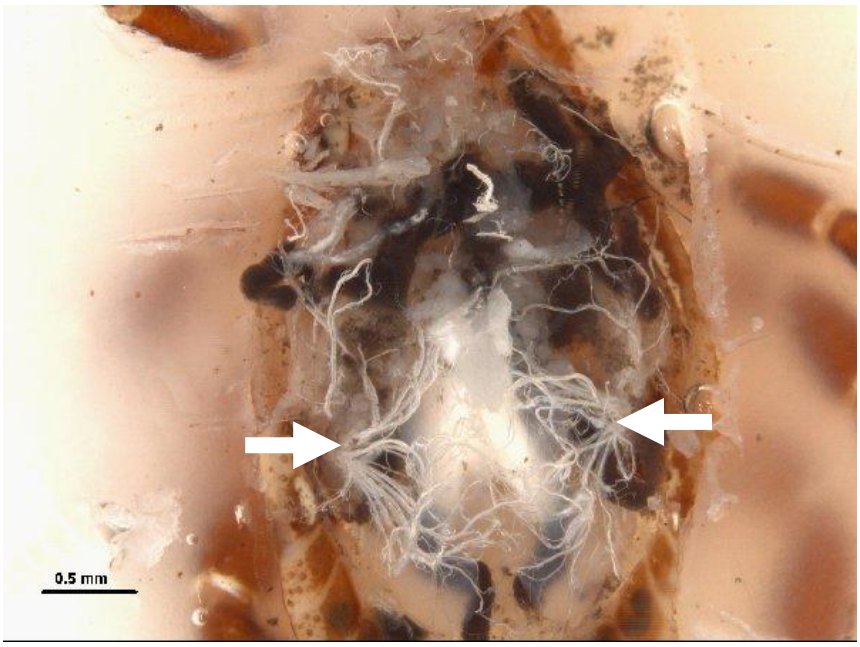


Figure 24. Step 10. Male – tracheae (arrows) originating from spiracular plates.

Figure 25. Step 10 (cont.) – Male tracheae close-up.

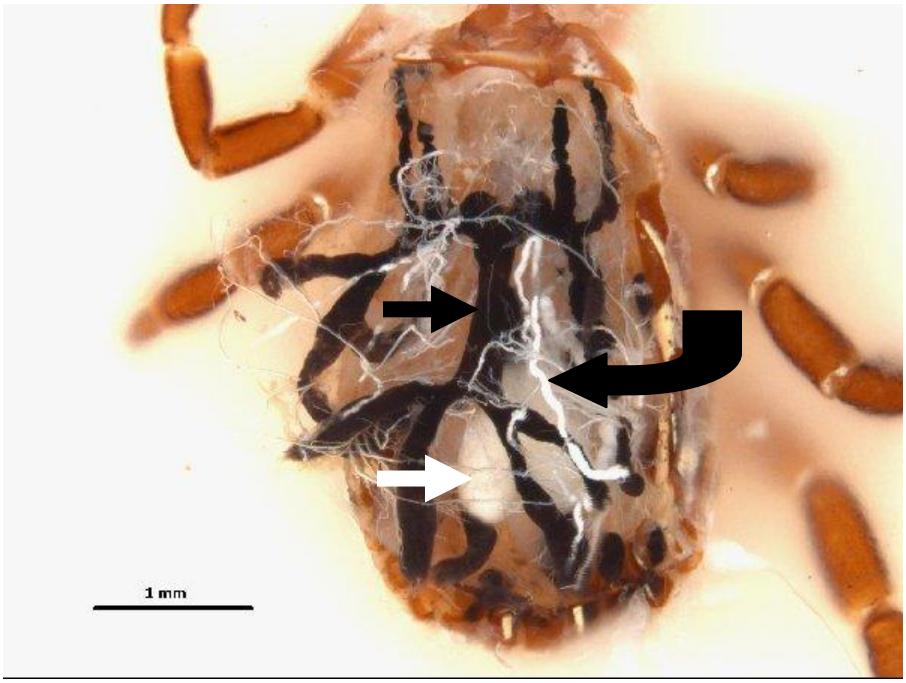


Figure 26. Step 11. Male – Malpighian tubules (curved arrow), rectal sac (white arrow), and midgut (black arrow).

