1-1-2012

Spotted Fever Group Rickettsiae, Rickettsia Parkeri And "Candidatus Rickettsia Andeanae", Associated With The Gulf Coast Tick, Amblyomma Maculatum Koch

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By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

August, 2012

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Title of Study: Spotted fever group rickettsiae, *Rickettsia parkeri* and "*Candidatus* Rickettsia andeanae", associated with the Gulf Coast Tick, *Amblyomma maculatum* Koch

Pages in Study: 116

Candidate for Degree of Doctor of Philosophy

The public health and veterinary importance of Gulf Coast ticks, *Amblyomma maculatum* Koch (1844) have become more apparent during the last several decades. In addition, new records of this three-host ixodid tick presently show a geographic distribution throughout much of the southern United States. *Rickettsia parkeri,* a spotted fever group rickettsia (SFGR) that is commonly found infecting the Gulf Coast tick, was only recently recognized as a human pathogen. Over the last decade, more than 20 human cases of disease caused by *R. parkeri* have been recognized in the Americas, all of which were similar in presentation to mild Rocky Mountain spotted fever. In addition, a novel, poorly characterized SFGR, "*Candidatus* Rickettsia andeanae", was recently identified in *A. maculatum* from Peru, United States, Chile and Argentina. As the recognition of *R. parkeri* as a pathogen and "*Ca.* R. andeanae" as an additional SFGR in *A. maculatum* only recently occurred, a general gap exists in our

understanding of the biology of these SFGRs. The overall objective of this dissertation was to contribute to our knowledge of SFGR infecting *A. maculatum*. In Chapter 3, we present a prevalence study of *R. parkeri* and *"Ca.* R. andeanae" in *A. maculatum* from Mississippi where we detected 15.2% *R. parkeri*-singly infected ticks and 3.1% total "*Ca.* R. andeanae" infected ticks of which 1.7% were co-infected with *R. parkeri*. In Chapter 4, we discuss finding four genetically different populations of *A. maculatum* from Mississippi infected with a homogenous population of *R. parkeri*, using Single Strand Conformation Polymorphism analysis. Those initial data relating to *"Ca.* R. andeanae" provided a foundation for studies described in Chapters 5 and 6. We report the first morphological study of *"Ca.* R. andeanae" using transmission electron microscopy in Chapter 5 and isolation of this SFGR in *A. maculatum* cell co-culture in Chapter 6. We anticipate that results presented in this dissertation will contribute to our understanding of the ecology of *A. maculatum* as a vector for the human pathogen, *R. parkeri,* and increase the current understanding of both *R. parkeri* and *"Ca.* R. andeanae" in *A. maculatum*.

Key words: *Amblyomma maculatum,* Gulf Coast tick, spotted fever group rickettsia, *Rickettsia parkeri*, America boutonneuse fever, "*Candidatus* Rickettsia andeanae", prevalence, population heterogeneity, single strand conformation polymorphism, transmission electron microscopy, embryonic tick cell line

DEDICATION

I dedicate this dissertation to my husband, Claudenir Ferrari, for being by my side during the great (and the not so great) days of pursuing a doctorate and a life abroad.

ACKNOWLEDGEMENTS

My father devoted over 35 years of his life (and still is) in assisting farmers with their crops and livestock as an extension agronomist. He imprinted on me the love for the natural sciences and, since the age of five, I said that I was going to be a veterinarian. As a veterinarian today, I could not be happier to have pursued this doctoral degree in Veterinary Sciences, working with parasites of humans and other animals. All of my experiences during this doctoral training were key for my professional growth and to me, the most comprehensive they could be. I have been changed personally too; I am much more respectful and in awe of nature. Once a city girl, I am now thrilled to have worked waist high in the woods near snakes, spiders, and whatnot during three hot and humid summers in the most remote corners of Mississippi! I am so grateful to all persons who inspired me and assisted me in making this dream to come true.

None of this dissertation work would have been possible if it were not for Dr. Andrea Varela-Stokes. As my major professor, she opened the door for a stranger abroad when few would and taught me how to be a competent and ethical scientist. I am indebted to her for showing me that I can do more than I thought I could (for “showing”, I mean it literally too, she goes on the bench and shows how to get the work done). Lots of times her help went beyond the

academic issues, and she has been it all: a motherly friend, career planner, counselor on family and international matters, you name it. When I started in the Ph.D. program, Dr. Stokes had just learned she was pregnant with Marco. I love seeing how grown he is now and hope to have the opportunity to mirror in her and her husband, John Stokes, as they double as researchers and parents.

I learned a lot from Dr. Jerome Goddard, mentor and advisor for my Minor in Entomology. Although I worked with ticks while as a veterinary student, Entomology was all very new to me when I started this Ph.D. I appreciate so much that Dr. Goddard shared with me his expertise which is as overwhelming as it is his love for the field. If Dr. Stokes gave me motherly advice, Dr. Goddard was like a father to me during these four years. All of the opportunities I had in the company of the Goddard's were amazing, especially in the company of the caring Rosella Goddard. I admire and have a great deal of respect towards the members of my committee. Many thanks to Drs. Christopher Paddock, Michael Caprio and Mark Lawrence for their mentorship, their precious time spent on projects together, and their kindness towards me. I will always be thankful to Dr. Joaquin Patarroyo (Federal University of Viçosa, Brazil), my advisor during veterinary school, and the people I had opportunity to work with there, especially Dr. Ana Paula Peconick.

The years at Mississippi State University were much happier because of the kind people I met there, especially at the Department of Basic Sciences, other departments of the College of Veterinary Medicine and the Department of Entomology. Special thanks to the Parasitology group: Drs. Kristine Edwards,

Linda Pote and Carla Panuska, for sharing their expertise in Parasitology and counseling me with life in general. I am so thankful to Gail Moraru for her support and friendship in and outside the “tick lab” (I am certain of her success in research, especially if it involves animals). Thanks are also due to John Stokes, Whitney Smith, Lauren Goltz and previous “tick lab” members, Ashley Castellaw, Erle Chenney, Diana Link and Alexandra Murphy, who brightened my day especially when everything was going wrong and lent a hand when needed.

I left everyone and everything I knew in Brazil in 2007 to study in the United States. I am thankful to have the companionship of a wonderful husband and our four-legged and first kid, Hannah, here with me. I am so happy to have had a great deal of support from family and friends who remained very involved in my life even though I was so busy and far away. Special thanks to my parents, Flávio and Iêda, my brothers, Vladimir and Tiago, and my grandmother, Maria Ozanália (*Obrigada pelo apoio a distância. Eu amo vocês!*).

TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

I. INTRODUCTION 1

II. HISTORICAL REVIEW 4

Amblyomma maculatum Ticks 4
Amblyomma maculatum as a Vector of Disease Agents 8
Biology of Spotted Fever Group Rickettsiae 9
Significance of SFGR 12
Rickettsia parkeri, an Emerging Human Pathogen 13
The Novel SFGR, "*Candidatus* Rickettsia andeanae" 17
Co-infection of SFGR in Ticks 18
Single Strand Conformation Polymorphism 19
Ultrastructural Studies of SFGR 21
Tick Embryonic Cell Lines 22

III. PREVALENCE OF *RICKETTSIA PARKERI* AND "*CANDIDATUS* RICKETTSIA ANDEANAE" IN GULF COAST TICKS FROM MISSISSIPPI 26

Abstract 26
Introduction 26
Materials and Methods 28
Tick Collection 28
DNA Extraction 29
Molecular Evidence of *Rickettsia parkeri* and "*Candidatus* R. andeanae" 29
Statistical Analysis 30

Results 30
Discussion 32
Acknowledgements 35

IV. POPULATION ANALYSIS OF *AMBLYOMMA MACULATUM* TICKS AND RICKETTSIA *PARKERI* USING SINGLE STRAND CONFORMATION POLYMORPHISM 37

Abstract 37
Introduction 38
Materials and methods 40
Tick Collection 40
DNA Extraction 41
Amblyomma maculatum and *Rickettsia parkeri* PCR 41
Non-isotopic SSCP Analysis of Amplicons 41
SSCP Patterns and Phylogenetic Analyses 42
Statistical Analysis 43
Results 43
Tick SSCP 43
Rickettsial SSCP 47
Discussion 48
Acknowledgements 53

V. ULTRASTRUCTURE OF "*CANDIDATUS* RICKETTSIA ANDEANAE" WITHIN THE GULF COAST TICK, *AMBLYOMMA MACULATUM* 59

Abstract 59
Introduction 60
Materials and Methods 61
Selection of "*Ca.* R. andeanae"-infected *A. maculatum* 61
Transmission Electron Microscopy (TEM) 63
Results 64
Discussion 66
Acknowledgements 68

VI. ESTABLISHMENT OF GULF COAST TICK CELL LINE AND ISOLATION OF "*CANDIDATUS* RICKETTSIA ANDEANAE" 71

Abstract 71
Introduction 72
Materials and Methods 74
Source of Tick Embryos 74
Establishment of *A. maculatum* Cell Line and Isolation of "*Ca.* R. andeanae 75
Passage of "*Ca.* R. andeanae" to ISE6 and Vero Cell Lines 76

Molecular Analyses 77
Quantitative Real-Time PCR 78
Results 80
Discussion 83
Acknowledgements 86

VII. CONCLUSIONS AND IMPLICATIONS 92

REFERENCES CITED 99

APPENDIX

A. SINGLE STRAND CONFORMATION POLYMORPHISM 114

LIST OF TABLES

3.1 PCR results by region for adult *Amblyomma maculatum* ticks in Mississippi, 2008 to 2010 36

3.2 PCR results by year for adult *Amblyomma maculatum* ticks collected in southern and northern Mississippi, USA 36

4.1 SSCP haplotypes and nucleotidic variation determined by sequencing of *Amblyomma maculatum* mitochondrial 16S rDNA from Mississippi. 55

4.2 Haplotype frequencies determined by SSCP analysis of *Amblyomma maculatum* mitochondrial 16S rDNA by sites of collection in Mississippi 56

6.1 PCR results of "*Ca.* R. andeanae"-species specific *rompA* fragment for several time points of rickettsial growth in the newly established *A. maculatum* cell line (GCT cells) and in challenged ISE6 and Vero cells..... 87

LIST OF FIGURES

2.1 Geographical range of Gulf Coast ticks and human reports of *R. parkeri* infection in the U.S. and Mississippi and sites of tick collection for chapters 3 and 4. 25

4.1 Gel patterns labeled A through D detected by PCR-SSCP of *Amblyomma maculatum* 16S mitochondrial rDNA gene fragment 57

4.2 Tamura-Nei neighbor-joining tree of *Amblyomma maculatum* 16S mitochondrial rDNA gene fragment from Mississippi, North Carolina, Oklahoma, Kansas and Texas ticks 58

5.1 Transmission electron micrographs depicting fine structure of rickettsia-like organisms in female *Amblyomma maculatum* 69

5.2 Transmission electron micrographs of rickettsia-like organisms in female *Amblyomma maculatum* tissues 70

6.1 Immunofluorescence antibody assay showing extracellular rickettsiae in *Amblyomma maculatum* cells including positive and negative controls of assay 87

6.2 Acridine orange staining showing "*Ca.* R. andeanae"-infected *Amblyomma maculatum*, ISE6 and Vero cells including uninfected cultures 88

6.3 Diff-Quik staining showing "*Ca.* R. andeanae"-infected *Amblyomma maculatum*, ISE6 and Vero cells including uninfected cultures 89

6.4 Ratio of "*Candidatus* R. andeanae" infection levels by host gene amounts based on QPCR results in three different cell lines 90

CHAPTER I

INTRODUCTION

Gulf Coast ticks (GCTs), *Amblyomma maculatum* Koch (1844), are ixodid three host ticks that are becoming increasingly recognized as public health and veterinary threats. Their distribution in the United States has shifted from what was historically accepted as a 100 mile northward stretch along the Gulf and Atlantic Coasts to presently occurring over much of the southern United States, including a population in Oklahoma and Kansas (Hooker and Bishopp 1912, Semtner and Hair 1973, Teel et al. 2010). In addition, GCTs, which were long established as pests of livestock, wildlife and canids, have become recognized as vectors for previously unrecognized disease agents in humans and canids during the last three decades.

The zoonotic agent, *Rickettsia parkeri,* is a spotted fever group rickettsia (SFGR) initially identified in *A. maculatum* in 1939, but not considered pathogenic to humans for approximately 65 years (Parker et al. 1939, Lackman et al. 1965). The index case for human infection with *R. parkeri* was a man from Virginia who initially presented to a clinic in 2002 with fever, body aches and skin reactions from a possible tick or other arthropod bites (Paddock et al. 2004). Since then, there have been over 20 reported human cases of disease caused by *R. parkeri*,

all of which were similar in presentation to mild Rocky Mountain spotted fever (RMSF) (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Paddock et al. 2010). *Rickettsia parkeri* is also emerging in South America, where there infected ticks were reported in Uruguay, Brazil, Argentina and Bolivia, and a case report of human infection was recently described in Argentina (Venzal et al. 2004, Silveira et al. 2007, Nava et al. 2008, Tomassone et al. 2010, Romer et al. 2011).

In addition to the human pathogen, *R. parkeri,* a second distinct SFGR has been identified in *A. maculatum.* "*Candidatus* Rickettsia andeanae" is a novel, poorly understood SFGR first detected by molecular assays in GCTs in Peru and more recently detected in GCTs from the United States, Chile and Argentina (Blair et al. 2004, Pacheco et al. 2007, Sumner et al. 2007, Paddock et al. 2010, Abarca et al. 2012). While this SFGR has not been detected in vertebrate hosts to date and its pathogenicity is unknown, investigations into the biology of *"Ca.* R. andeanae" have been limited due to its relatively recent recognition.

The overall objective of this dissertation was to contribute to our knowledge of SFGR infecting *A. maculatum.* Our goals were to ultimately raise awareness of *A. maculatum* as a vector and *R. parkeri* infection as an emerging rickettsiosis, particularly in the state of Mississippi, as well as increase our knowledge of the novel SFGR, *"Ca.* R. andeanae", in *A. maculatum*. Specifically, this work identified genetic heterogeneities in populations of GCTs and *R. parkeri* in Mississippi using Single-Strand Conformation Polymorphism (SSCP) analysis.

These results shed light on tick movement in Mississippi by defining populations present in this state and additionally define genetic heterogeneity of *R. parkeri* detected in GCTs. Further, we anticipate that our results will help fill a gap in knowledge regarding *"Ca.* R. andeanae" through our characterization of the fine structure of this SFGR using transmission electron microscopy and through its isolation in *A. maculatum* cell co-culture. We anticipate that these studies will provide a foundation for future work elucidating infectivity and pathogenicity of *"Ca.* R. andeanae" as well as evaluating interactions between this SFGR and the known pathogen, *R. parkeri* in *A. maculatum.*

The following specific aims are proposed for this dissertation:

1. Determine the infection rates of *R. parkeri* and *"Ca.* R. andeanae" in adult *A. maculatum* from sites in North and South Mississippi.
2. Evaluate genetic heterogeneity in populations of adult *A. maculatum* from sites in Mississippi and determine if *R. parkeri* infecting these ticks are genetically different.
3. Characterize the fine structure and tissue tropism of "*Ca.* R. andeanae" in unfed *A. maculatum* adults using transmission electron microscopy.
4. Develop an *A. maculatum* embryonic cell line for isolation of "*Ca*. R. andeanae" and to provide a tick cell line from an additional tick species for future research.

CHAPTER II

HISTORICAL REVIEW

Amblyomma maculatum Ticks

Gulf Coast ticks (GCTs), *Amblyomma maculatum* Koch (Acari: Ixodidae), was first described in 1844 by a German acarologist, Carl Ludwig Koch. The type specimen was from “Carolina” and the type host is unknown (Cooley and Kohls 1944). In general, ticks are effective vectors of a variety of agents comprising bacteria, protozoa and viruses. Tick-borne diseases in humans are only second in importance to those transmitted by mosquitoes, largely because of malaria. As a family, the Ixodidae, also known as hard ticks due to the presence of a hard scutum on their dorsum, include the most clinically relevant ticks (Sonenshine 1991). *Amblyomma maculatum* is an established pest of livestock in the United States. This tick has been implicated with cases of tick paralysis (Paffenbarger 1951, Espinoza-Gomez et al. 2011), and more recently has been identified as an important vector of medical and veterinary infectious agents in the United States (Uilenberg 1982, Mathew et al. 1998, Paddock et al. 2004).

In North America, *A. maculatum* can be easily distinguished from other tick species partly because it is one of the largest species to occur on this continent and has recognizable morphological characteristics, such as an ornate

dorsum and long mouthparts (Hooker 1908, Bishopp and Hixson 1936, Cooley and Kohls 1944). However, difficulty arises with identification of tick species within the general *A. maculatum* group occurring in the Western hemisphere. Ticks in this group belong to the subgenus *Anastosiella* and include the following species: *A. maculatum*, *A. triste*, and *A. tigrinum* (Estrada-Pena et al. 2005). Although the three species are very similar in appearance, there is little overlap in the geographical distribution of the Neotropical and Nearctic species *A. maculatum* with the two typically Neotropical ticks, *A. triste* and *A. tigrinum* (Estrada-Pena et al. 2005, Mertins et al. 2010).