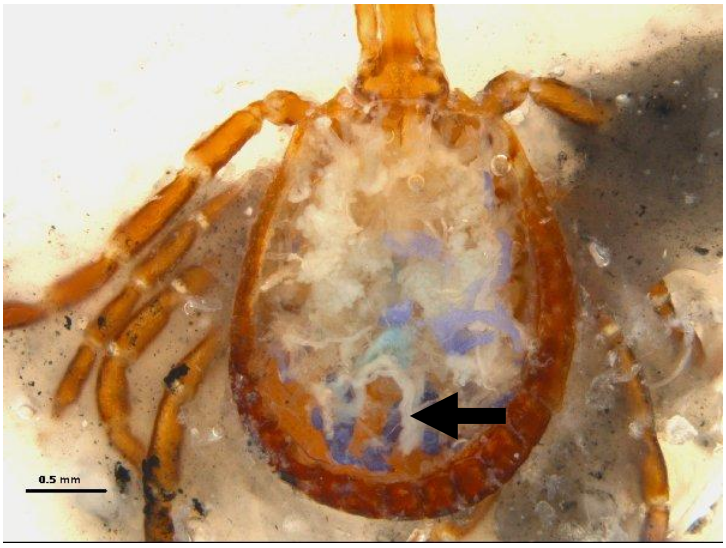


Figure 27. Step 12. Gut is removed and ovaries are apparent (arrow) in a female *Amblyomma americanum* tick.

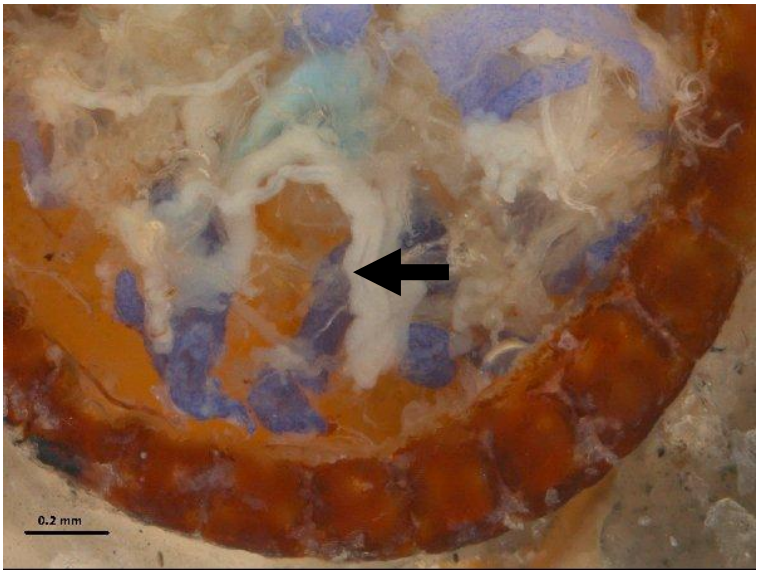


Figure 28. Step 12 (cont.). Close-up of previous tick with gut removed to reveal ovaries (arrow) in a female *Amblyomma americanum* tick.

Acknowledgements

We gratefully acknowledge Joe MacGown, Department of Entomology & Plant Pathology, Mississippi State University for his assistance in taking photographs 4-12 and to Erle Chenney, Department of Basic Sciences, Mississippi State University for his assistance in preparing the Petri dishes. Funding provided by the Department of Entomology & Plant Pathology and the College of Veterinary Medicine, Mississippi State University. This article has been approved for publication as Journal Article No. J-11498 of the Mississippi Agriculture and Forestry Experiment Station, Mississippi State University.

CHAPTER VI

DISTRIBUTION OF *RICKETTSIA PARKERI* IN SELECT TISSUES OF EXPERIMENTALLY-INFECTED AND FIELD-COLLECTED GULF COAST TICKS (*AMBLYOMMA MACULATUM*)

Abstract

Salivary glands, midgut, Malpighian tubules, and ovaries were dissected from reared *Amblyomma maculatum* (Gulf Coast ticks) injected with either *Rickettsia parkeri* or phosphate-buffered saline (PBS); similar tissues were dissected from hemolymph-positive, field-collected ticks. All ticks were analyzed by indirect-fluorescent antibody (IFA). The PBS-injected ticks were IFA negative while spotted fever group (SFG) rickettsiae were detected by IFA in 100% of the salivary glands and ovaries and 78% and 75% of midgut and Malpighian tubules, respectively of *R. parkeri*-injected ticks. Nearly 22% (10/46) of the field-collected ticks were hemolymph positive. Of those, SFG rickettsiae were detected by IFA in 80% of the salivary glands, 67% of the ovaries and 60% in the midgut and Malpighian tubules. This is the first study to assess the distribution of *R. parkeri* in select tissues in *A. maculatum* ticks.

Introduction

In 1937, R. R. Parker and coworkers discovered an organism distinct from *Rickettsia rickettsii* in *Amblyomma maculatum*, Gulf Coast ticks (GCT), collected from cattle in Texas (Parker et al. 1939). The organism was eventually named *R. parkeri* in honor of Dr. Parker (Lackman et al. 1965). The agent was referred to as a non-pathogen until 2002 when the first case of infection due to *R. parkeri* was confirmed in a Virginia man (Paddock et al. 2004). Parker

speculated this organism might cause a Rocky Mountain spotted fever (RMSF)-like illness, which he referred to as "maculatum" infection (Parker 1940) because he discovered it in *A. maculatum* ticks. Since that report, the number of rickettsiosis cases attributable to *R. parkeri* has risen to twelve (Paddock et al. 2008) with four of those cases occurring in Mississippi residents.

Rickettsiae are transmitted both transstadially and transovarially in their tick vectors (Azad and Beard 1998) and *R. parkeri* has been studied in an artificially-infected colony of *A. americanum* (lone star ticks) (Goddard 2003), however detection of *R. parkeri* in specific GCT tissues has not been evaluated. Elucidating the spotted fever group rickettsiae tissue distribution is vital in discovering transmission mechanisms as well as novel approaches to tick-borne rickettsial disease control, but few studies have addressed this issue. An exception is the characterization by real-time polymerase chain (PCR) reaction of the distribution of rickettsial infection in *A. americanum* ticks in specific tissues (Zanetti et al. 2008). In that study, no specific tissue was consistently observed to have the greatest rickettsial burden throughout the feeding event when the salivary glands, gut, and ovaries were examined (Zanetti et al. 2008). The purpose of the current study was to document the distribution of SFG rickettsiae in specific tissues of the GCT and to compare the distribution in colony-reared GCT to the distribution in field-collected, SFG-positive GCT.

Materials and Methods

All work was conducted in a biosafety level-2 (BSL-2) laboratory[6]. Engorged nymphal *A. maculatum* ticks obtained from a laboratory-reared colony at Oklahoma State University

[6] Biological use authorization approved by the Mississippi State University institutional environmental health and safety committee.

(Stillwater, OK) were injected percutaneously with *R. parkeri*[7] or with phosphate-buffered saline (PBS, pH 7.4) at 5 days post-engorgement as previously described (Goddard 2003). Ticks were first immersed in 70% ethanol, dabbed with 70% ethanol or given no surface treatment (Table 2). Ticks were reared to adults for use in infection and transmission studies (CHAPTER III) and a subset was set aside for this study. Eighteen of the *R. parkeri*-injected GCT were analyzed by PCR and hemolymph tests to determine whether they were infected.

Ten PBS-injected ticks and nine *R. parkeri*-injected ticks were dissected as previously described (Edwards et al. 2009). For comparison, forty-six adult *A. maculatum* ticks were collected by drag cloth from a site near Moss Point, Mississippi during August, 2009. Ten of these ticks (21.7%) were hemolymph positive and subsequently were dissected for tissue analysis.

For all ticks (*R. parkeri*-injected, PBS-injected, and field-collected), tissue from salivary glands, midgut, Malpighian tubules, and ovaries (in females) were applied to individual slide wells for each tick. Each slide was then incubated with a 1:100 dilution of human anti-*R. rickettsii,* washed, and then incubated with a 1:20 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-human antibodies (CDP). After washing and counterstaining with eriochrome black-T, slides were visualized with epifluorescence.

Results

In the PBS treatment group, seven female and three male (10 total) ticks were dissected. For each PBS-injected tick, samples were collected from salivary glands, midgut,

[7] *Rickettsia parkeri* (strain Tate's Hell) was isolated in Vero E6 cells from a female *Amblyomma maculatum* tick collected in Franklin county, Florida, in 2005 (Christopher D. Paddock, CDC). The inoculum consisted of the 4th passage of this isolate in Vero E6 cells. Cytospins of the isolate made at the time of inoculation showed numerous rickettsiae by IFA staining with anti-*R. rickettsii* human antibodies.