Historically, *A. maculatum* in the United States were rarely found more than 100 miles inland from the Gulf of Mexico, hence their common name (Bishopp and Hixson 1936). Ticks occurring outside the historical range were first reported in the mid-20th century (Cooley and Kohls 1944, Semtner and Hair 1973, Goddard and Norment 1983). Since then, there have been incidental collections of *A. maculatum* as far north as Iowa and Maine (Rand et al. 2007, Teel et al. 2010). Established tick populations occur from the eastern part of Texas along the Gulf and Atlantic Coasts as far north as Virginia, and include originally distinct populations in Oklahoma and Kansas which have now coalesced between the two states, and a “hot spot” in Arkansas (Semtner and Hair 1973, Teel et al. 2010, Trout et al. 2010b). Some scientists have suggested that these recent inland populations were colonized with the movement of infested cattle from the Gulf Coast region around 1973 (Semtner and Hair 1973, Goddard and Norment 1983). However, Teel (2010) recently proposed that the

inland population founded the population along the Gulf and Atlantic coast, as the former was more ancient. In Mississippi, GCTs have a more sporadic distribution compared to other more commonly found ticks in this state and are mostly found in the southern and central regions of Mississippi (Goddard and Paddock 2005). A map illustrating current range of *A. maculatum* in the southern U.S. and specifically in Mississippi is shown in Figure 2.1. Outside the United States, *A. maculatum* is found in Mexico, some Caribbean Islands, Colombia, Venezuela and Peru (Bishopp and Trembley 1945, Mendoza-Uribe and Chavez-Chorocco 2004, Teel et al. 2010).

Amblyomma maculatum is a three host tick, which means that each developmental stage will leave the current host, molt to the next stage off the host, and then seek a new host on which to feed. *Amblyomma* ticks possess long mouthparts and inflict painful bites. Ticks of this genus are also aggressive. After sensing hosts they actively move towards them in a behavior called “hunting” that is used in addition to a passive behavior known as “questing”, whereby they crawl up vegetation and wave their forelegs to facilitate clinging to host fur (Hooker 1908, Sonenshine 1993). In general, ticks use cues like carbon dioxide released from breathing, ammonia from waste, heat radiated from the host, and the sounds, vibration and shade produced by the host, for both active and passive host-seeking behaviors (Sonenshine 1993).

Eggs of GCTs are laid in July and August and hatch by early fall with larval stages that often overwinter. Most activity of larval, or “seed”, *A. maculatum* ticks will occur from July until late November, and larvae may

continue to be active during warm winters. Nymphal activity for GCTs mainly occurs during late winter and the spring. Adult GCTs are very resistant to desiccation and can be found from late spring until early fall, although highest activity occurs during the hot months of July and August (Bishopp and Hixson 1936, Hixson 1940, Goddard and Paddock 2005).

Unlike some ticks, such as *Rhipicephalus sanguineus* and *A. tuberculatum,* that mostly feed on dogs and the gopher tortoise, respectively (Cooney and Hays 1972, Rhodes and Norment 1979), none of the life stages of GCT are very host specific. In nature, a wide host range has been reported. Immature GCTs have been described attached to several ground dwelling bird species, predominantly bobwhite quail and meadowlarks, as well as small mammals, especially rodents (Bishopp and Hixson 1936, Koch and Hair 1975). The adult GCT is a known pest of cattle and dogs; they are also frequently found on other large mammals, such as goats, sheep, and horses (Hooker and Bishopp 1912, Bishopp and Hixson 1936). This species will occasionally be found attached to people (Felz et al. 1996, Felz and Durden 1999, Goddard 2002). In birds and rodents, immature ticks tend to attach to the head and neck while adult ticks are often seen in the ears of larger mammals.

Damage to the ear produced by large numbers of ticks is a particular problem in cattle where inflammation and cartilage destruction cause the ears to thicken and droop in a condition known as “gotch ear” (Hooker and Bishopp 1912, Bishopp and Hixson 1936, Edwards et al. 2010). The parasitized animal is less efficient at keeping flies off by flicking its ears, potentially allowing further

damage to the ear. In the past this damage also led to detrimental infestations with the primary screwworm fly, *Cochliomyia hominivorax* (formerly *C. americana*, or *Callitroga americana*) (Bishopp and Hixson 1936). Parasitism by the GCT and the gotch ear condition has been reported in other livestock as well as cattle for over a century (Hooker 1908, Hooker and Bishopp 1912, Edwards et al. 2010).

Amblyomma maculatum as a Vector of Disease Agents

In the early 1900's, important diseases transmitted by other ticks to animals and humans were becoming recognized, such as bovine babesiosis transmitted by *Rhipicephalus (Boophilus) annulatus* and Rocky Mountain spotted fever transmitted mainly by *Dermacentor* spp. (Hooker 1908, Sonenshine 1993). However, at that time, there was no description of biological agents within the GCT. It was not until 1939 that a rickettsia, *Rickettsia parkeri*, was identified in *A. maculatum* (Parker et al. 1939). Although it was found to be pathogenic to guinea pigs, its zoonotic potential was unclear and at that time was only known as the "maculatum agent" (Parker et al. 1939). The first case of human disease was reported within the last decade by Paddock *et al.* (Paddock et al. 2004).

In 1997, a protozoan named *Hepatozoon americanum* was identified that is transmitted to dogs by the ingestion of *A. maculatum* (Vincent-Johnson et al. 1997, Mathew et al. 1998). The disease caused by this protozoan, American canine hepatozoonosis, is characterized by severe leukocytosis, myositis, and proliferation of periosteal bone, becoming very painful and potentially fatal if

treatment is delayed. Disease prognosis in canids may be improved if treated early, however treatment is long lasting. If left untreated, prognosis was guarded to poor in most cases (Potter and Macintire 2010). American canine hepatozoonosis was first described in Texas in 1978 (Craig et al. 1978) and now is becoming more broadly recognized due to the increasing distribution of the tick vector, improved diagnostic assays, and heightened awareness of the disease among veterinarians and researchers (Baneth 2011).

In the 1800s, cattle imported into the Caribbean Islands from Africa brought with them *A. variegatum* ticks that were infected with *Ehrlichia ruminantium,* which cause heartwater disease in livestock (Uilenberg 1982). Heartwater is a severe, fatal ehrlichiosis of African domestic ruminants, and wild ruminants, resulting in economic losses to farmers. After heartwater was determined to be transmitted at least experimentally by *A. maculatum*, concern over potential introduction of the agent into the United States arose (Uilenberg 1982, Mahan et al. 2000). Heartwater-infected ticks could enter this country inadvertently through animal trade or deliberately by pernicious release as a bioterror weapon (Barre et al. 1987).

Biology of Spotted Fever Group Rickettsiae

The order Rickettsiales comprises small obligate intracellular alphaproteobacteria in genera such as the *Rickettsia*, *Ehrlichia*, *Wolbachia*, *Neorickettsia* and *Holospora*, and is supported by molecular characteristics of these genera, including 16S ribosomal RNA phylogeny (Fredricks 2006). Of

public health and veterinary relevance are bacteria in the families Rickettsiaceae (*Rickettsia* spp.) and Anasplasmataceae (*Anaplasma* and *Ehrlichia* spp.). The family Rickettsiaceae consists of gram negative, aerobic, rod-like microparasites that can be found intracytoplasmic, and sometimes intranuclear, in both vertebrates and arthropods (Fredricks 2006). *Rickettsia* spp. adhere to the host cell membrane by rickettsial outer membrane adhesion proteins, called rOmpA and rOmpB. In addition, the SFGR use host cell machinery to produce a cytoskeleton of actin which is used to move within the cytoplasm, for nourishment and reproduction within the cytoplasm, and for exiting en route to a new cell (Walker 2007). Proteomic analysis of the recently identified human pathogen, *R. parkeri,* has shown similarities to pathogenic rickettsiae as it possesses the same proteins involved in actin-based motility (Pornwiroon et al. 2009).

Traditionally, members of the family Rickettsiaceae were classified in three groups: spotted fever group, typhus group and scrub typhus group. In 1965, Lackman created subgroups in the spotted fever group rickettsiae (SFGR) based on their antigenic properties in complement fixation tests (Lackman et al. 1965). For instance, antibodies against *R. conorii*, the agent of boutonneuse fever in Africa and Europe, were more reactive with the “maculatum agent” (*R. parkeri*) antigen than were *R. rickettsii* antibodies; thus, he grouped *R. parkeri* and *R. conorii* together, and separated them from the Rocky Mountain spotted fever (RMSF) agent. The only member of the original scrub typhus group, previously known as *R. tsutsugamushi*, is now in a new genus, *Orientia*, which is closely related to the genus *Rickettsia* (Tamura et al. 1995).

Recently, a multi-genic approach proposed separating the spotted fever group into two different clades: a spotted fever group that includes the tick-associated rickettsiae (such as *R. rickettsii*, *R. conorii*, *R. parkeri*) and a transitional group (*R. akari* and *R. felis*), transmitted by mites and fleas (Gillespie et al. 2007). *Rickettsia prowazekii* and *R. typhi* remained in the typhus group, while a fourth clade of non-pathogenic rickettsiae was assigned which is called the ancestral group because it is basal to the other three, includes *R. belli* and *R. canadensis* (Gillespie et al. 2007). Molecular studies have confirmed over 20 *Rickettsia* species as SFGR in the original classification, including *R. rickettsii, R. conorii, R. parkeri, R. africae, R. peacockii* (Fournier et al. 1998, Raoult et al. 2005, Fournier and Raoult 2007, Socolovschi et al. 2009). In the past two decades several other proposed SFGR have been identified but not fully studied, as is the case of "*Candidatus* Rickettsia andeanae" (Blair et al. 2004). The designation of rickettsial species has become a dynamic and controversial topic. As genetic and genomic approaches for rickettsial taxonomy rapidly advance, a plea for including phenotypic and epidemiologic criteria in taxonomic classification and nomenclature was recently made (Merhej and Raoult 2011). Respectively, example of such criteria to be included are temperature of optimum growth and type of host that bacterium is commonly found associated with. Descriptive studies like those described in this dissertation provide information that will contribute to future efforts that revisit the "species" definition.

Significance of SFGR

Spotted fever rickettsioses have been considered the most important neglected emerging infections in the world (Walker 2007). The importance of pathogenic SFGR like *R. rickettsii, R. prowazekii* and *R. conorii* is clear because life-threatening diseases can develop and some scientists suggest that these pathogens can also be weaponized for bioterrorism; on the other hand, little is known regarding the overall health importance of other SFGR (Azad and Radulovic 2003, Walker 2007).

The most severe spotted fever rickettsiosis in the Western Hemisphere, RMSF, appears to be resurging in North and South America (Openshaw et al. 2010). An example of the resurgence occurred between 2002 and 2004 in Arizona. During this time, 13% of children and up to 70% of dogs were seropositive for exposure to *R. rickettsii* and two fatal cases of RMSF occurred in a community previously non-endemic for RMSF (Demma et al. 2006). The incidence of RMSF in 1997-2002 was reported to be approximately 2.2 cases per million persons (Chapman 2006) and as of 2008, the CDC reported 2563 confirmed or probable cases of RMSF in the United States, an increase of 15% from the previous year (CDC 2008). While additional cases of RMSF are likely to have occurred, they may not always be reported due to insufficient or non-specific laboratory testing (Dahlgren et al. 2012).

Severe morbidity or death may arise when patients do not seek health care during the acute phase of RMSF or through misdiagnosis by physicians and administration of an incorrect treatment. Despite the fact that antibiotics in the

tetracycline class successfully treat RMSF, if left untreated, this disease can be fatal in up to a third of patients (Dalton et al. 1995). A review of fatal cases of RMSF from 1990 until 2007 showed that the risk groups for disease are children five to nine years old, American Indians, immunosuppressed patients and delaying in treatment of severe cases (Dahlgren et al. 2012).

Rocky Mountain spotted fever is the most severe of the spotted fever rickettsioses in the Americas, highlighting the medical and veterinary importance of correctly identifying and understanding pathogenic SFGR. However, similarities in clinical presentation between RMSF and other spotted fever rickettsioses and the inability of current routine laboratory techniques to differentiate between these rickettsial diseases recently prompted the CDC to announce that RMSF cases should now be reported as a “spotted fever rickettsiosis” (CSTE 2009, CDC 2010). The most current, although provisional, annual data reports a total of 2154 cases (confirmed or probable) of spotted fever group rickettsioses cases in 2011, using the updated case definition (CDC 2011).

Rickettsia parkeri, an Emerging Human Pathogen

In 1939, a rickettsial agent in the spotted fever group, colloquially called “maculatum agent”, was reported in the GCT (Parker et al. 1939, Lackman et al. 1965). For over 60 years the “maculatum agent” was considered only pathogenic to guinea pigs, causing fever and scrotal reaction in this host (Parker 1940). Lackman et al. eventually gave Parker’s “maculatum agent”, the species name,

R. parkeri (Lackman et al. 1965), based on sufficient antigenic differences to other SFGR.

In the last 20 years over a dozen *Rickettsia* spp., whether previously described or not, have been implicated as agents of animal and human disease (Parola and Raoult 2001, Raoult 2010). This includes *R. parkeri*, which was considered non-pathogenic until the first human case of *R. parkeri* infection was diagnosed in 2002 (Paddock et al. 2004). This index case was a man in Virginia who presented to a clinic with fever, body aches and skin reactions, including multiple eschars, or areas of necrosis, at sites suspected to be tick bites. The patient had flu-like symptoms, typical of spotted fever rickettsiosis like RMSF, but not as severe. Since that initial case report, there have been approximately 20 total reported *R. parkeri* rickettsiosis cases (Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Romer et al. 2011). Figure 2.1 provides the geographical distribution of 15 out of more than 20 human cases that have been reported to date.

Despite systemic similarities to mild RMSF in humans, the presence of eschars in *R. parkeri* infection more closely resembles boutonneuse fever caused by *R. conorii*, a SFGR present in Europe and Africa. Given this, Goddard suggested the moniker “American boutonneuse fever” (Goddard 2004). In addition, *R. parkeri* is more closely related phylogenetically to *R. conorii, R. africae* and *R. sibirica,* than to *R. rickettsii* based on sequences of *rompA, rompB* and citrate synthase (*gltA*) genes (Fournier et al. 1998, Roux and Raoult 2000, Goddard 2009, Paddock et al. 2010).

Human cases of *R. parkeri* infection are not restricted to the United States, or to the range of *A. maculatum,* and have been suggested to occur in South America where *R. parkeri* was detected in other *Amblyomma* spp., namely *A. triste* in Brazil, Uruguay, Argentina (Venzal et al. 2004, Silveira et al. 2007, Nava et al. 2008) and *A. tigrinum* in Bolivia (Tomassone et al. 2010). Serological evidence of human rickettsiosis suggestive of *R. parkeri* infection was described in Uruguay (Conti-Diaz et al. 2009). And, most recently, two confirmed cases of *R. parkeri* infection, and other additional probable cases have been reported from Argentina (Romer et al. 2011).

In the United States, another *Amblyomma* sp., *A. americanum,* the lone star tick, has been found naturally infected with *R. parkeri* (Goddard and Norment 1986). Experimental transmission of *R. parkeri* with *A. americanum* has also been successful using a guinea pig model (Goddard 2003). These ticks, which are widespread in the southeastern and south-central U.S., can transmit the agent both transovarially and transstadially. When experimentally placed on guinea pigs, infected *A. americanum* successfully transmitted the pathogen, causing mild fever and scrotal reactions in exposed animals (Goddard 2003). Recently, one study found lone star ticks naturally infected with *R. parkeri* at a very low prevalence (1/418 ticks sampled in Georgia and 1/446 ticks sampled in Tennessee) (Cohen et al. 2009), while other studies did not detect *R. parkeri* in any sampled *A. americanum* (Mixson et al. 2006b, Castellaw et al. 2010, Heise et al. 2010). Further work is needed to better understand the role of the lone star tick as a vector of *R. parkeri*.

Prevalence data are important for both disease diagnosis and pest control assessment by physicians and public health authorities. Although current prevalence data mainly rely on assays that detect DNA of pathogens, historical evidence for *R. parkeri* in GCTs also exists. Specifically, *R. parkeri* (formerly referred to as the "maculatum agent") was initially reported from GCTs in Mississippi in the 1950's using detection by complement fixation tests (Philip and White 1955). To the best of our knowledge, prior to work presented in this dissertation, two limited surveys using PCR assays provided data evaluating presence of SFGR in GCTs in Mississippi. In those studies, 11% to 40% of *A. maculatum* were infected with *R. parkeri* (1/9 ticks sampled from Copiah county, and 2/8 and 25/62 ticks sampled from Jackson and Oktibbeha counties, respectively) (Sumner et al. 2007, Paddock et al. 2010). This infection rate is consistent with data supported by studies in other states. In Virginia and North Carolina, *R. parkeri* DNA was detected in approximately 42% and 20-33% of sampled *A. maculatum*, respectively (Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011). Conversely, only about 1% of *Dermacentor* ticks are usually found infected with *R. rickettsii* in nature (Paddock 2009, Stromdahl et al. 2011). Since spotted fever rickettsiosis cases occur in Mississippi (Mississippi State Department of Health 2011) and misdiagnosis of RMSF is not uncommon based on serology and clinical signs, it is important to understand and monitor the occurrence of *R. parkeri* in *A. maculatum* ticks occurring in the state.