Malpighian tubules, and ovaries (in females) for a total of thirty-seven tissues. There was no evidence of spotted fever group (SFG) rickettsiae in 100% (37/37) of tissue samples dissected from these PBS-injected *A. maculatum* ticks (Table 3).

Eighteen GCT were injected with *R. parkeri* and analyzed by PCR and hemolymph tests. Eighty-three percent (15/18) had evidence of organism by PCR amplification of the *rompA gene*. Gimenez staining of hemolymph demonstrated organism in 86% (16/18) of the same ticks. Two female and seven male (9 total) ticks were dissected and a total of twenty-eight tissue samples were collected from nine *R. parkeri*-injected GCT. There was evidence of SFG rickettsiae in both ovarian samples (2/2), 100% (9/9) of the salivary gland tissue samples, and 78% and 75% of midgut and Malpighian tubule tissues, respectively, from these ticks (Table 3).

In the field-collected tick group, six female and four male ticks were hemolymph-positive 21.7% (10/46). These ticks were dissected, and thirty-six tissue samples were collected from these ten ticks. Evidence of SFG rickettsiae was demonstrated by IFA in (8/10) 80% of the tissue samples dissected from the salivary glands, 67% (4/6) of the tissue samples dissected from the ovaries, and 60% (6/10) of the tissues dissected from the midgut and Malpighian tubules, respectively (Figure 29 and Table 3) . Although the SFG rickettsiae detected in the field-caught *A. maculatum* ticks were not identified, they were likely *R. parkeri* based on previous studies of SFG associated with *A. maculatum* ticks (Sumner et al. 2007).

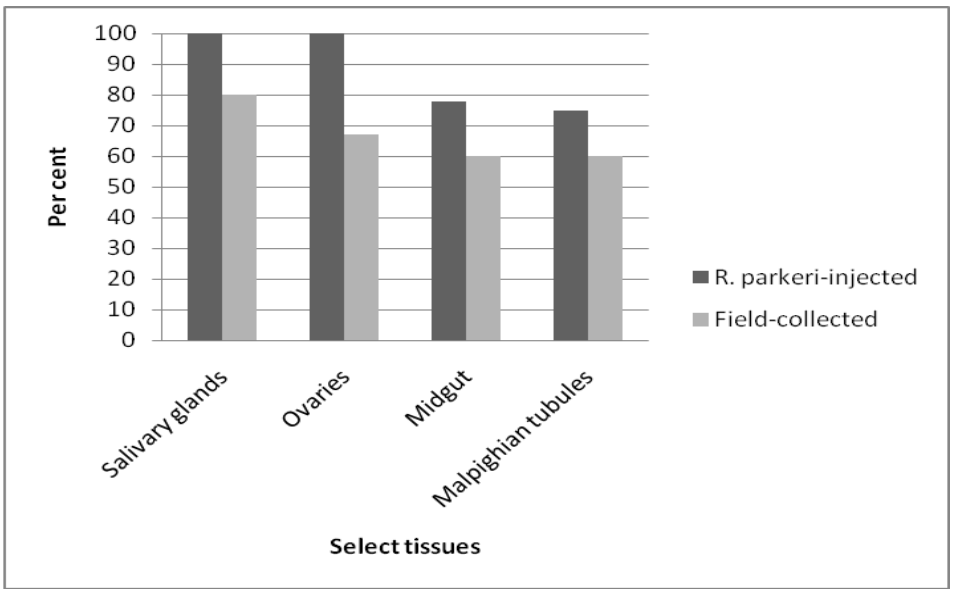


Figure 29. Comparison of percent positive by immunofluorescent antibody assay to spotted fever group rickettsiae in colony-reared *Rickettsia parkeri*-injected *Amblyomma maculatum* ticks and field-collected, hemolymph-positive *A. maculatum* ticks.

Discussion

To our knowledge, this is the first study to examine the tissue distribution of SFG rickettsiae in GCT. The infection rate of 21.7% (10/46) in the field-collected GCT is consistent with that of other studies. For example, Sumner et al. (Sumner et al. 2007) estimated infection prevalence to be 11-12% in GCT collections from Florida and Mississippi. Also, Paddock (personal communication) found *R. parkeri* infection rate as high as 40% in the *A. maculatum* ticks tested in Mississippi. This study suggests that *R. parkeri* infection in ticks is systemic, including hemolymph, salivary glands, midgut, Malpighian tubules, and ovaries. The results provide support for the hypothesis that GCT themselves may play a role as a reservoir in both artificially- and naturally-infected GCT for this rickettsia. The study also suggests that field-collected GCT have a similar tissue distribution of SFG rickettsiae to laboratory-reared GCT

infected by percutaneous injection, although the levels of SFG rickettsial infection appear to be lower in naturally-infected GCT.

Table 2. Mean days to molt and percent molted for different treatment groups in Gulf Coast ticks (*Amblyomma maculatum*) reared for study in calves experimentally exposed to *Rickettsia parkeri,* 2008.

Injection	Treatment	Number Of ticks	Mean days to molt	Range of days
R. parkeri	None	67	24.39	22-28
	Dabbed†	67	24.78	22-28
	Immersed*	65	24.21	23-31
	Total molted	199/210 (95%)		
PBS+	None	25	24.88	22-30
	Dabbed†	24	25.71	23-31
	Immersed*	23	25.26	23-28
	Total molted	72/75 (96%)		

† Dabbed with 70% EtOH
* Immersed for 30 seconds in 70% EtOH
+ Phosphate-buffered saline

Table 3. Indirect fluorescent antibody assay comparing tissue samples dissected from Gulf Coast ticks (*Amblyomma maculatum*) experimentally-infected with *Rickettsia parkeri* (2008) and field-collected Gulf Coast ticks positive for spotted fever group rickettsiae at a 1:64 dilution (positive [+] or negative [-]).

Injection	Gender	Salivary glands	Midgut	Malpighian tubules	Ovaries
R. parkeri	Female	+	-	-	+
	Female	+	+	+	+
	Male	+	-	-	
	Male	+	+	+	
	Male	+	+	+	
	Male	+	+	+	
	Male	+	+	+	
	Male	+	+	-----	
	Male	+	+	+	
Total tissues	28				
Total ticks	9				
Infection rate		100% (9/9)	78% (7/9)	75% (6/8)	100% (2/2)
PBS[†]	Female	-	-	-	-
	Female	-	-	-	-
	Female	-	-	-	-
	Female	-	-	-	-
	Female	-	-	-	-
	Female	-	-	-	-
	Female	-	-	-	-
	Male	-	-	-	
	Male	-	-	-	
	Male	-	-	-	
Total tissues	37				
Total ticks	10				
Infection rate		0%	0%	0%	0%
Field-collected					
	Female	+	-	-	+
	Female	+	+	-	+
	Female	+	+	+	+
	Female	+	+	+	+
	Female	+	+	-	-
	Female	-	-	-	-
	Male	+	+	+	
	Male	+	+	+	
	Male	-	-	+	
	Male	+	-	+	
Total tissues	36				
Total ticks	10				
Infection rate		80% (8/10)	60% (6/10)	60% (6/10)	67% (4/6)

[†]Phosphate-buffered saline

CHAPTER VII

CONCLUSIONS AND IMPLICATIONS

All of the objectives of this study were met, and in some instances, surpassed. In the experimental study in calves, we determined that Gulf Coast ticks (GCT), (*Amblyomma maculatum)* successfully fed to repletion on the ears of calves. We also accumulated substantial evidence that the exposed calves were *R. parkeri*-infected by documenting rickettsemia, antibody production, and evidence of rickettsial transmission (by immunohistochemstry) in some of the calves. We defined the condition "gotch" ear in cattle and demonstrated, for the first time, that it occurs in tick-infested calves whether or not they are rickettsemic, and whether or not the ticks are infected with *R. parkeri*. Calves did not appear to become clinically ill for the duration of the study as determined by hematologic and clinical parameters, except for calves with ticks, all of which developed "gotch" ear.

We documented the presence of GCT on cattle from some sale barns in Mississippi but we did not demonstrate evidence of rickettsemia by PCR for any of the cattle. Our assessment took place at one point in time providing only a "snapshot", so we could only evaluate the sera as either positive or negative for a particular dilution. We first tested cattle sera at a 1:64 dilution and found them all seronegative for SFG rickettsiae, then, when we retested at a 1:32 dilution, we found that nearly 50% of the cattle had evidence of exposure to SFG rickettsiae. We also performed an IFA assay for antibodies at a 1:32 dilution on the sera of the experimental calves. Some calves showed seroconversion at this dilution as early as DPI-2. Since we were testing for IgG antibodies, we thought it unlikely that seroconversion could occur that soon after

exposure. This was a carefully controlled study and we were confident the calves were all previously negative to SFG rickettsiae as evidenced by PCR screening prior to experimental exposure. Therefore, it is difficult to explain the early seroconversion in calves. It is noteworthy that both negative control calves were negative by IFA at both the 1:32 and the 1:64 dilutions in a blinded evaluation.