The Novel SFGR, "*Candidatus* Rickettsia andeanae"

Recently, a novel SFGR was described in *A. maculatum* and *Ixodes boliviensis* from the Peruvian Andes, hence its proposed name, "*Candidatus* Rickettsia andeanae" (Blair et al. 2004). Later it was detected in *A. maculatum* from the southeastern United States (Sumner et al. 2007). Prevalence of the novel SFGR ranged from 2% in Mississippi and Florida in one study (Paddock et al. 2010) to approximately 5% in Mississippi, Georgia and Florida (Sumner et al. 2007). In Virginia, it was about 1-1.5% (Fornadel et al. 2011, Wright et al. 2011) and 3.8% from North Carolina (Varela-Stokes et al. 2011). Interestingly, four out of 35 ticks found attached to humans were positive by PCR to "*Ca.* R. andeanae" in Oklahoma and Kansas (Jiang et al. 2011).

Most of our current knowledge regarding "*Ca.* R. andeanae" comes from molecular detection in infected ticks. To date, no vertebrate has been found infected with this novel rickettsia. Initial attempts to isolate *"Ca.* R. andeanae" in Vero cells, C6/36 mosquito cells, and ISE6 (*Ixodes scapularis*) tick cells were unsuccessful (Blair et al. 2004, Sumner et al. 2007, Paddock et al. 2010). Similarly, the "East Side" agent, *R. peacockii*, a known endosymbiont in *Dermacentor andersoni* was initially difficult to cultivate. Only after several unsuccessful attempts in several mammalian cell lines was *R. peacockii* ultimately isolated (Niebylski et al. 1997b). Most recently, "*Ca.* R. andeanae" was propagated in mammalian and *Drosophila* cells (Luce-Fedrow et al. 2011). However, the stability of this isolate in these cell lines was unclear and these

culture systems may not be ideal for future studies requiring organism for experimental infections.

To our knowledge, the potential for transmission of or disease by "*Ca*. R. andeanae" has not been explored. Interestingly, there has been an increase of spotted fever rickettsioses cases in Tennessee (224 cases in 2008), where an epidemiological survey found no ticks out of over 1500 ixodid ticks infected by *R. rickettsii*. The authors suggested other antigenically related SFGR may be responsible for disease in the area (Moncayo et al. 2010). While the studies presented in this dissertation do not evaluate infectivity or pathogenicity of *"Ca.* R. andeanae" in vertebrate hosts, they do provide a basis for future studies that explore these and other questions regarding this novel rickettsia.

Co-infection of SFGR in Ticks

Rickettsiae have an intimate relationship with their tick vectors, which also often function as reservoirs. Transstadial and transovarial transmission, i.e. to successive developmental stages and from female ticks to offspring, respectively, are important strategies for rickettsial survival outside a vertebrate host (Burgdorfer and Brinton 1975). Interestingly, certain SFGR have been shown to compete inside the tick, using what is called transovarial interference, and natural co-infection of these members of SFGR within individual ticks is not common (Azad and Beard 1998). In *Dermacentor andersoni*, the endosymbiont *R. peacockii* is frequently found infecting female tick ovaries where it replicates and is vertically transmitted, but blocks the replication of *R. rickettsii* in ovaries,

thus preventing transmission of *R. rickettsii* to tick progeny (Burgdorfer et al. 1981, Burgdorfer 1988). Eventually fewer *D. andersoni* ticks are infected with *R. rickettsii* in that population. Another example of the phenomenon was more recently described between the nonpathogenic species, *R. rhipicephali* and *R. montanensis,* where infection with one species prevented infection by the other in *D. variabilis* (Macaluso et al. 2002). Despite the rarity of co-infections described possibly because surveys of ticks typically used one broad-range assay to detect any SFGR, recently a male GCT was reported simultaneously infected with *R. parkeri* and "*Ca.* R. andeanae" in North Carolina (Varela-Stokes et al. 2011). Also, SFGR co-infection has been reported in *A. americanum* co-infected with *R. amblyommii* and *R. rickettsii* (Berrada et al. 2011) and in *D. occidentalis* with *R. rhipicephali* and *R. bellii* (Wikswo et al. 2008, Berrada et al. 2011). These reports warrant further study of rickettsial co-infection, particularly to determine how simultaneous infections with sympatric rickettsiae may affect their individual maintenance in nature and transmission to vertebrates from ticks.

Single Strand Conformation Polymorphism

Analysis of genetic variation has been useful for systematic, evolutionary, and epidemiological studies of infectious agents and their vectors (Gasser and Chilton 2001). For instance, single strand conformation polymorphism (SSCP) has proven to be a powerful tool for disease surveillance and control strategies in Lyme disease, cryptosporidiosis, and parasitic helminthes (Guttman et al. 1996, Anderson and Norris 2006, Jex et al. 2007, Simsek et al. 2011). In addition,

SSCP has become more user-friendly with a non-isotopic SSCP method that utilizes larger amplicons of sizes up to 500bp (Hongyo et al. 1993, Gasser and Chilton 2001, Gasser et al. 2007). SSCP relies on the principle that the mobility of denatured, single strands of DNA in a non-denaturing gel is highly dependent on chemical composition and number of nitrogenous bases (Orita et al. 1989). Thus, DNA sequences differing by single base pair mutations can be identified as unique gel profiles, which are also different haplotypes, based on numbers of bands and length of migration in electrophoresis.

There are few reports describing genetic variation analyses in GCT populations. In a recent study, considerable population variability was detected in GCT populations in the southern United States (Ketchum et al. 2009). The authors found that ticks from Kansas were more heterogeneous than ticks from Oklahoma and Texas and suggested a need for additional studies to determine reasons for these differences and implications (Ketchum et al. 2009). In Chapter 4, we present data from our study to identify haplotypes occurring in selected areas of Mississippi using SSCP. This study was similarly based on a fragment of the tick 16S mitochondrial DNA, chosen because it has been shown to be good indicator of variability (Black and Piesman 1994, Anderson et al. 2004, Trout et al. 2010a).

Genetically distinct populations of ticks may also differ in vector competency, such that their capacity to acquire, maintain and transmit pathogens may vary (Reichard and Kocan 2006). For example, Qiu *et al*. found that when genetic variability of the tick vector, *Ixodes scapularis,* was low, infection rates

with *Borrelia burgdorferi*, the agent of Lyme disease, were higher (Qiu et al. 2002). We hope to use our SSCP data to contribute to the knowledge of *R. parkeri* and *A. maculatum* genetic heterogeneity in Mississippi. Interestingly, a single study of rickettsial genetic variation based on SSCP of *rompA* gene showed no variability in *R. montanensis,* suggesting that *D. variabilis* ticks in the study area were infected with a single SFGR haplotype (Ammerman et al. 2004). Intergenic spacer regions, which are noncoding sequences that are under less selection pressure, were significantly more variable than coding genes and split or remnant genes and more useful in typing *Rickettsia conorii* (Fournier et al. 2004) and *R. rickettsii* isolates (Karpathy et al. 2007). Thus, as the *rompA* gene sequence may not be very informative due to low variability, an alternative approach may be to analyze intergenic spacer regions (Fournier et al. 2004, Karpathy et al. 2007).

Ultrastructural Studies of SFGR

Rickettsiae are small (0.3-0.5 x 0.8-2 μm), rod-shaped bacteria that have cell walls and lack flagella (Fredricks 2006). The cell wall of rickettsiae is similar to other gram negative bacteria at the light microscopy level, but at the ultrastructural level, there is a trilaminar cell wall consisting of the cytoplasmic membrane, and an inner and outer leaflet surrounded by an electron lucent layer, called a “halo zone” or slime layer (Hayes and Burgdorfer 1979, Silverman 1991).

Transmission electron microscopy (TEM) has been frequently utilized to elucidate the relationship of ticks and SFGR (Silverman 1991, Simser et al. 2002,

Kurtti et al. 2005). Hayes and Burgdorfer reported *R. rhipicephali* broadly infecting the tissues of *Rhipicephalus sanguineus* ticks, but maximum concentrations were found in salivary glands, Malpighian tubules and ovaries (Hayes and Burgdorfer 1979). A similar distribution that included the tick midgut was described for the SFGR, *R. honei,* in *Aponomma hydrosauri*, a reptilian tick. That *R. honei* was also identified in ovaries suggested it was transovarially transmitted (Whitworth et al. 2003). In chapter 5, TEM is used to study the fine structure of *"Ca.* R. andeanae" as well as to provide ultrastructural descriptions of tissue tropism in *A. maculatum,* which may generate hypotheses to test aspects of pathogenicity and transmission.

Tick Embryonic Cell Lines

Tick cell lines have been utilized in research for over 60 years aiding in isolation and study of several arboviruses, bacteria and protozoa (Bell-Sakyi et al. 2007). Tick cell lines have been key in isolating pathogens such as the Crimean-Congo Hemorrhagic Fever virus, the agent of a serious zoonosis especially problematic in Africa and in the Middle East (Bell-Sakyi et al. 2011), *R. felis*, an emerging human pathogen and potential bioterror weapon carried by the cat flea (Pornwiroon et al. 2006), and *Ehrlichia ruminantium* (Bell-Sakyi et al. 2000). Tick cell lines have also been valuable for studies of host-vector-pathogen interactions, proteomics and genomics investigations as well as the production of vaccines (Bell-Sakyi et al. 2007, Richards 2011). Primary cultures may be established from molting nymphs and ovarian tissues, but the easiest and most

widely utilized process is through utilization of tick embryos (Bell-Sakyi et al. 2007).

Rickettsiae are obligate intracellular bacteria that are incapable of growing in eukaryotic cell-free medium. In vitro cultivation of SFGR is possible using a variety of cell lines, including Vero (African green monkey kidney cells), L-929 (murine aneuploid fibrosarcoma cells), HEL (human erythroleukemia cells) and MRC5 (human fetal lung fibroblast cells) (Dumler and Walker 2005). However, as most SFGR are maintained within ticks in nature, tick embryonic cell lines have been established to aid in isolating many of these SFGR. For example, isolation of the fastidious tick endosymbiont, *R. peacockii* required establishment of a *D. andersoni* cell line (Simser et al. 2001). There are currently over 57 tick cell lines from 13 ixodid and one argasid tick species, however no cell line from the GCT has been established to date (Bell-Sakyi et al. 2007, Bell-Sakyi et al. 2011).

As discussed earlier, the public health and veterinary importance of GCTs have been increasing with the awareness that GCTs may harbor and transmit a human rickettsia and canine protozoan that were only recognized in the last few decades. Studies of organisms harbored by this tick species, including the poorly characterized SFGR, *"Ca.* R. andeanae", will benefit from establishment of an *A. maculatum* cell line. The pathogenicity of *"Ca.* R. andeanae" is currently unknown, and future studies to evaluate both pathogenicity and infectivity to vertebrates will require a stable isolate. The most natural environment for the propagation of a tick-associated bacterium for studying its biology would be cells from the native tick host, *A. maculatum*. As shown, ehrlichiae grown in vertebrate

or tick cells showed different levels of protein expression at the p28/p30-Omp locus (Singu et al. 2006). Similarly, in *R. conorii* grown in mammalian compared to insect cells, transcription of the SpoT3 gene, a gene involved in adaptation of various arthropod-borne organisms to different environments, ceases during growth in the insect cells (Rovery et al. 2005). In Chapter 6, we present the establishment of a new embryonic cell line from naturally infected *A. maculatum* with "*Ca.* R. andeanae" and isolation of the rickettsia, both of which will contribute to our understanding of GCT-associated organisms as well as *"Ca.* R. andeanae".

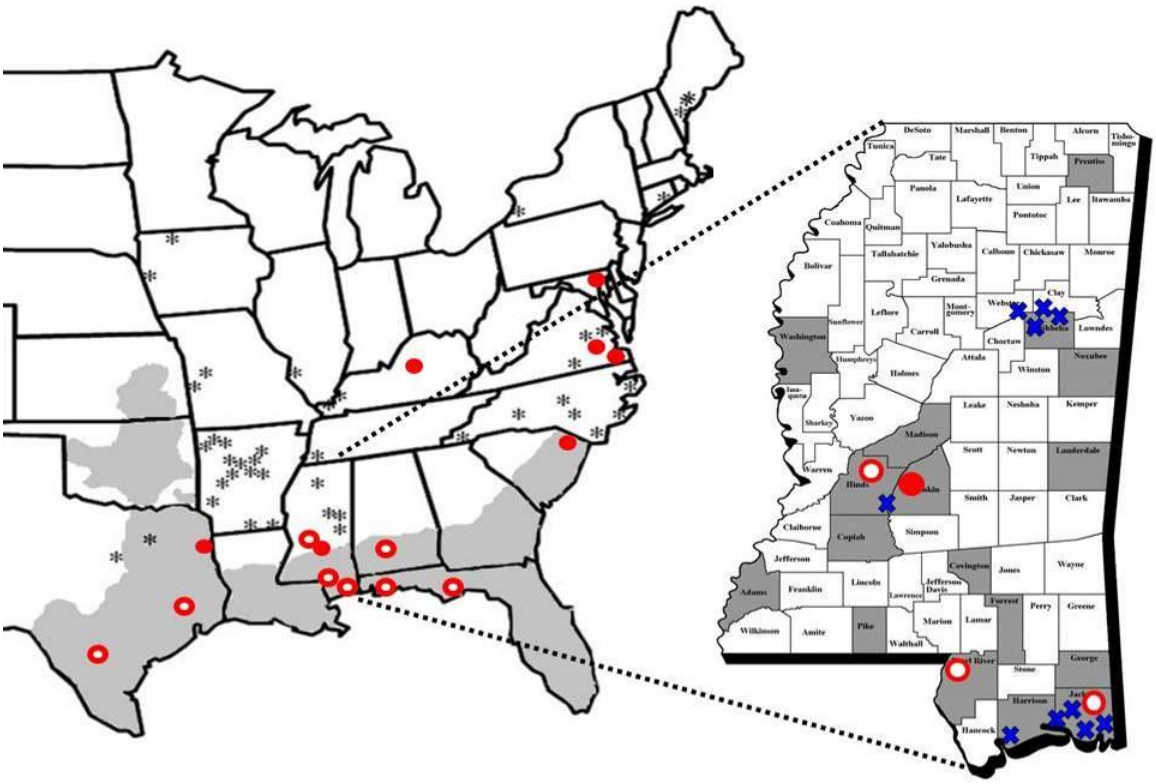


Figure 2.1 Geographical ranges of Gulf Coast ticks and human reports of *R. parkeri* infection in the U.S. and Mississippi and sites of tick collection for chapters 3 and 4

NOTE: Left map shows the most established distribution of Gulf Coast ticks in the U. S. (gray area) and the asterisks are published collections of these ticks in incidental reports (may or may not reflect permanent populations) (Teel et al. 2010). Also in the U. S. map are 15 of the reported cases of *Rickettsia parkeri* rickettsiosis (filled circles, confirmed cases; hollow circles, probable;(Paddock et al. 2004, Paddock et al. 2008, Cragun et al. 2010). The inset map shows the Mississippi counties where Goddard and Paddock (Goddard and Paddock 2005) reported occurrence of *Amblyomma maculatum* in the shaded counties as well as the previously reported cases of rickettsiosis by *R. parkeri* (Paddock et al. 2008). Right map also shows the sites of GCT collection for chapters 3 and 4 of this dissertation ('x' marks). Figures adapted with permission.

CHAPTER III

PREVALENCE OF *RICKETTSIA PARKERI* AND "*CANDIDATUS* RICKETTSIA ANDEANAE" IN GULF COAST TICKS FROM MISSISSIPPI

Abstract

During 2008 through 2010, 707 adult Gulf Coast ticks (*Amblyomma maculatum*) were collected from ten sites in Mississippi. Of these, 15.2% were infected with the human pathogen, *Rickettsia parkeri,* and 1.4% of Gulf Coast ticks were singly infected with "*Candidatus* Rickettsia andeanae", a recently described species of unknown pathogenicity. Additionally, 1.7% of ticks were co-infected with *R. parkeri* and "*Ca.* R. andeanae", demonstrating that co-infections occurred at a rate higher than single infections of *"Ca.* R. andeanae" in Gulf Coast ticks. To our knowledge this is the largest collection of Gulf Coast ticks tested for these rickettsiae from one state. Studies to examine the role of "*Ca.* R. andeanae" as a potential pathogen and to evaluate the ecological relationships among *R. parkeri* and "*Ca.* R. andeanae" in Gulf Coast ticks are warranted.

Introduction

Gulf Coast ticks*, Amblyomma maculatum* Koch, are currently found in most of the central and southeastern United States, (Figure 2.1) (Semtner and

Hair 1973, Teel et al. 2010, Trout et al. 2010b). *Amblyomma maculatum* ticks have attracted attention in the past decade because *Rickettsia parkeri*, a spotted fever group *Rickettsia* (SFGR) species transmitted by *A. maculatum*, was recently identified as a cause of human disease (Paddock et al. 2004). More than 20 cases of this infection have been described from the U. S. and Argentina (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Paddock et al. 2010, Romer et al. 2011). Disease due to *R. parkeri* infection is similar to, but milder than, Rocky Mountain spotted fever, caused by *R. rickettsii*. Misdiagnosis of Rocky Mountain spotted fever with other SFG rickettsioses, including *R. parkeri* infection, may occur due to clinical similarities among spotted fever rickettsioses and serological cross-reactivity among SFGR using current diagnostic tests (Paddock et al. 2008).

Another SFGR species, "*Candidatus* Rickettsia andeanae", was recently found in *A. maculatum.* This novel rickettsia was first identified in *A. maculatum* and *Ixodes boliviensis* from the Peruvian Andes and later identified in Gulf Coast ticks in Mississippi, Florida, Georgia, Kansas, Oklahoma and Virginia (Blair et al. 2004, Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Jiang et al. 2011, Luce-Fedrow et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011). Recently, an isolate from an *A. maculatum* tick was propagated in three cell lines collected in Virginia (Luce-Fedrow et al. 2011). As no cases of infection in humans or other vertebrates have been described, the pathogenicity of *"Ca.* R. andeanae" is currently unknown.

Mississippi is located centrally in the range of the Gulf Coast tick distribution and reported cases of *R. parkeri* rickettsiosis in the U. S., with at least one confirmed and three probable cases of *R. parkeri* rickettsiosis from this state (Paddock et al. 2008). Here we report the results of a three-year collection of questing adult *A. maculatum* from ten sites in Mississippi to more fully ascertain the prevalence of *R. parkeri* and *"Ca.* R. andeanae" in these ticks.

Materials and Methods

Tick Collection

From June to September of 2008, 2009 and 2010, adult Gulf Coast ticks were collected using a 1 m^2 muslin drag cloth to sweep ticks from vegetation in ten sites in Mississippi (Figure 2.1). Sample sizes were selected based on a prevalence estimate of tick-borne rickettsiae (including *Ehrlichia* spp., *Rickettsia parkeri,* and *Anaplasma* spp.) in unfed ticks, a 5% precision, and 95% confidence level using the formula by Daniel (Daniel 1999). We used a lower prevalence of 5% in calculations, resulting in a sample size of 73, and chose a minimum of 70 Gulf Coast ticks as the goal per site for a total of 700 ticks. Sites were selected from counties in Mississippi previously identified as supporting populations of Gulf Coast ticks (Goddard and Paddock 2005), including the towns of Starkville in Oktibbeha county, Mathiston in Choctaw county, Byram in Hinds county, Moss Point, Pascagoula and Gautier in Jackson county, and Pass Christian in Harrison county. Adult ticks were identified morphologically to species and sex and preserved in 70% ethanol until DNA extraction.

DNA Extraction

Genomic DNA was extracted using Illustra™ Tissue & Cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA). Individual ticks were minced using a sterile scalpel blade in proteinase K and lysis buffer from the kit and prepared following the manufacturer's instructions. Amplifiable DNA was assessed by a PCR assay targeting a 299-bp fragment of the tick mitochondrial 16S rRNA gene (Black and Piesman 1994).

Molecular Evidence of *Rickettsia parkeri* and "*Candidatus* R. andeanae"

A nested PCR assay that amplifies a portion of the rickettsial outer membrane protein A gene (*rompA*) (Paddock et al. 2004) was used to screen ticks for molecular evidence of any SFGR species. Tick extracts that tested positive by this assay were subsequently tested in species specific assays, using primers to amplify a 447-bp fragment of the *R. parkeri rompA* gene (Varela-Stokes et al. 2011) and 408-bp *rompA* gene fragment of "*Ca.* R. andeanae" (Paddock et al. 2010). All PCR products were stained with ethidium bromide and electrophoresed in a 2% agarose gel. All PCR assays included a positive control of DNA extracted from cultured *R. parkeri* (Tate's Hell strain) or "*Ca.* R. andeanae"-infected Gulf coast ticks, confirmed previously by PCR and sequencing. Water was used as a negative control for all assays. PCR products were purified using Montage PCR Centrifugal Filter Device (Millipore, Bedford, MA, USA) and sequenced by Eurofins MWG Operon (Huntsville, AL, USA).

Consensus sequences were generated by ClustalX2 alignment and identified using GenBank BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Analysis

Proportions of ticks infected with SFGR, by region and year, were compared separately by Fisher's Exact test followed by pairwise comparisons with a Bonferroni adjustment, using PROC FREQ, SAS for Windows, V9.2 (SAS Institute, Cary, NC). A p-value of less than or equal to 0.05 was considered to be significant for all analyses unless otherwise noted. An index of co-infection (IC) was calculated to determine if the level of co-infections of *A. maculatum* ticks with *R. parkeri* and "*Ca.* R. andeanae" was significantly higher than expected due to chance alone, using the formula: IC = ((O – E)/N) x 100, where O = observed co-infection numbers; E = expected occurrence of co-infection due to chance alone and N = total number of ticks infected by either or both microorganisms (Ginsberg 2008).

Results

In this study, most *A. maculatum* ticks were collected in July and August of each sampled year in areas exposed to full sun during hours (9am-3pm) of peak temperature. Along the coast, typically ticks were collected in pine savannahs, containing mostly wiregrass (*Aristida stricta*), gallberry (*Ilex glabra*), and invasive torpedograss (*Panicum repens*). In the central and northern Mississippi sites, most ticks were collected in areas containing golden rod (*Solidago* spp.),

Johnson grass (*Sorghum halepense*), Bermuda grass (*Cynodon dactylon*), Fescue (*Festuca* spp.) and dallisgrass (*Paspalum dilatatum*) (Goddard et al. 2011). Our goal was to collect 70 ticks per site, however this was not possible in 2008 and additional collections were made in 2009 and 2010 to fulfill this goal. A total of 707 adult *A. maculatum* ticks were obtained during the three-year sampling period, comprising 350 in 2008, 194 in 2009 and 163 in 2010.

Mitochondrial 16S rDNA was detected in 698 (98.7%) of the tick extracts. Of 698 ticks with evidence of successful DNA extraction as demonstrated by the tick mitochondrial PCR assay, 128 (18.4%) were positive for SFGR DNA including 106 (15.2%) singly infected with *R. parkeri* and 10 (1.4%) that were singly infected with "*Ca.* R. andeanae" (Table 3.1). Interestingly, 12 (1.7%) ticks were co-infected with *R. parkeri* and "*Ca.* R. andeanae". All 22 ticks singly or co-infected with "*Ca.* R. andeanae" as determined by species-specific PCR assay were confirmed by sequencing. For the 12 co-infected ticks, PCR products of *R. parkeri* from the species-specific assay were also purified and sequenced to confirm the PCR findings.

The majority (94.6%) of tick specimens could be segregated into those collected from northeastern Mississippi (N = 260) and those from coastal Mississippi (N = 409). There was no significant difference in the prevalence of ticks infected singly with *R. parkeri* between northern and southern Mississippi locales (p-value = 0.13) (table 3.1). However, the southern sites had significantly more ticks singly infected with "*Ca.* R. andeanae" than sites from the northern region of the state (p-value = 0.03). The prevalence of co-infected ticks in the

southern sites was nearly significant higher compared to the northern (p-value = 0.06). Prevalences were also compared among the three years of collection for North and South sites using Fisher's exact test (Table 3.2). Only the prevalence of singly infected ticks with *R. parkeri* was statistically different among the years (p-value = 0.01) and the prevalence of *R. parkeri* in 2010 was significantly greater than in 2009 (p-value = 0.003, $\alpha/3 = 0.02$). We determined an overall index of co-infection with *R. parkeri* and "*Ca.* R. andeanae" of 6.5, suggesting that the numbers of co-infections were greater than expected due to chance alone.

Discussion

Gulf Coast ticks occurring throughout Mississippi, particularly along the Gulf Coast, have been shown to be commonly infected with *R. parkeri* (Sumner et al. 2007, Paddock et al. 2010). We found an overall prevalence of 18.4% in sampled Gulf Coast ticks infected with any SFGR species, where 15.2% of ticks were singly infected with *R. parkeri* and 1.7% had both *R. parkeri* and "*Ca.* R. andeanae". Typically, *R. parkeri* has been found in moderate prevalence rates. Ticks collected in Georgia, Florida, Kentucky, Oklahoma and South Carolina and Mississippi have been detected with an average prevalence rate of 11.5% (Sumner et al. 2007). However, two recent studies detected *R. parkeri* infecting about 42% of *A. maculatum* collected in Virginia (Fornadel et al. 2011, Wright et al. 2011) and 20-33% in North Carolina (Varela-Stokes et al. 2011). High prevalence rates (over 20%) as reported from Virginia and North Carolina have

been found in Jackson and Oktibbeha counties in Mississippi in a previous study (Paddock et al. 2010) and in this study from the same two counties. Although we did not collect consistently at all ten sites during the three year study period, we observed a fluctuation in prevalence of *R. parkeri*-infected ticks at sites between different years. The infection rate observed in this study, equivalent to approximately one *R. parkeri*-infected Gulf Coast tick for every 6 ticks tested, suggests that it may not be uncommon for humans to encounter infected Gulf Coast ticks in Mississippi. Thus, physicians should consider including *R. parkeri* rickettsiosis when formulating a differential diagnosis for febrile patients who have nonspecific symptoms accompanied by dermatological reactions, such as a rash, or particularly an eschar at the site of a tick bite; history of tick bite may or may not be present (Paddock et al. 2008).

"*Candidatus* R. andeanae" is an uncharacterized SFGR occurring in Gulf Coast ticks, first described in Peru (Blair et al. 2004). In studies from the U. S., a cumulative infection prevalence of 3% has been reported from questing adult *A. maculatum* ticks collected in Mississippi, Florida, Georgia and Virginia (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Wright et al. 2011). We also identified "*Ca.* R. andeanae" infection in approximately 3% of Mississippi Gulf Coast ticks; with approximately half of these representing single infections and the others as a co-infection with *R. parkeri*. To our knowledge, except for the recent report of one co-infected Gulf Coast tick in North Carolina (Varela-Stokes et al. 2011), there are no other reports of ticks co-infected with *R. parkeri* and "*Ca.* R. andeanae". Tick surveys for SFGR agents characteristically use one

broad-range assay to determine the infecting species. Had we used this type of protocol, it is likely that only *R. parkeri* or "*Ca.* R. andeanae" infections would have been identified in each of the 12 tick specimens that were co-infected with both agents. Although simultaneous infection of individual ticks with two *Rickettsia* sp. is rarely reported, and the transovarial interference phenomenon (Azad and Beard 1998) contributes to this rarity, there are several mechanisms to produce co-infected ticks (Burgdorfer and Brinton 1975, Burgdorfer 1988). Co-infections with *Rickettsia* spp. were recently reported involving *R. rhipicephali* and *R. bellii* in *Dermacentor occidentalis*, and *R. amblyommii* and *R. rickettsii* in *Amblyomma americanum* (Wikswo et al. 2008, Berrada et al. 2011). Our report and others underscore the importance of using multiple assays to identify SFGR co-infections in ticks. The biological consequences of rickettsial co-infections remain to be determined; however, our findings suggest that *R. parkeri*-"*Ca.* R. andeanae" co-infections occur in Gulf Coast ticks more frequently than expected by chance alone.

Although the pathogenic status of "*Ca.* R. andeanae" has not yet been investigated, the first report of this novel SFGR was from two infected ticks occurring in a region with an outbreak of febrile people where two patients had died (Blair et al. 2004). Jiang *et al.* (Jiang et al. 2011) showed that four ticks removed from people in Oklahoma and Kansas were also positive for the bacterium. The potential for human infection with *"Ca.* R. andeanae" is unknown, however human infections have been demonstrated with other SFGR that were previously thought to be non-pathogenic (Raoult and Roux 1997). We observed

that the majority of the infected ticks with "*Ca.* R. andeanae", whether singly or co-infected with *R. parkeri,* were from southern Mississippi in comparison with the northern sites. Interestingly, there have been four reports of *R. parkeri* rickettsiosis that have occurred in the same southern Mississippi region where we collected ticks for this study (Paddock et al. 2008). In addition to the human disease potential of *"Ca.* R. andeanae", the impact of co-infection in Gulf Coast ticks with both *"Ca.* R. andeanae" and *R. parkeri* on disease transmission of the latter known pathogen is also unknown. The recent isolation of "*Ca.* R. andeanae" may aid in elucidating the relationship of both SFGR and pathogenicity of the novel rickettsia (Luce-Fedrow et al. 2011).

Acknowledgments

We would like to acknowledge the assistance of Gail Moraru, Diana Link, Claudenir Ferrari, Marcia Carvalho and Ryan Lawrence with tick collecting at occasions as well as Dr. Robert Wills for assisting with statistical analyses and Erle Chenney and Whitney Smith for contributing to the molecular analysis of ticks.

This work was supported by a SECEBT (Southeastern Center for Emerging Biologic Threats) grant awarded to Dr. Varela-Stokes in 2009 and funding from the College of Veterinary Medicine at Mississippi State University.

This chapter is under revision for manuscript submission as "Ferrari, F.A.G., Goddard J., Paddock C., Varela-Stokes A. 2012. *Rickettsia parkeri* and

"*Candidatus* Rickettsia andeanae" in Gulf Coast ticks, Mississippi. Emerging Infectious Diseases".

Table 3.1 PCR results by region for adult *Amblyomma maculatum* ticks in Mississippi, 2008 to 2010

Sites in MS	Total no. ticks	No. (%; 95% CI) SFG rompA positive	No. (%; 95% CI) *R. parkeri* positive only	No. (%; 95% CI) "*Ca.* R. andeanae" positive only	No. (%; 95% CI) co-infected
North	257	49 (19.1*; 14.5-24.4)	48 (18.7†; 14.1-24)	0 (0‡)	1 (0.4§; 0-2.1)
Central	38	4 (10.5)	1 (2.6)	2 (5.3)	1 (2.6)
South	403	75 (18.6*; 14.9-22.8)	57 (14.1†; 10.9-17.9)	8 (2.0‡; 0.9-3.9)	10 (2.5§; 1.2-4.5)
Total	698	128 (18.4)	106 (15.2)	10 (1.4)	12 (1.7)

* p-value= 0.9187; † p-value=0.1275; ‡ p-value=0.0257; § p-value=0.0578 (comparison of prevalences from northern and southern sites only).

Table 3.2 PCR results by year for adult *Amblyomma maculatum* ticks collected in southern and northern Mississippi, USA

Year	Total no. ticks	No. (%, 95% CI) SFG rompA positive	No. (%, 95% CI) *R. parkeri* only positive	No. (%, 95% CI) "*Ca.* R. andeanae" only positive	No. (%, 95% CI) co-infected
2008	343	68 (19.8¶; 15.7-24.4)	54 (15.7*; 12.0-20.0)	7 (2.0‡; 0.8-4.2)	7 (2.0†; 0.8-4.2)
2009	161	21 (13.0¶; 8.3-19.2)	16 (9.9*; 5.8-15.6)	1 (0.6‡; 0-3.4)	4 (2.5†; 0.7-6.2)
2010	156	35 (22.5¶; 16.1-29.8)	35 (22.4*; 16.1-29.8)	0 (0‡)	0 (0†)

¶ p-value=0.0713; * p-value=0.0097; ‡ p-value=0.1359; † p-value=0.1381

CHAPTER IV

POPULATION ANALYSIS OF *AMBLYOMMA MACULATUM* TICKS AND *RICKETTSIA PARKERI* USING SINGLE STRAND CONFORMATION POLYMORPHISM

Abstract

Gulf Coast ticks, *Amblyomma maculatum*, are expanding into areas not previously reported in the United States and are emerging threats for public and veterinary health. Genetic analyses of the Gulf Coast tick and the zoonotic agent it transmits, *Rickettsia parkeri,* can provide a better understanding of the dynamics of this tick-pathogen system and implications for disease transmission. To assess genetic variation of tick and rickettsial populations, we collected adult *A. maculatum* from ten sites in Mississippi, four in the northern, one in central and five in the southern part of the state. PCR amplicons from tick mitochondrial 16S rRNA and *rompA* genes as well as five intergenic spacer regions were evaluated for genetic variation using single strand conformation polymorphism analysis. Frequencies of the four tick haplotypes were not significantly different among regions of Mississippi, but within sites there were differences in distribution that can be explained by the high migration rate estimated. Phylogenetically, one tick haplotype was basal to the other three and

comparisons to a similar genetic variation study from ticks in Texas, Oklahoma and Kansas were drawn. Interestingly, in this study we report finding no genetic variation among *R. parkeri* infecting these ticks. Future studies that include a larger selection of intergenic spacer regions may be able to distinguish potential genetic heterogeneity between *R. parkeri* populations.