A possible explanation is that early seroconversion might have been due to the massive doses of *R. parkeri*-infected Vero cells we injected into the inoculated calves and into the *R. parkeri*-infected ticks we placed on the calves. Each calf designated for inoculation was injected with 0.3 ml of 2.63 $X10^6$ *R. parkeri*-infected Vero cells/ml at each route. A study of related rickettsiae (*R. conorii*) in cattle showed that the rate of seroconversion is dose-related (Kelly et al. 1991). In that study, all of the cattle were inoculated with either 3,000 organisms (LD [low dose]) or 100,000 organisms (HD [high dose]). The HD cattle showed an IgG response (titer ≥ 1:40) by DPI-7 and LD cattle by DPI-15. The dose used in our study was higher than their higher dose by a factor of nearly 30. Also, we demonstrated that experimentally-infected GCT had a higher infection rate in each tissue tested by IFA than in corresponding tissues of naturally-infected GCT.

By re-assaying sera of sale barn cattle at a 1:32 dilution, we sought to determine the level of SFG antibodies (if any) in cattle ***naturally*** exposed to *R. parkeri*-infected ticks. We also screened some of the sera at a 1:16 dilution but there appeared to be too much background fluorescence to accurately assess the slides.

We found in the experimental study that *R. parkeri*-infected GCT are capable of transmitting organism to naïve calves. We also demonstrated that *R. parkeri*-infected ticks can be found on cattle in nature and at least some of the sale barn cattle demonstrated antibodies reactive with SFG rickettsiae. Coupling that information with the fact that GCT naturally-infected

with *R. parkeri* have the highest rate of infection in the salivary glands, we conclude that it is likely at least some of the sale barn cattle have been exposed to *R. parkeri* through the bite of infected GCT.

We demonstrated that some GCT removed from sale barn cattle were indeed infected with *R. parkeri,* lending substantial credence to the hypothesis that the ticks act as reservoirs as well as vectors of the pathogen. It may be that cattle are refractory to infection with *R. parkeri,* but provide the GCT with a blood meal and increase their distribution by movement of GCT-infested cattle. This in turn increases exposure of wildlife and people to *R. parkeri*-infected ticks, whether or not the cattle themselves become infected. If cattle are refractory to infection with *R. parkeri,* it may actually potentiate the role of the GCT as a vector of the pathogen. That is, *R. parkeri* may be found in ticks in nature infesting otherwise healthy cattle unimpaired to movement, thereby providing a source of infection to people and wildlife from the infected ticks on them.

Before this study began, the distribution of *R. parkeri* in select tissues of the GCT had not been examined. A previous study demonstrated that *R. parkeri* is transmitted both transovarially and transstadially in *A. americanum* (the lone star tick) (Goddard and Norment 1983). Our study validates that study and in addition demonstrates the systemic nature of the infection in GCT. Clearly, the infection rate in ticks, as well as the high percentage of infection demonstrated in salivary glands, supports the hypothesis that the ticks are capable of transmitting infection via saliva.

In summary,

1. Calves became transiently rickettsemic when exposed to *R. parkeri* but did not become clinically ill during this study
2. Calves developed "gotch" ear when infested with GCT, with or without organism, and with or without evidence by PCR of rickettsemia
3. GCT infected with *R. parkeri* are found on sale barn cattle in Mississippi
4. Sale barn cattle are exposed to SFG rickettsiae likely via infected ticks
5. GCT may be systemically infected with *R. parkeri* providing several potential means of vectoring the pathogen including transovarially and transstadially
6. Lesions at the site of tick attachment on the sale barn cattle were in keeping with very early signs of "gotch" ear according to our definition of the condition

These questions we posed had not been asked or answered before and are important when assessing the effects of ticks and rickettsiae on livestock. The results of this study have important implications. For example, in the development of prevention and management approaches for "gotch" ear management, emphasis should be placed on tick control rather than antibiotic treatment. These results also provide vital knowledge for assessing the risk of ABF ricketttsiosis in people and critical information necessary for making accurate and reliable distinctions between ABF, RMSF, and other rickettsioses.