Introduction

Genetic analyses contribute to an understanding of systematics, phylogenetics and the epidemiology of arthropod vectors and the infectious agents they transmit (Gasser and Chilton 2001). Large population studies have contributed to our understanding of common tick-borne disease vectors in North America, specifically, *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis* (Qiu et al. 2002, Mixson et al. 2006a, Krakowetz et al. 2010). However, other North American tick vectors have not been as well studied. *Amblyomma maculatum,* the Gulf Coast tick (GCT), serves as the primary vector for *Rickettsia parkeri,* an agent causing spotted fever rickettsiosis similar to Rocky Mountain spotted fever (Paddock et al. 2004). *Amblyomma maculatum* was historically documented along the Gulf and Atlantic coasts; however, populations have spread and this species is currently found in most of the central and southeastern United States, including Oklahoma, Kansas and Arkansas (Figure 2.1) (Semtner and Hair 1973, Teel et al. 2010, Trout et al. 2010b). Since the disease and vector potential of *R. parkeri* and *A. maculatum*, respectively, were overlooked for nearly 70 years, and have only recently been studied more

intensively, many gaps in our understanding of this tick-pathogen system still exist. A single genetic study using limited numbers of GCTs found that *A. maculatum* populations from Kansas were more heterogeneous than those from Oklahoma and Texas, possibly due to differences in wildlife influence or livestock movement among these states (Ketchum et al. 2009). As the public health importance of *A. maculatum* rises, genetic analyses using large populations of this tick species would help us understand the dynamics of this tick-pathogen system and implications for disease transmission to humans.

Genetically distinct populations of ticks may possess distinct vector competency, such that their capacity to acquire, maintain and transmit pathogens may vary in different regions (Reichard and Kocan 2006). Qiu *et al*. found that the tick vector, *Ixodes scapularis,* and the agent of Lyme disease, *Borrelia burgdorferi*, had an intrinsic relationship related to their co-evolution over time and spatial distribution in the United States. The genetic variability of the southern tick population may be a cause of low *Borrelia* infection in the South (Qiu et al. 2002). Over 20 cases of spotted fever rickettsiosis due to *R. parkeri* infection have been reported in the U.S. and Argentina (Paddock et al. 2004, Whitman et al. 2007, Paddock et al. 2008, Cragun et al. 2010, Paddock et al. 2010, Romer et al. 2011). In Mississippi, approximately one out six ticks (15.2%) was found infected with *R. parkeri,* however a prevalence as high as 40% has also been reported (Paddock et al. 2010). Genetic analyses may aid defining heterogeneous populations of *A. maculatum* as well as the rickettsial pathogen it transmits.

Mississippi is located in the center of both the Gulf Coast tick distribution and the range of reported cases of *R. parkeri* rickettsiosis in the U. S. Thus, Mississippi is an ideal location to study population genetics of this tick and bacterium. Single strand conformation polymorphism (SSCP), a commonly used tool for analyses of genetic variation, has assisted in the surveillance and design of control strategies for Lyme disease, cryptosporidiosis, and parasitic helminthes (Guttman et al. 1996, Anderson and Norris 2006, Jex et al. 2007, Simsek et al. 2011). Here we report the results of a three-year collection of questing adult *A. maculatum* from ten sites in Mississippi that analyzed the genetic heterogeneity of tick and rickettsial populations from sites in Mississippi using SSCP.

Materials and Methods

Tick Collection

Adult *A. maculatum* were collected as part of a previous study (Ferrari, F.A.G et al., submitted to Emerging Infectious Diseases journal) from vegetation in ten sites ('x' marks in the inset map, Figure 2.1) during the summers of 2008, 2009 and 2010. Sites were selected from counties in Mississippi previously identified as supporting populations of Gulf Coast ticks (Goddard and Paddock 2005). In addition, 25 adult *A. maculatum* from a previous study in North Carolina (Varela-Stokes et al. 2011) were used as an SSCP outgroup for tick and rickettsial studies. Adult ticks were morphologically identified to species and sex and preserved in 70% ethanol until DNA extraction.

DNA Extraction

Genomic DNA was extracted using Illustra™ Tissue & Cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA). We first minced individual ticks using a sterile scalpel blade in proteinase K and lysis buffer from the kit and then continued the DNA extraction following the manufacturer's instructions.

***Amblyomma maculatum* and *Rickettsia parkeri* PCR**

PCR products were generated for fragments of tick 16S mitochondrial rRNA (Black and Piesman 1994) and rickettsial *ompA* (Paddock et al. 2004, Varela-Stokes et al. 2011) genes as described for all tick DNA extracts (FAGF, unpublished data). Additionally, five primers targeting rickettsial intergenic spacer regions (ISRs) were chosen. We selected *mppA-purC*, *rpmE*-tRNAfMet, RR1240-*tlc*5, RR0155-*rpmB and cspA-ksgA* as they were the most informative regions identified in previous studies (Fournier et al. 2004, Karpathy et al. 2007). All PCR amplicons were subjected to cold SSCP analysis, described below.

Non-isotopic SSCP Analysis of Amplicons

Our cold SSCP protocol followed suggestions by the manufacturer of the gels and gel apparatus with some adaptations (Elchrom®). For each positive sample, 8.4uL of PCR product was added to 3.6 µl of denaturing loading mix prepared right before use and mixed well. The mix is composed of 150µl of formamide, 1.5µl of 1M NaOH and 3.9µl of each product-specific primer. A few grains of bromphenolblue were added to the mix for better visualization. Samples

were then denatured by heating at 95°C for 5.5 minutes, snap cooled on ice for 3 minutes and rapidly loaded into precast GMA™ gels (Elchrom Scientific AG, Cham, Switzerland). Electrophoresis took place in an Origins™ gel apparatus (Elchrom Scientific AG) in cool (8˚C) pumping buffer at 6 V/cm for 15h to 17h, depending on the fragment size, as suggested by the manufacturer. After that, gels were stained with ethidium bromide in a shaker for 40 minutes and photodocumentation was performed using ChemiDoc XRS+ from Bio-Rad (Hercules, CA). All gels included a PhiX molecular weight ladder.

SSCP Patterns and Phylogenetic Analyses

All gel pictures were analyzed visually to determine gel migration patterns, defined by the number of bands and migration distance in the gel. Recurring patterns were assigned the same letter. At least four representatives of each SSCP pattern visualized in the tick 16S mitochondrial rRNA, *rompA* or ISR gene targets were bidirectionally sequenced to compare phenotype on SSCP gels with the nucleotide sequence. For sequencing, PCR products were purified using Montage PCR Centrifugal Filter Device (Millipore, Bedford, MA, USA) and sequenced by Eurofins MWG Operon (Huntsville, AL, USA). Consensus sequences were generated using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) or Vector NTI 9.0 software (Invitrogen, Carlsbad, CA) and were adjusted manually as needed by visual inspection. *MEGA* version 5 was used to construct phylogenetic trees of *A. maculatum* 16S mtRNA gene amplicons based on Maximum parsimony and

Neighbor joining methods (bootstrap, N = 1000). A fragment of the 16S mitochondrial rDNA of *Amblyomma americanum* was used as outgroup sequence, obtained from NCBI/GenBank (accession number L34313.1).

Statistical Analysis

The program HIERFSTAT (Goudet 2005) was used to estimate the F-statistics for proportion of variance between sites within regions (F_{SR}), between sites overall (F_{ST}), and between the two regions grouping the sites (F_{RT}). Using G statistics, we reassigned samples to either regions or sites (G_{region} or G_{site}) randomly, resampled 1000 times and re-calculate the test statistic for each resample (Goudet 2005). To calculate dispersal between sites, the equation $F_{ST} = 1/(4Nm+1)$ was used, where Nm is the number of migrants exchanged by a population per generation (Wright 1965). A p-value of less than or equal to 0.05 was considered to be significant for all analyses unless otherwise noted.

Results

Tick SSCP

A total of 698 adult *A. maculatum* ticks collected from the 10 Mississippi study sites (Figure 2.1) had amplifiable DNA and were used for genetic analysis. Of these, 654 samples yielded apparent bands in SSCP gel electrophoresis. GCTs from the ten sites analyzed in Mississippi demonstrated four SSCP patterns labeled A through D (Figure 4.1). Sequencing of twenty samples produced high quality chromatograms and only these were used for further

analyses (Table 4.1). There were five single nucleotide polymorphic sites in the sequence alignment (298 bp), and these represented one transitional mutations, three transversional mutations, and an indel (insertion/deletion). Interestingly, there was a hyper variable region of 5bp in the reverse primer flanking region that was very informative, including one transversional and four transitional mutations (Table 4.1). In addition to the four sequences represented by four haplotypes, three unique sequences were identified that could not be matched to a unique SSCP gel pattern. The nucleotide constitution for these three samples is given in table 4.1 and they were included in the phylogenetic analysis as Mississippi C2, C3 and D2, as the sequences were most close related to the sequences of patterns C and D (Figure 4.2). Noteworthy, out of 10 samples sequenced but not included in the haplotype analysis due to poor quality chromatograms, four sequences produced reliable chromatograms except at exactly this hyper variable 5bp region of the reverse primer (not included in haplotype analysis). Additionally, 22 GCTs from North Carolina were analyzed by SSCP. North Carolina ticks presented matching band patterns to Mississippi haplotypes A, B, and C. Of the six North Carolina samples sequenced, at least one from each A, B, and C haplotype matched identically to respective haplotypes from Mississippi. However, like sequence results from Mississippi ticks, we identified two additional unique sequences in North Carolina tick samples that could not be matched to a unique SSCP pattern. These also differed from all Mississippi tick sequences.

The tick SSCP haplotype frequencies seen in ten sites collected over a three year period are summarized in table 4.2. Nucleotide sequences will be deposited in the NCBI GenBank. Thirty samples total were sequenced. To assess whether gel patterns in a smaller population of *A. maculatum* ticks from North Carolina differed from those seen in Mississippi samples, we performed SSCP using the same conditions for analysis of the tick mitochondrial 16S rRNA gene as was used for Mississippi ticks. Twenty two out of 25 ticks from North Carolina yielded band patterns in tick SSCP gels that matched Mississippi haplotypes A, B and C; three samples failed to show bands on SSCP analysis.

A phylogenetic tree using Neighbor-joining analysis demonstrated a monophyletic relationship among all haplotypes from this study of Mississippi and North Carolina ticks, and including haplotypes from a similar study of GCTs from Oklahoma, Kansas and Texas (Ketchum et al. 2009) as shown in Figure 4.2. Maximum parsimony analysis produced similar topography, therefore only the Neighbor-joining tree will be discussed. Mississippi haplotype D is most similar to haplotype C from Oklahoma and Kansas (Ketchum et al. 2009), which were basal to all other haplotypes. Mississippi and North Carolina haplotypes A, B, and C appears to have diverged from haplotypes A, B, D, E, F, and G found in the previous study (Ketchum et al. 2009) although they are a monophyletic group.

We performed statistical analyses on SSCP data of ticks collected from the two main regions in Mississippi, northern and southern (N=229 and 387 ticks, respectively), that comprised the majority of ticks that yielded bands on SSCP

analysis (94.2%). We tested the occurrence of genetic variability following a hierarchical genetic structure by regions (North or South Mississippi) and within sites per regions. Using the program HIERFSTAT (Goudet 2005), we estimated the F-statistics for proportion of variance between sites within regions (F_{SR}), between sites overall (F_{ST}), and between the two regions grouping the sites (F_{RT}). Based on the estimates, there was no variance between populations at the regional scale (F_{RT} = -0.001029765) and some variability was seen between sites within regions (F_{SR} = 0.02311502) and between sites overall (F_{ST} = 0.02210905). The F-statistics results were supported by G-statistics, as follows. Resampling 1000 times, reassigning sites to regions randomly and recalculating the test statistic for each resample, we found no support for differentiation between the northern and southern samples (G_{region} p-value = 0.572). In 1000 resamples for variation within regions, there is strong support for the presence of genetic differences between sites within regions (G_{Site} p-value = 0.001). Additionally, we estimated dispersal between sites within the two main regions of Mississippi. Based on the equation by Wright, we estimate that approximately 10.57 migrants per generation are exchanged between sites (Wright 1965).

Despite the small number of samples, we included the haplotype frequencies observed using SSCP analysis of 22 North Carolina ticks for preliminary analysis as a third region with a single site. As a region, North Carolina was not significantly different (G_{region} p-value = 0.94) from the other two regions in Mississippi after 1000 permutations. The test for differentiation between sites was significant (G_{site} p-value less than 0.001 after 1000

permutations). Results from North Carolina ticks agree with data from Mississippi.

Rickettsial SSCP

Of the 698 GCTs with amplifiable 16S rDNA, 106 were singly positive for *R. parkeri,* and were used in SSCP analyses for genetic variability of this rickettsia. No difference in migration pattern was observed in 33 *R. parkeri*-positive GCTs analyzed by SSCP electrophoresis using the *rompA* PCR amplicons. The lack of variability observed is consistent with a previous study using *rompA* in *R. montanensis* in *D. variabilis* ticks (Ammerman et al. 2004). Five intergenic spacer regions were subsequently chosen as potentially more informative regions than *rompA* due to a higher degree of genetic variability (Fournier et al. 2004, Karpathy et al. 2007). All 106 *R. parkeri* singly infected GCTs had DNA amplified by PCR for the five ISRs fragments. Still no difference in migration pattern was observed among three of the ISRs: *cspA-ksgA*, RR0155-*rpmB* and *mppA-purC*. For RR1240-*tlc*5, we saw one sample with a slightly different gel pattern. Consensus sequences from this sample compared to two other samples showed no nucleotide differences. SSCP patterns using the *rpmE*-tRNAfMet PCR amplicon demonstrated a slightly different pattern in three *R. parkeri*-infected tick samples, however they had nucleotide sequences identical to the sequences from other samples with the common gel pattern. For comparison, we ran 25 samples of Gulf Coast ticks from North Carolina using

rpmE-tRNAfMet and we found no variation as well. We will deposit nucleotide sequences in the NCBI GenBank prior to journal submission.

Discussion

Mississippi is ideally located for population studies of both *A. maculatum* and *R. parkeri*. Genetic analysis tools such as SSCP coupled with sequencing can be used to determine population heterogeneity of arthropods and pathogens transmitted by them, and, in some cases, genetic variability may demonstrate altered vector-pathogen interactions (Gasser and Chilton 2001, Reichard and Kocan 2006). Here we describe four haplotypes defined by four unique SSCP patterns and sequences from *A. maculatum* in Mississippi. For comparison, we studied the genetic variability of a smaller scale of GCTs from North Carolina. We relied on a fragment of the tick 16S mitochondrial DNA for haplotype analysis, a gene target that has been previously described as a good indicator of variability (Black and Piesman 1994, Anderson et al. 2004, Trout et al. 2010a). In addition, no genetic variability was reported among *R. parkeri* populations infecting Mississippi ticks or the smaller tick collection from North Carolina.

SSCP relies on the principle that the mobility of the single strand of DNA in a non-denaturing gel is highly dependent on chemical composition and number of nitrogenous bases (Orita et al. 1989). Thus, DNA sequences differing by single base pair mutations can be identified as unique gel patterns, based on numbers of bands and length of migration in electrophoresis and potentially representing different haplotypes. Noteworthy, three different sequences (haplotypes C2, C3

and D2) in the tick SSCP conditions in this study were not associated with unique SSCP gel patterns. Similarly, additional unique sequences were also observed with North Carolina ticks (haplotypes A2 and C2). For rickettsial SSCP of samples from Mississippi, two ISRs (RR1240-*tlc*5 and *rpmE*-tRNAfMet) presented four samples total with slightly different gel patterns but no difference in nucleotide sequence. While SSCP can theoretically differentiate between single nucleotide differences, some differences in the number or patterns of bands on SSCP gels may be due to inconsistencies in running conditions. Additional optimization of running conditions will be essential in future analyses using SSCP.

A hierarchical statistic software, HIERFSTAT (Goudet 2005), was used to estimate the proportion of haplotype frequencies among subpopulations of Gulf Coast ticks collected in Mississippi. In this study, the majority of sampled ticks (94%) were collected in nine out of 10 sites belonging to two regions in Mississippi, North and South. The individual sites within North and South regions had large enough populations that allowed for hierarchical studies of populations of GCTs to be performed. F statistics results showed no difference when comparing all sites from northern Mississippi from those from southern Mississippi; this was also supported by resampling methods (G statistics).

Despite the lower sample size of North Carolina ticks, it was interesting that ticks from this distant state possessed three SSCP patterns also observed in Mississippi ticks. Additionally, observed haplotype frequencies in North Carolina were not statistically different from those in the two regions in Mississippi. Similar

results of no genetic variability were reported with nine populations of *A. americanum* ticks in Georgia (Mixson et al. 2006a) and *D. variabilis* ticks from four localities in the Canadian prairies (Krakowetz et al. 2010). Notably, the Mississippi northern population is apparently established within the last 30 years (Goddard and Norment 1983) and not likely to be in drift/migration equilibrium. The similarity observed in this study between the northern and southern populations suggests that at least at some point in time, a substantial number of migrants likely founded (or expanded into) the northern area. Founder events could be due to movement of cattle herds or occasional “catastrophic" events such as hurricanes. Historically, it was accepted that the GCT distribution was restricted to 100 miles inland from the Gulf Coast and southern region of the Atlantic Coast (Bishopp and Trembley 1945). However, the northbound expansion of that distribution has been reported as far North as Maine (Teel et al. 2010). In Mississippi specifically, the northbound expansion in range has been observed since 1983, with occasional GCTs (six ticks reported in 1982) collected 200-250 miles inland from the Gulf Coast in the state (Goddard and Norment 1983). This study reports a relatively large collection of this tick in Northern Mississippi. Furthermore, the lack of genetic variability between the northern population (approximately 230 miles from the coast) and the coastal southern population supports establishment of *A. maculatum* in areas farther north than the historical range. However, genetic differences were significant within the individual sites of northern and southern Mississippi regions. The nine individual sites reflect small populations that will likely reach equilibrium between drift and

migration quickly. We estimated that approximately 10.57 migrants per generation are exchanged between these sites (Wright 1965). Migration may be the result of phoretic movement of ticks on wildlife they are commonly found infesting like deer, cotton rats and birds as well as some anthropogenic movement (i.e. cattle sales barns).

In a similar SSCP study of *A. maculatum*, Ketchum et al. described seven haplotypes (A through G) occurring in Texas (two haplotypes), Oklahoma (three haplotypes), and Kansas (four haplotypes in one site and three in another site) (Ketchum et al. 2009). These findings are comparable to the variability seen in this study in Mississippi (North and South Mississippi, both four haplotypes) and North Carolina (one region, three haplotypes). In the phylogenetic analysis by Ketchum et al., the haplotype occurring in Kansas and Oklahoma only (C) was basal to the other six described, including all Texan haplotypes (A and D) (Ketchum et al. 2009). Teel et al. (2010) stated that the lack of more ancient haplotypes in samples from Texas coupled with the higher genetic variability observed in Kansas (3 or 4 haplotypes) suggested that the Kansas population could have founded the U.S. coastal populations (Teel et al. 2010). In our study, phylogenetic analysis supports a monophyletic relationship among all haplotypes seen in Mississippi, North Carolina, Oklahoma, Kansas and Texas (Figure 4.2). Moreover, Mississippi and North Carolina haplotypes ABC diverged from their haplotypes ABDEFG. The Mississippi population included a more ancestral haplotype, D, which appears closely related to haplotype C from Oklahoma and Kansas; both our D haplotype and C identified by Ketchum et al. (2009) were

basal to all other haplotypes. Based on our results, we propose that alternatively to a movement from Kansas to the Gulf Coast, it is possible that independent introductions of ticks from source populations from Central or South America may have occurred to form these distinct populations in Kansas and in the coastal population. From a historical perspective, reports of GCTs occurring along the Gulf and Atlantic Coasts and 100 miles inland from there date back to the turn of the 20th century (Hooker and Bishopp 1912). Resolving this question would require a broad sampling of potential source populations and a larger U.S. sampling.

SSCP has contributed to disease surveillance and the design of control strategies for Lyme disease, cryptosporidiosis, and parasitic helminthes (Guttman et al. 1996, Anderson and Norris 2006, Jex et al. 2007, Simsek et al. 2011). Although evaluating *R. parkeri* genetic variation in the context of *A. maculatum* variation may lead us to test hypotheses regarding differences in *R. parkeri* pathogenicity or aspects of the tick-pathogen relationship, in our study, we found no genetic variable populations of *R. parkeri* in Mississippi. A previous study of *R. montanensis* in *Dermacentor* ticks using SSCP of the *rompA* gene also showed no variability and it was suggested that ticks in the area of study were infected with a single haplotype (Ammerman et al. 2004). In addition to limited *rompA* analyses, we evaluated genetic variability of ISRs, which are noncoding sequences under less selection pressure and thus should be more variable than other genes (Fournier et al. 2004). For *R. conorii* and *R. rickettsii,* a selection of multiple ISRs was most informative in evaluating genetic variability

(Fournier et al. 2004, Karpathy et al. 2007). We chose five of the most variable ISRs identified for *R. conorii* and *R. rickettsii*. No genetic variation was observed in *R. parkeri* within Mississippi *A. maculatum* or within a smaller population analyzed from North Carolina. We observed slight differences in gel migration in two of the intergenic spacer regions (RR1240-*tlc*5 and *rpmE*-tRNAfMet), however sequences from two other representatives were identical.

Additional investigations of *R. parkeri* genetic variability may benefit from a larger selection of ISRs and further optimization of SSCP conditions. Other factors to consider include the possibility of sampling infected *A. maculatum* from a larger geographical area and include *R. parkeri* from different sources, such as infected *A. americanum* ticks and vertebrate hosts. In addition, all Gulf Coast ticks in this study were adults. By including immature stages, additional heterogeneity may be revealed since *A. maculatum* immatures use a greater variety of hosts (including avian species) that may cover an even larger geographical range. Thus, while our findings contribute to the growing knowledge of this tick-rickettsia system, the study has also revealed additional factors and questions that could be pursued in future studies to better understand *A. maculatum* and *R. parkeri* populations.

Acknowledgments

This work is funded by a SECEBT (Southeastern Center for Emerging Biologic Threats) grant awarded to Dr. Varela-Stokes in 2009 and funding from the College of Veterinary Medicine at Mississippi State University.

This chapter is under revision for manuscript submission as “Ferrari, F.A.G., Goddard J., Caprio, M., Mixson-Hayden, T., Paddock C., Varela-Stokes A. 2012. Population Analysis of *Amblyomma maculatum* ticks and *Rickettsia parkeri* using Single Strand Conformation Polymorphism. Vector-Borne and Zoonotic Diseases”.

Table 4.1 SSCP haplotypes and nucleotidic variation determined by sequencing of *Amblyomma maculatum* mitochondrial 16S rDNA from Mississippi

Sample name, region and year collected	Gel pattern	Reverse primer flanking region (1-49bp)		Consensus (50bp-298bp)			
		41bp	45-49bp	53bp	108bp	124bp	139bp
Female1 South 08	A	A	GCTGT	T	T	A	A
Male4 South 08	A	A	GCTGT	T	T	A	A
Male3 South 08	A	A	GCTGT	T	T	A	A
Female1 North 09	A	A	GCTGT	T	T	A	A
Female2 South 09	A	A	GCTGT	T	T	A	A
Female1 South 08	B	A	ACAGC	T	T	A	A
Male3 North 09	B	A	ACAGC	T	T	A	A
Female12 North 09	B	A	ACAGC	T	T	A	A
Female21 North 09	B	A	ACAGC	T	T	A	A
Male3 South 08	C	A	ACAGC	T	-	A	A
Male15 South 09	C	A	ACAGC	T	-	A	A
Female1 North 08	*	A	GCAGC	T	-	A	A
Male8 South 08	*	A	GCAGC	T	-	A	A
Male1 South 09	*	A	GCTGC	T	-	A	A
Female8 South 09	*	A	GCTGC	T	-	A	A
Female1 South 08	D	T	ATAAT	A	T	T	G
Female12 Central 09	D	T	ATAAT	A	T	T	G
Female1 North 09	D	T	ATAAT	A	T	T	G
Male2 South 08	*	T	ATTAT	A	T	T	G
Male1 Central 08	*	T	ATTAT	A	T	T	G

* Reporting as unique sequences, but no gel pattern could be stipulated. Consensus of reverse and forward sequences consisted of a region between position 50bp-298bp. Position 1 to 49bp of the reverse primer flanking region was included in analysis for being very informative for samples that presented supporting high quality chromatogram.

Table 4.2. Haplotype frequencies determined by SSCP analysis of *Amblyomma maculatum* mitochondrial 16S rDNA by sites of collection in Mississippi

Sites per region of MS	Haplotypes				Number of haplotypes	Sample size
	A	B	C	D		
South 1	0.67	0.04	0.24	0.04	4	70
South 2	0.71	0.04	0.17	0.07	4	69
South 3	0.72	0.08	0.13	0.07	4	72
South 4	0.75	0.01	0.13	0.12	4	103
South 5	0.56	0.10	0.15	0.19	4	73
Central 1	0.66	0.05	0.03	0.26	4	38
North 1	0.59	0.00	0.30	0.11	3	27
North 2	0.75	0.09	0.06	0.09	4	77
North 3	0.67	0.02	0.09	0.23	4	66
North 4	0.83	0.12	0.02	0.03	4	59

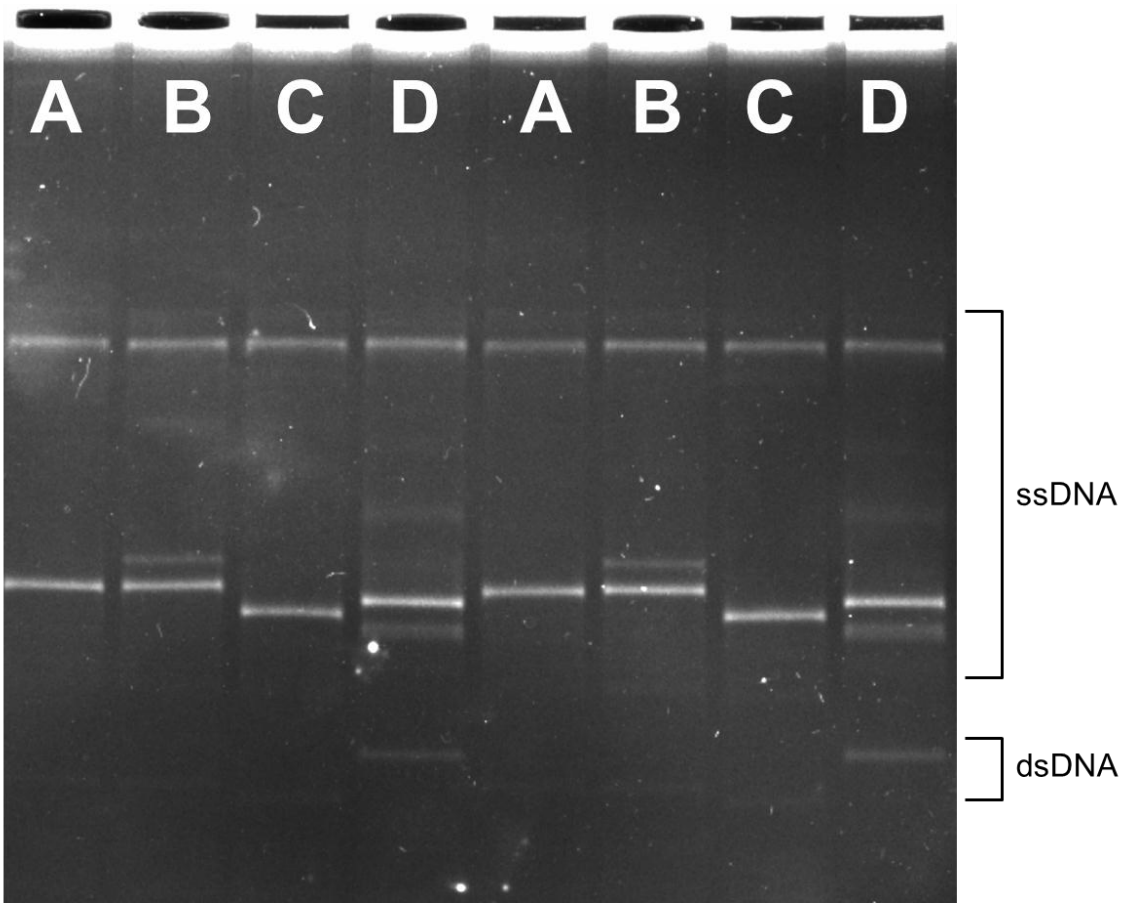


Figure 4.1 Gel patterns labeled A through D detected by PCR-SSCP of *Amblyomma maculatum* 16S mitochondrial rDNA gene fragment

NOTE: Single strand DNA, ssDNA and double strand DNA, dsDNA.

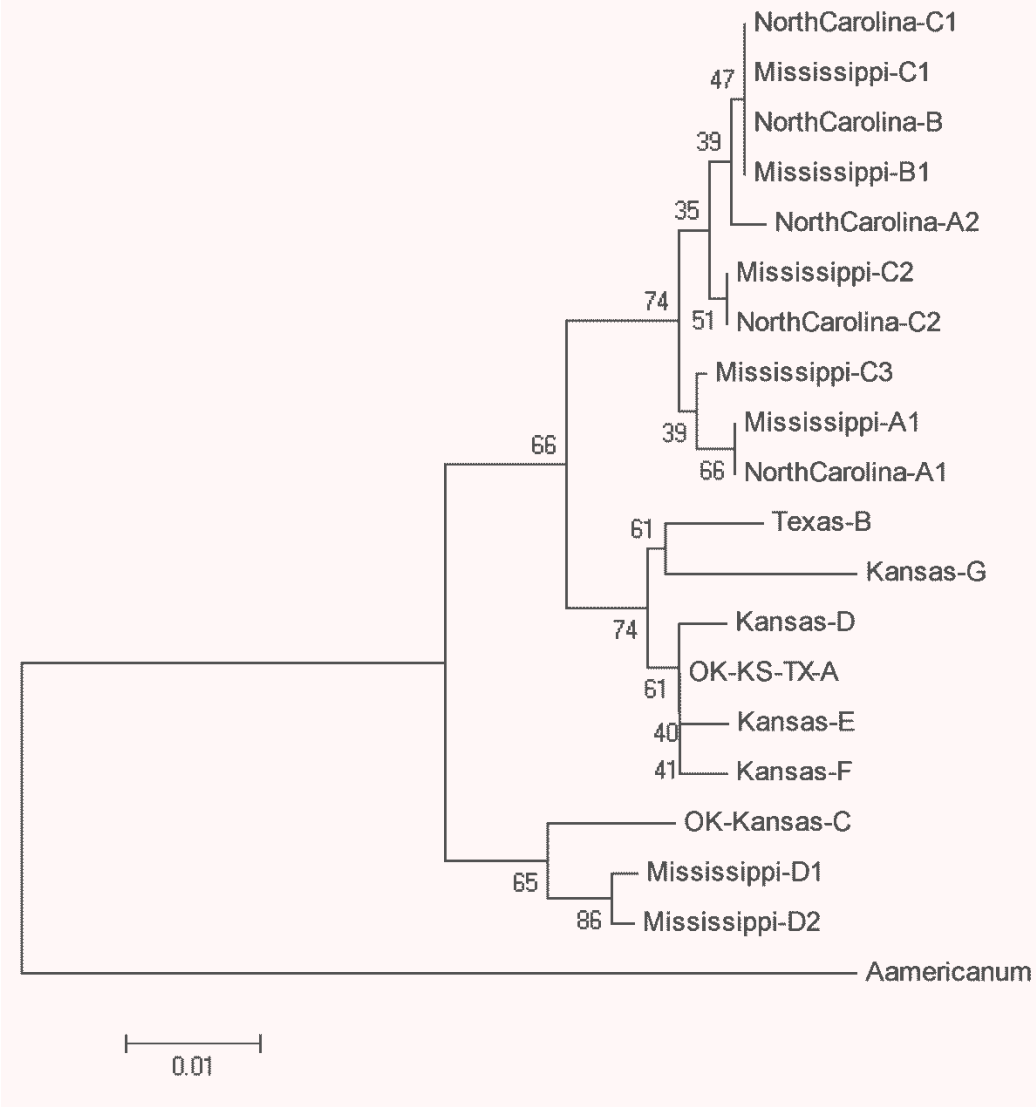


Figure 4.2 Tamura-Nei neighbor-joining tree of *Amblyomma maculatum* 16S mitochondrial rDNA gene fragment from Mississippi, North Carolina, Oklahoma, Kansas and Texas ticks

NOTE: Phylogenetic analysis included from this study four sequences of haplotypes A through D from Mississippi (Mississippi A-D) plus three sequences called Mississippi C2, C3 and D2 (with unique sequences but not different gel pattern), three sequences of haplotypes A-C from North Carolina and two unique sequences called “North Carolina A2 and C2”. Additionally, sequences of seven haplotypes observed in a similar study from Texas, Oklahoma and Kansas were included (Ketchum et al. 2009). *Amblyomma americanum* represents the outgroup. Numbers at internal nodes are bootstrap values based on a 1000 bootstrap replicates. The scale bar represents the number of nucleotide substitutions per site.

CHAPTER V

ULTRASTRUCTURE OF "*CANDIDATUS* RICKETTSIA ANDEANAE" WITHIN THE GULF COAST TICK, *AMBLYOMMA MACULATUM*

Abstract

Gulf Coast ticks, *Amblyomma maculatum,* occur in the central and southern United States, and are vectors of *Rickettsia parkeri*, a spotted fever group rickettsia (SFGR) causing mild human rickettsiosis similar to Rocky Mountain spotted fever. A novel SFGR, "*Candidatus* Rickettsia andeanae", was recently also detected in Gulf Coast ticks though it has not yet been well-studied and is not well-characterized at this time. Here we describe "*Ca.* R. andeanae" within the tissues of *A. maculatum* ticks using transmission electron microscopy. In ultrathin sections of unfed *A. maculatum* adult females, we found evidence of bacteria with morphological features consistent with SFGR, including small size (approximately 0.3 × 0.9 µm), a halo zone (electron-lucent layer around the bacterium), and a trilaminar cell wall. In female ticks, bacteria were present in granular salivary glands and ducts, foregut, Malpighian tubules, nerve trunks and reproductive tissue. These findings contribute to the current understanding of this recently identified rickettsia in *A. maculatum*.