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APPENDIX A

HEMOLYMPH TEST

(Burgdorfer 1970)

1. Obtain tick hemolymph by amputating pretarsus of first leg of the tick and applying to slide (Figure 25)
2. Air dry smear and fix by passing slide through flame 2-3 times
3. Filter working solution of basic fuschin onto slide and let stand for 5-7 minutes
4. Wash thoroughly in beaker of running tap water
5. Immerse slide in malachite green for 6-10 seconds
6. Wash in tap water thoroughly
7. Immerse slide again in malachite green for 6-10 seconds
8. Wash thoroughly in tap water
9. Blot dry and examine

APPENDIX B

GIMENEZ STAINING

(Gimenez 1964)

1. Stock solution (carbol basic fuschin)
    a. Mix 100 ml 10% basic fuschin in 95% EtOH (10 G basic fuschin in 100 ml 95% EtOH)
    b. Add 250 ml 4% aqueous phenol (10 ml phenol in 250 ml distilled water (H_2O)
    c. Add 650 ml distilled H_2O
2. Stock buffers
    a. 0.2 M NaH_2PO_4 (2.84 G in 100 ml distilled H_2O)
    b. 0.2 M Na_2HPO_4 (2.76 G in 100 ml distilled H_2O)
3. Basic fuschin (working solution)
    a. 4 ml stock and 10 ml 0.1 M phosphate-buffered-saline (PBS) pH = 7.45
    b. Prepare buffer solution as follows:
        i. 3.5 ml 0.2 M NaH_2PO_4 (70 ml)
        ii. 15.5 ml Na_2HPO_4 (310 ml)
        iii. 19 ml distilled H_2O (380 ml)
    c. Working solution should be filtered immediately. Remains suitable for 48 hours and then should be discarded.
4. Malachite green oxalate: 0.8% solution in distilled H_2O + up to 100

APPENDIX C

IMMUNOFLUORESCENT ANTIBODY TEST (IFA)

Protocol 1 – IFA for cattle serum screening at 1:32 dilution

1. Remove 12-well, antigen-coated slides from -80°C freezer (antigen is Tate's Hell *R. parkeri*)
    a. Allow slides to thaw and air dry (~ 15 min)
    b. Fix slides in acetone for 15 min (minimum) at -20°C (place in freezer); air dry
    c. Label necessary
2. Obtain 1X PBS*
3. Place a 40 µl primary antibody (cattle serum) diluted to 1:32 onto each well of slide. Add 12.5 µl sample to 187.5 µl PBS to achieve the 1:32 dilution.
    a. Place 40 µl serum diluted to 1:32 from negative control calf #519 on one well of each slide for a negative control
    b. Place 40 µl serum diluted to 1:32 from 10 head of sale barn cattle on each slide
    c. Place 40 µl anti-*Rickettsia rickettsii* human serum in one well of each slide for a positive control
4. Place each slide in an incubating (Petri) dish with pipette tips to elevate and filter paper underneath moistened with H_2O.
5. Incubate slides for 30 min (37°C).
6. Wash slides after incubation by immersing in Coplin jars as follows:
    a. Twice in fresh PBS for 5 min each time.
    b. Once in dH_2O for 5 min.
7. Allow to dry. In meantime, thaw FITC-labeled secondary antibody (do not expose to light) and dilute in PBS.

8. Add a 40 µl diluted FITC conjugate to each slide well.

    a. The following example is for 6 slides with 10 head of sale barn cattle and 1 negative control for each slide and a 1/100 dilution of anti-bovine FITC

        i. 6X11 = 66 plus 2 extra is 68
        ii. 68X40 µl samples = 2720 µl
        iii. 1/100 = x/2720 -> X = 27.2
        iv. 2692.8 µl PBS and 27.2 µl anti-bovine FITC

    b. The following example is for 6 slides with 3 positive controls using anti-human FITC at a 1/20 dilution

        i. 6X40µl samples = 240 µl
        ii. 1/20 = x/240 -> X = 12
        iii. 228 µl PBS and 12 µl anti-human FITC

9. Place slides in incubating dishes and incubate for another 30 min (37°C).

10. Repeat the washes from step #6, except counterstain if necessary for last dH_2O wash.

11. To counterstain, add 20 µl eriochrome black T to 50 ml of dH2O and pour into jar.

12. Allow slides to dry fully in a slide box (do not expose much to light- will quench dye).

13. Apply 2 drops VectaShield to each end and place cover slip over being sure that the VectaShield is dispersed completely over all wells.