Introduction

Gulf Coast ticks (GCT), *Amblyomma maculatum* Koch (1844), occur in the central and southern United States and may be infected with *Rickettsia parkeri* and "*Candidatus* Rickettsia andeanae", two spotted fever group rickettsiae (SFGR) (Teel et al. 2010, Jiang et al. 2011). "*Candidatus* Rickettsia andeanae" was first identified in *A. maculatum* and *Ixodes boliviensis* from the Peruvian Andes, hence its proposed species name (Blair et al. 2004). Since then it has been detected in approximately 2-5% of adult *A. maculatum* sampled in Mississippi, Florida, Georgia, Kansas, Oklahoma and Virginia (Blair et al. 2004, Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Jiang et al. 2011, Luce-Fedrow et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011). Unlike *R. parkeri,* which is a known human pathogen, infection in humans or other vertebrates by "*Ca.* R. andeanae" has not been described and the pathogenicity of this SFGR is currently unknown. In addition, *"Ca.* R. andeanae" has not yet been fully characterized. However, the recent report of "*Ca.* R. andeanae" isolation in three different cell lines and establishment of additional isolates in the future should help expand our understanding of *"Ca.* R. andeanae" and evaluate potential pathogenicity of this rickettsia in humans (Luce-Fedrow et al. 2011).

In a previous study, we identified DNA of "*Ca.* R. andeanae" in approximately 3% of Gulf Coast ticks collected throughout Mississippi, approximately half of which were co-infected with *R. parkeri* (Ferrari, F.A.G., unpublished data). In a study by Edwards et al. (2011), SFGR were detected in salivary glands, ovaries, Malpighian tubules and midgut from field-collected *A.*

maculatum as well as *A. maculatum* experimentally infected with *R. parkeri* using an immunofluorescence assay (Edwards et al. 2011). In order to further investigate *"Ca.* R. andeanae" in *A. maculatum,* we utilized transmission electron microscopy (TEM), an established technique used in evaluating the fine structure of SFGR and to aid in elucidating the relationships of SFGR with tick hosts (Silverman 1991). In the current study, we describe the fine structure and tissue tropism of "*Ca.* R. andeanae" by TEM in unfed adult *A. maculatum* to contribute to our understanding of this novel SFGR.

Materials and Methods

Selection of "*Ca.* R. andeanae"-infected *A. maculatum*

Fifty adult laboratory-reared *A. maculatum* ticks were purchased from Oklahoma State University. This source of ticks was chosen because we previously identified "*Ca.* R. andeanae" in ticks from this colony by PCR assay (Moraru, G. and Varela-Stokes, A., unpublished data). Adult ticks were cleaned to remove any external contaminants using 70% ethanol, and then dried and their legs removed for DNA extraction. Genomic DNA was extracted from tick legs using the Illustra™ Tissue & Cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ). A fragment of the tick 16S mitochondrial rRNA gene was amplified by PCR to confirm successful DNA extraction (Black and Piesman 1994). The DNA extracts were screened for the presence of SFGR using a nested PCR assay to amplify the rickettsial outer membrane protein A (*rompA*) gene. Ticks positive for SFGR DNA by this PCR assay were further

tested using separate species-specific primers targeting regions of the *rompA* gene specific to *R. parkeri* and "*Ca.* R. andeanae" (Paddock et al. 2010, Varela-Stokes et al. 2011). Amplicons of "*Ca.* R. andeanae" *rompA* gene from the four ticks analyzed by TEM were submitted for sequencing as well. PCR products were purified using Montage PCR Centrifugal Filter Device (Millipore, Bedford, MA, USA) and sequenced by Eurofins MWG Operon (Huntsville, AL, USA). Consensus sequences were generated by ClustalX2 alignment and identified using GenBank BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All PCR assays included a positive control of DNA extracted from cultured *R. parkeri* (Tate's Hell strain) or "*Ca.* R. andeanae"-infected Gulf coast ticks respectively, confirmed previously by PCR and sequencing. To verify other bacteria potentially found in ticks, PCR assays to amplify the following gene fragments were attempted using the respective primers: the *ftsZ* homolog of *Wolbachia* spp. (alpha Proteobacteria) found in arthropods (ftsZ reverse and forward primers), elongation factor G, *fusA,* of *Coxiella* spp. (fusA reverse and forward primers) and 16S rRNA of *Francisella* spp. (Fr153FO.1/Fr1281RO.1 primers) (Noda et al. 1997, Barns et al. 2005, Jasinskas et al. 2007). For each assay, PCR reactions included a positive control. For *Wolbachia* spp., we used DNA extracted from *Aedes albopictus* mosquito known to be infected with *Wolbachia* by previous PCR. Field caught *Amblyomma americanum* previously determined by PCR to be infected with *Coxiella* were used. DNA from cultured *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS) was used as positive control for *Francisella*. All PCR assays also included negative (water) controls. We

electrophoresed all PCR products on 2% agarose gels stained with ethidium bromide, and visualized gels under ultraviolet light.

Transmission Electron Microscopy (TEM)

Two female and two male *A. maculatum* ticks that tested positive by PCR for "*Ca.* R. andeanae" and negative for *R. parkeri* were prepared for TEM. Tick bodies were fixed in Karnovsky's fixative (4% glutaraldehyde; 4% paraformaldehyhde) containing 1% DMSO made up in 0.1 M phosphate buffer at pH 7.2. Ticks stayed in fixative for 1 hour at room temperature and were then rinsed several times with 0.1 M buffer and stored in the refrigerator. Then, they were post-fixed in 2% osmium tetroxide (also in 0.1M phosphate buffer) and rinsed with water. After an overnight *en bloc* stain with 2% aqueous uranyl acetate, samples were further dehydrated in a graded ethanol series (35%, 50%, 70%, 95%, and 100%). Using acetone as a transitional fluid, specimens were embedded in Spurr's resin (Electron Microscopy Sciences, Hatfield, PA) and cured at 70°C overnight.

Semi-thin (0.5 µm) and ultra-thin (60-90 nm) sections were cut with a Reichert-Jung Ultracut E ultramicrotome. Semi-thin sections, stained with 1% toluidine blue, were used to locate areas of interest for TEM observations. Ultra-thin sections were stained with alcoholic uranyl acetate and lead citrate and observed on a JEOL JEM-100 CXII (JEOL USA, Peabody, MA) at 80 kV. One male and one female *A. maculatum* ticks from Oklahoma State University tick colony that tested negative by PCR to all bacterial assays described above were

used as negative controls for TEM studies. Negative control tick bodies were processed in an identical manner as the *"Ca.* R. andeanae"-infected tick bodies.

Salivary glands, Malpighian tubules, ovaries and midgut tissues of Gulf Coast ticks were reported previously as heavily infected with SFGR using an immunofluorescence antibody assay (Edwards et al. 2011). In addition, previous work with Hayes and Burgdorfer (1979) also focused primarily on these specific tissues except digestive tissues (Hayes and Burgdorfer 1979). Thus, in the current study, salivary glands, Malpighian tubules, and digestive and reproductive organs, were most thoroughly examined under TEM in both infected and control ticks.

Results

Bacteria morphologically consistent with *Rickettsia* species were found in the two male and two female Gulf Coast ticks that tested positive for *"Ca.* R. andeanae" and negative for *R. parkeri* when evaluated by molecular assays specific for these SFGR. Sequencing of the four ticks analyzed by TEM using the "*Ca.* R. andeanae" *rompA* gene resulted in 100% identity to "*Ca.* R. andeanae" *rompA* fragment. Neither of the female ticks had molecular evidence of infection with a *Wolbachia*, *Coxiella* or *Francisella* species. Both male ticks were PCR-negative for *Wolbachia*. Both males had a faint PCR product evident by gel electrophoresis for the *Coxiella* spp. PCR and one male had a PCR product for *Francisella* spp. PCR assay. However, repeated PCR assays revealed inconsistent results and insufficient DNA was available for sequencing the

sample positive for *Francisella* species. A PCR product amplified from one male tick was identical in sequence to *Coxiella* species. Although *Francisella* spp. are wolbachiae-like, large bacteria approximately 1.2 µm in diameter (Niebylski et al. 1997a), *Coxiella* sp., are morphologically identical to rickettsiae. Thus, even if present in low numbers in the male ticks as suggested by the inconsistent weak PCR amplicons (Dumler and Walker 2005), we excluded male ticks from further analysis in this study due to the possible presence of these organisms.

In female specimens, small, rod-like bacteria, approximately 0.27-0.35 µm × 0.7-0.985 µm in size were often surrounded by a halo zone, the electron-lucent layer around the organism correspondent to the bacterial capsule. A trilaminar cell wall was also observed, which is consistent with the cytoplasmic membrane and the inner and outer membranes present in the cell wall of SFGR (Figure 5.1a). Bacteria at different physiological stages were observed. There is evidence suggestive of multiplication by binary fission (Figure 5.1b) and of older cells, represented by smaller and more electron dense bacteria (Dumler and Walker 2005). Figure 5.1c shows bacteria in the reproductive organs of a female tick where some bacteria show extensions of the cell membrane. We found no evidence of bacteria in tick bodies from negative control specimens, which were examined to the same extent as "*Ca.* R. andeanae"-infected tick bodies. However, examiners were not blinded to which samples came from ticks with PCR positive and negative for "*Ca.* R. andeanae" *rompA*.

Female ticks contained evidence of bacteria in various tissues including granular salivary glands and ducts, the muscle layer of the foregut (Figure 5.2b),

connective tissue near trachea, Malpighian tubules (Figure 5.2a), nerve trunks and in epithelial cells of ovaries and oocytes where they were found both intra- and extracellularly (Figure 5.2c). Clusters of at least 10 bacteria were seen in the reproductive organs, with the largest group comprising approximately 100 bacteria (Figure 5.2d). No bacteria were observed in the female midgut.

Discussion

Results described in this study provide an ultrastructural characterization of bacteria within tissues of female *A. maculatum,* positive by PCR for *"Ca.* R. andeanae". The bacteria reported here are most likely "*Ca.* R. andeanae", given that PCR assays for *"Ca.* R. andeanae" produced strong bands confirmed by sequencing, while PCR assays for *Rickettsia parkeri*, *Wolbachia spp.*, *Coxiella spp.* and *Francisella spp.* were negative. To our knowledge, this is the first ultrastructural description of "*Ca.* R. andeanae" in tissues of the primary tick host, *A. maculatum.*

The organisms suspected to be "*Ca.* R. andeanae" in this study shared morphological similarities with other SFGR. SFGR in the family Rickettsiaceae are described as rod-shaped, small bacteria (0.3-0.5 × 0.8-2 μm), that have trilaminar cell walls, lack flagella and obligately intracellular, mostly intracytoplasmic and sometimes found intranuclear (Fredricks 2006). The characteristic trilaminar cell wall seen in the bacteria in this study were consistent with that of rickettsiae, consisting of an inner cytoplasmic membrane, and thick

inner and thin outer leaflets, surrounded by a thick electron lucent layer, also called a slime layer or halo zone (Hayes and Burgdorfer 1979, Silverman 1991).

Within the female body, bacteria were distributed unevenly with large numbers of bacteria in reproductive tissue, moderate numbers in salivary glands and ducts, and few bacteria in the foregut, Malpighian tubules and nerve trunks. The distribution and numbers of bacteria seen may have been different if a greater number of ticks and ticks at different physiological stages (e.g. feeding versus unfed, immatures versus adults) were examined, as previously described in *R. tsutsugamushi* in *Leptotrombidium pallidum* mites (Urakami et al. 1994).

To date, no animal or humans have been found infected with "*Ca.* R. andeanae"; identification of this rickettsia has been limited to tick specimens and infectivity to vertebrates is unknown. *Rickettsia peacockii*, a known endosymbiont of *Dermacentor andersoni* ticks, is found only in female ticks, primarily distributed in ovarian tissues, and is not found in midgut, Malpighian tubules and salivary glands (Niebylski et al. 1997b). By comparison, the causative agent of Rocky Mountain spotted fever, *R. rickettsii*, infects all tissues of the tick host (Burgdorfer and Brinton 1975). Cytopathic effects observed with pathogenic *R. rickettsii* may include heterochromatin aggregation at nuclear membrane, loss of cytosol, ribosomes, mitochondria and endoplasmic reticulum, as well as alterations to the integrity of nuclear and cellular membranes (Hayes and Burgdorfer 1982). We did not identify any of these cellular alterations in the ticks infected with "*Ca.* R. andeanae".

Until recently, *"Ca.* R. andeanae" could not be isolated in cell culture, a characteristic similar to the fastidious nature of tick endosymbionts like *R. peacockii* (Simser et al. 2001, Paddock et al. 2010). The isolation of *"Ca.* R. andeanae" (Luce-Fedrow et al. 2011) coupled with this ultrastructural description of the novel SFGR in tissues of *A. maculatum* will assist in targeted studies of this organism in ticks and vertebrate hosts, and particularly in efforts to evaluate the pathogenicity of this rickettsia in vertebrates.

Acknowledgements

We thank Gerald Baker, Amanda Lawrence and William Monroe (Institute for Imaging and Analytical Technologies, Mississippi State University) for the analytical and technical assistance with transmission electron microscopy; Jason Rasgon (Johns Hopkins University) and David Cross (Mississippi State University) for providing wolbachiae samples and Whitney Smith for her kind assistance in the lab.

This work was supported by intramural funding through Mississippi State University, College of Veterinary Medicine.

This chapter is under revision for manuscript submission as "Ferrari, F.A.G., Goddard J., Paddock C., Varela-Stokes A. 2012. Ultrastructure of "*Candidatus* Rickettsia andeanae" within the Gulf Coast Tick, *Amblyomma maculatum*. Applied and Environmental Microbiology".

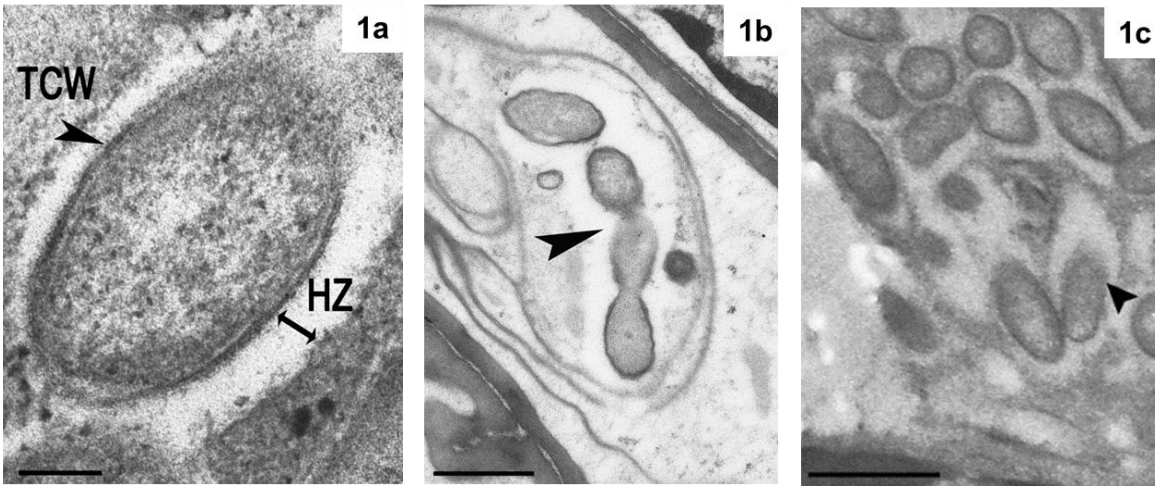


Figure 5.1 Transmission electron micrographs depicting fine structure of rickettsia-like organisms in female *Amblyomma maculatum*

NOTE: 1a consists of single bacterium, approximately 250nm (cross section), presenting a trilaminar cell wall (TCW) and halo zone (HZ); scale bar = 100nm. 1b: Four bacteria in the cytoplasm of the female reproductive organ. Dividing bacteria (arrowhead); scale bar = 1µm.1c: Over 15 bacteria in the cytoplasm of the female reproductive organ. An example of bacterium with extended cell membrane depicted at the arrowhead; scale bar = 1µm.