14. View with UV scope

*1X PBS recipe

a. Reagents
    i. 8.0g NaCl
    ii. 0.2g KCl
    iii. 1.44g Na_2HPO_4
    iv. 0.24g KH_2PO_4

b. Add reagents to 500 ml dH_20 to dissolve, and then add dH_2O up to 1000 ml.

c. Adjust pH to 7.2

Protocol 2 – IFA for ticks

Dissect ticks (Chapter V) and spot tissue onto well of slides

1. Store slides at −20°C until ready to stain
2. A separate antigen-coated slide from the -80°C freezer (antigen is Tate's Hell *R. parkeri*) was used as a positive control
3. Warm slides to room temperature
4. Put slides in acetone in freezer for 15 minutes
5. Load each antigen field with 40 µl of anti-*Rickettsia rickettsii* human serum diluted 100-fold in PBS
6. Incubate at 37°C for 35 min
7. Wash slides three times for 5 min each; twice in PBS and once in dH_2O; air dry
8. Pipette 40 µl of 1:20 fluorescein isothiocyanate-conjugated anti-human IgG onto each field
    a. This example is for 50 wells with 40 µl per well
    b. 50X40µl samples = 2000 µl
    c. 1/20 = x/2000 -> X = 100
    d. 1900 µl PBS and 100 µl anti-human FITC
9. Incubate the slides again at 37°C for 35 min
10. Repeat the washes from step #8, except counterstain if necessary for last dH_2O wash as described above
11. After drying, cover the slides with 2 drops VECTASHIELD® and place cover slip over slide being sure the VECTASHIELD® is dispersed completely over all wells
12. View the slides with ultraviolet microscope

APPENDIX D

POLYMERASE CHAIN REACTION TECHNIQUE (PCR)

Protocol 1 – nested PCR for amplification of *rompA* gene (Sumner et al. 2007)

For the primary stage of each assay, 10 µl of extract and primers 190–70 and 190–701 were used. For the nested reaction, 1 µl of completed primary reaction was used as template with primers 190-FN1 (5′-AAG CAA TAC AAC AAG GTC-3′) and 190-RN1 (5′-TGA CAG TTA TTA TAC CTC-3′). Thermacycler parameters for the primary stage consisted of an initial denaturation period of 5 minutes at 95°C, followed by a standard 3-step cycling profile consisting of 40 cycles at 95°C for 30 seconds, 60°C for annealing for 30 seconds and a 1 minute extension period at 72°C. The cycling profile was followed by a final extension period at 72°C for 5 min. For the nested stage, the same thermacycler parameters were used. The primary product (10 µl) was separated by electrophoresis in 2% agarose gels containing ethidium bromide.

Protocol 2 – nested PCR for amplification of *17kDa* gene (Paddock et al. 2004)

For the primary stage of each assay, 5 µl of extract and primers R17122 and R17500 were used. For the nested reaction, 1 µl of completed primary reaction was used as template with primers TZ15 and TZ16 were used. Thermacycler parameters for the primary stage consisted of an initial denaturation period of 5 minutes at 95°C, followed by a standard 3-step cycling profile consisting of 40 cycles at 95°C for 30 seconds, 57°C for annealing for 30 seconds, and a 5-minute extension period at 72°C. For the nested stage, the annealing temperature was changed to 58°C and the number of cycles was reduced to 30. The primary product (10 µl) was separated by electrophoresis in 2% agarose gels containing ethidium bromide.

Protocol 3 - nested PCR for amplification of *rompA* gene (Sumner et al. 2007)

For the primary stage of each assay, 5 µl of extract and primers 190–70 and 190–701 were used. For the nested reaction, 2 µl of completed primary reaction was used as template with primers RN1 and FN1. Thermacycler parameters for the primary stage consisted of an initial denaturation period of 2 minutes at 94°C, followed by a standard 3-step cycling profile consisting of 40 cycles of 15 seconds at 94°C, 30 seconds at 60°C, 45 seconds at 72°C, followed by a 5-minute extension period at 72°C. For the nested stages, thermacycler parameters consisted of an initial denaturation period of 2 minutes at 94°C, followed by a standard 3-step cycling profile consisting of 30 cycles of 15 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 72°C, and a 5-minute extension period at 72°C. The primary product (5 µl) was separated by electrophoresis in 2% agarose gels containing ethidium bromide.