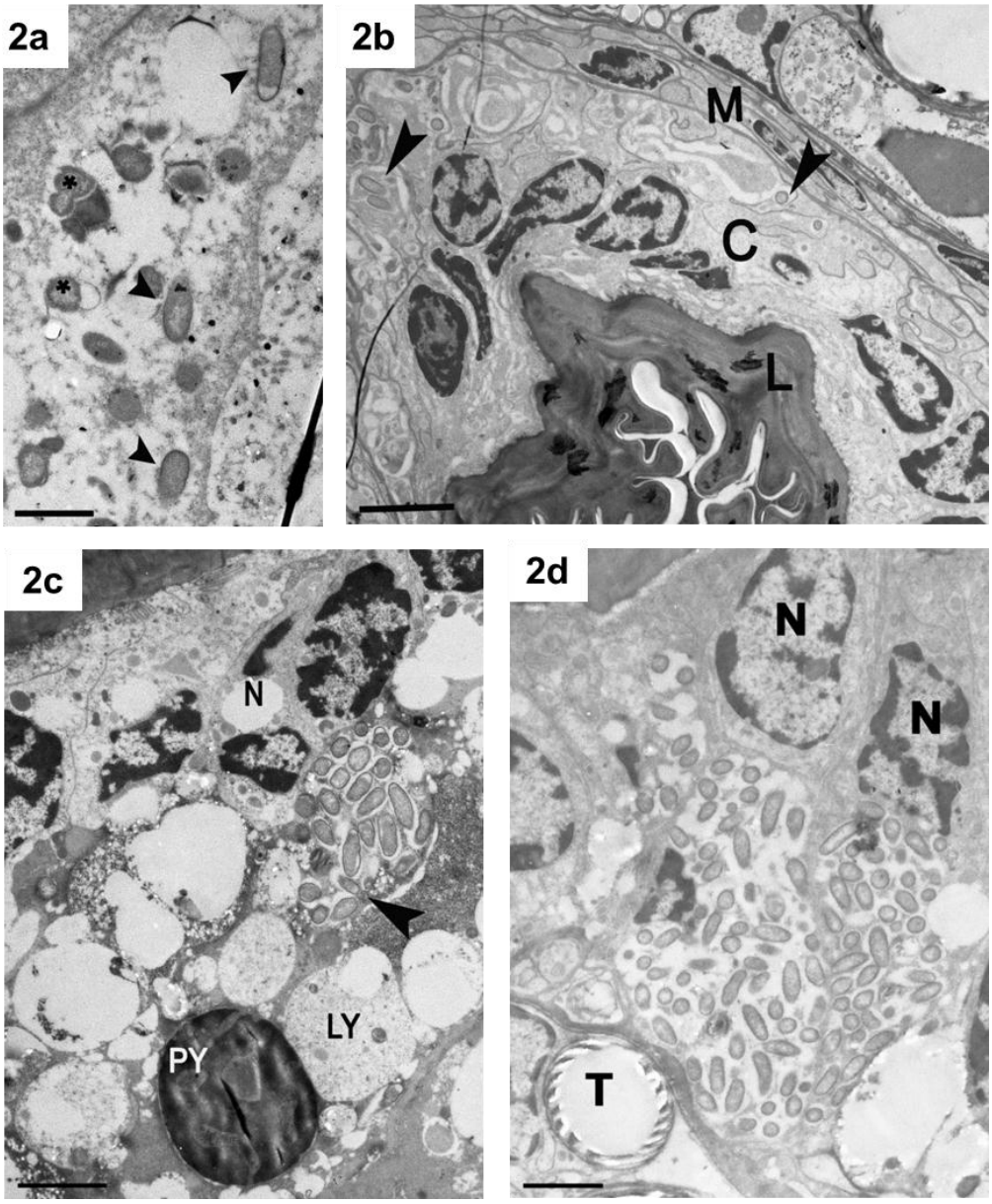


Figure 5.2 Transmission electron micrographs of rickettsia-like organisms in female *Amblyomma maculatum* tissues

NOTE: 2a: Bacteria (arrowheads) in the Malpighian tubule wall among excretory granules (asterisks); scale bar = 1μm. 2b: Scattered occurrence of at least 10 bacteria (arrowheads) between the foregut connective tissue (C) and its muscle layer (M), none at the foregut mucosa lining (L); scale bar = 5μm. 2c: Cluster of over 20 bacteria (arrowhead), both in cross section and as rods in longitudinal section, in reproductive system intracellularly near epithelial cell nuclei (N), among protein yolk (PY) and lipid yolk (LY); scale bar = 2μm. 2d: Over 100 bacteria intracellularly in the female reproductive system; nuclei (N) and trachea (T); scale bar = 2μm.

CHAPTER VI

ESTABLISHMENT OF GULF COAST TICK CELL LINE AND ISOLATION OF "*CANDIDATUS* RICKETTSIA ANDEANAE"

Abstract

Gulf Coast ticks, *Amblyomma maculatum* are increasing in public health awareness as the vectors of a recently recognized human pathogen, *Rickettsia parkeri.* More recently, these ticks were also found to harbor a novel spotted fever group rickettsia in Peru first, then the United States, Chile and Argentina. To date, little is known about "*Candidatus* Rickettsia andeanae" and its pathogenicity to vertebrates has not yet been explored, partly due to the lack of an isolate. Although the isolation of *"Ca.* R. andeanae" was recently described in several cell lines, its stability in these cells was not clear. A stable isolate is essential for studies testing the infectivity of this novel rickettsia in vertebrates. In addition, it allows for a better understanding of the basic biology of *"Ca.* R. andeanae", a more complete genetic characterization that paves the way for formal designation of species status. Here we report the establishment of a new cell line from *A. maculatum* ticks that were naturally infected with "*Ca*. R. andeanae" and describe the propagation of *"Ca.* R. andeanae" in this cell line

and subsequent passage to ISE6 and Vero cells, in which propagation was limited.

Introduction

Rickettsiae are Gram-negative, obligate intracellular bacteria, of which some can be pathogenic to animals. Most bacteria in the spotted fever group of rickettsiae (SFGR) are maintained in nature within ticks, which function as hosts for the bacteria as well as vectors for transmission of rickettsiae to vertebrates during a tick blood meal (Dumler and Walker 2005). In vitro cultivation of SFGR is possible using a variety of cell lines, including Vero, L-929, HEL and MRC5 cells (Dumler and Walker 2005). However, several tick embryonic cell lines have been established to aid in isolating fastidious bacteria, including *Rickettsia* spp., that fail to grow in mammalian cell lines like those above mentioned (Bell-Sakyi et al. 2000). Tick cell lines have also been valuable for proteomics and genomics studies as well as the production of vaccines (Bell-Sakyi et al. 2007, Richards 2011). Finally, the ability to establish tick cell lines offers an approach to isolating, characterizing, and investigating host-pathogen relationships for novel rickettsiae in their natural tick host (Bell-Sakyi et al. 2007). This approach has been successful in the propagation of *R. peacockii,* a fastidious endosymbiont that was eventually isolated in cells from its natural tick host, *Dermacentor andersoni* (Niebylski et al. 1997b, Simser et al. 2001).

In 2004, a novel SFGR was identified in *A. maculatum* and *Ixodes boliviensis* ticks in the Peruvian Andes (Blair et al. 2004). "*Candidatus* Rickettsia

andeanae" has now been reported from *A. maculatum* (Gulf Coast tick) collected in Mississippi, Florida, Georgia, Kansas, Oklahoma and Virginia (Blair et al. 2004, Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Jiang et al. 2011, Luce-Fedrow et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011). Current knowledge of "*Ca.* R. andeanae" is mainly limited to surveys detecting the bacterium in ticks by PCR assays. In addition to harboring *"Ca.* R. andeanae", *A. maculatum* is the known vector of *R. parkeri*, a SFGR first isolated in 1939 and considered non-pathogenic until the first human infection was diagnosed in 2002 (Parker et al. 1939, Paddock et al. 2004). Although no human (or other vertebrate) infections of *"Ca.* R. andeanae" have been reported to date, thorough studies, such as experimental animal infections, require a stable isolate to evaluate infectivity and pathogenicity of this rickettsia. Recently, "*Ca.* R. andeanae" was propagated in three different cell lines including two mammalian and one insect cell lines (Luce-Fedrow et al. 2011). However, since none of those cultures used cells from sources that may be found naturally infected with *"Ca.* R. andeanae", their usefulness for characterizing *"Ca.* R. andeanae" further is unknown, and may not reflect the natural system. Tick-borne bacteria may differentially express essential proteins in different environments. For example, *Ehrlichia chaffeensis* propagated in tick cells expressed a different set of proteins than when grown in macrophages; similarly, transcription levels for the *spoT3* gene differed for *R. conorii* grown in mammalian compared to insect cells (Rovery et al. 2005, Singu et al. 2006). Additionally, previous attempts to grow "*Ca.* R. andeanae" in Vero cells, in an

arthropod cell line (C6/36), and in *Ixodes scapularis* tick cell line (ISE6), were unsuccessful (Blair et al. 2004, Sumner et al. 2007, Paddock et al. 2010). Thus, the viability of this novel rickettsia may not be optimal in traditional cell lines.

An embryonic cell line developed from the natural tick host species, *A. maculatum,* may provide a more suitable cell system to isolate "*Ca.* R. andeanae". A stable isolate from the natural tick host will aid in future investigations of *"Ca.* R. andeanae" that enable us to determine its pathogenicity and to further characterize the rickettsia using genomic and proteomic approaches. Last, studies of other organisms, specifically *R. parkeri*, a known pathogenic rickettsia found in *A. maculatum,* should benefit from the establishment of the new cell line from *A. maculatum* reported here.

Materials and Methods

Source of Tick Embryos

To establish Gulf Coast tick cell lines, both naturally infected with "*Ca.* R. andeanae" and not infected with this rickettsia, we used embryonic cells from engorged female *A. maculatum.* Eight fully engorged, gravid *A. maculatum* females were purchased from laboratory-reared colonies at Oklahoma State University (OSU; n=4) and Texas A&M University (TAMU; n=4). Tick sources were chosen because we previously identified *A. maculatum* from OSU colonies to be infected and those from TAMU colonies to be uninfected with "*Ca.* R. andeanae" by PCR assay (G.M. M. and A.S.V-S. unpublished data). Upon arrival, females were allowed to oviposit in sterile multi-well plates in a humidity

chamber with humidity maintained at approximately 92% using a saturated potassium nitrate solution. Portions of each egg mass were collected to be tested by PCR.

Establishment of *A. maculatum* Cell Line and Isolation of "*Ca.* R. andeanae"

After tick embryos reached about 75% of their development time, approximately 100mg of eggs from individual females were removed for establishment of cultures. We surface disinfected egg samples using 3 minute washes on a rocker as follows: 0.5% household bleach containing a drop of Tween 80, 70% ethanol, twice in sterile phosphate buffer saline (PBS; pH 7.4) and last wash in modified Leibovitz's medium (L-15B300), (Munderloh and Kurtti 1989, Munderloh et al. 1999). We included an additional disinfection with 0.1% benzalkonium chloride for the egg masses produced by TAMU ticks, which were processed 15 days after OSU egg masses were processed. After egg samples were disinfected, we added 5ml of L-15B300 medium supplemented with heat inactivated 20% fetal bovine serum and 10% tryptose phosphate broth, and the addition of antibiotics (100U/ml of penicillin and 100mg/ml of streptomycin sulfate, Sigma-Aldrich, St. Louis, MO) and antifungals (10mg/mL of amphotericin, Sigma-Aldrich, St. Louis, MO). Eggs were crushed in this medium using a sterile glass rod and then centrifuged at 100x g for 1 minute to separate embryos from egg shells. Using approximately 5 mL of supernatant, we seeded a 12.5cm^2 flask, added 10 mL of medium to the pellet and seeded two other flasks. Flasks

were initially incubated at 33°C for tick cell growth and were moved to 28.5°C to favor rickettsial growth after three months when tick cell monolayers have become more established (U. G. Munderloh, personal communication). Antibiotics initially added to establish cultures were discontinued after the third week. Cell media was replaced weekly for uninfected cells established from TAMU ticks or bi-weekly in infected cells established from OSU ticks; passages were made every other week or as needed. When confluent, 10-50% of the cells were transferred to other flasks by scraping cells off the flask and pipeting to new media for a final volume of 5ml. Alternatively, cells were frozen in L15B300 medium, 10% DMSO and 20% fetal bovine serum, placed in cryovials, and stored at -80°C in a Nalgene Mr. Frosty freezing container (Sigma-Aldrich, St. Louis, MO) overnight, then stored in ultra-low freezer. Cultures were observed weekly for confluency using an inverted microscope. We evaluated cultures for rickettsia infection by cytospins of spent media made approximately every week and stained with acridine orange or Diff-Quik (Dade Behring, Newark, DE). Culture samples were also tested approximately bi-monthly by PCR and QPCR assays and once by immunofluorescence assay following a protocol using human anti-*R. rickettsii* antibodies described by Edwards et al. (Edwards et al. 2011) for evidence of rickettsia.

Passage of *"Ca.* R. andeanae" to ISE6 and Vero Cell Lines

ISE6 (*Ixodes scapularis* embryonic) cells (provided by U. G. Munderloh, University of Minnesota) were maintained at 33°C (during the first month, then

28.5°C thereafter) in L15B300 medium supplemented with heat inactivated 20% fetal bovine serum and 10% tryptose phosphate broth. Vero cells were maintained at 37°C, 5% CO_2 in MEM plus 10% fetal bovine serum. Vero and ISE6 cells were challenged using approximately 0.2 mL of media and cells removed by pipetting or scraping from primary cultures and centrifuged to concentrate inoculum. When challenged, both ISE6 and Vero received amphotericin during the first month.

Molecular Analyses

For genomic DNA extraction from egg masses and cultures at various time points, we used an Illustra™ Tissue & Cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA). Samples of approximately 50 mg of eggs were minced using a sterile pestle per sample in proteinase K and lysis buffer from the kit following the manufacturer's instructions for tissues. For the culture time points, at least 10 mL of spent media per cell lines were collected in 15 ml centrifuge tube then centrifuged at 2500 rpm for five minutes. After the supernatant was discarded, we followed kit manufacturer protocol for remaining cells. For egg masses, we first screened for evidence of any SFGR DNA using a nested PCR protocol with SFGR-wide primers targeting the rickettsial *ompA* (*rompA*) gene (Paddock et al. 2004). Positive samples were subsequently tested by PCR assay using species specific *rompA* primers for "*Ca.* R. andeanae" and *R. parkeri* (Paddock et al. 2010, Varela-Stokes et al. 2011). For culture samples taken at specific time points, we used only the species-specific *rompA* assays. All

PCR assays included a positive control of DNA extracted from cultured *R. parkeri* (Tate's Hell strain) or "*Ca.* R. andeanae"-infected Gulf coast ticks, confirmed previously by PCR and sequencing. Water was used as a negative control for all assays. All PCR products were stained with ethidium bromide and electrophoresed in a 2% agarose gel. PCR products selected for sequencing were purified using Montage PCR Centrifugal Filter Device (Millipore, Bedford, MA, USA) and sequenced by Eurofins MWG Operon (Huntsville, AL, USA). Consensus sequences were generated by ClustalX2 alignment and identified using GenBank BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative Real-Time PCR

We evaluated relative growth of *"Ca.* R. andeanae" over time from samples of spent media collected from naturally infected established *A. maculatum* cell cultures, tetracycline-treated *A. maculatum* cell cultures, ISE6 cell cultures and Vero cell cultures. Primers Rx-190-F and Rx-190-R, previously designed for detection of *"Ca.* R. andeanae" by QPCR (Paddock et al., 2010) were used at 300nM concentrations in a similar Brilliant II SYBR Green (Agilent Technologies, Santa Clara, CA) assay. A template volume of 2 µL was used in a final reaction volume of 20 µL. In order to take into account differences in rickettsial levels due to variability in collection of samples, number of host cells present, and other factors, we measured relative rickettsial levels as a ratio of rickettsial PCR product ("CaRa") to host gene ("HG"). For HGs, we selected primers 16S+2 and 16S-1 to amplify an approximately 298-bp portion of the tick

16S mitochondrial rRNA gene (Black and Piesman 1994) in *A. maculatum* and ISE6 cell cultures. For Vero cell culture samples, we used primers 17F and 106R (Tennant et al. 2011) to amplify a 90-bp portion of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Ten-fold serial dilutions using DNA template from known positive samples (*A. maculatum* cell culture, ISE6 cell culture, an adult *A. maculatum* from Mississippi that was positive for *"Ca.* R. andeanae" by PCR and sequencing) were included in all reactions to generate standard curves. For each cell culture type, *A. maculatum* cells, ISE6 cells and Vero cells, duplicate samples were included for host cell and "*Ca.* R. andeanae" standards as well as samples from culture flasks. The standard curve was used to evaluate reaction efficiency and calculate relative values for the amount of rickettsial and host genes present in order to calculate CaRa/HG ratios. Thermal cycler parameters were modified from Paddock et al. (Paddock et al. 2010) after empirically determining that an annealing temperature of 54ºC was suitable to efficiently amplify rickettsial DNA at the same time as host cell DNA. Thus, amplification of *"Ca.* R. andeanae" occurred concurrently with amplification of the specific host cell target, whether this was targeting 16S mitochondrial rRNA or GAPDH genes. A standard dissociation curve was included in the thermal cycle program and all assays were performed using a Stratagene MX3005P QPCR system (Agilent Technologies, Santa Clara, CA).

Results

For establishment of the *A. maculatum* embryonic cell line, we allowed female ticks to oviposit in humidity chambers for 25 days to a month after onset of oviposition. At this point most of the egg mass contained embryos, not fully formed larvae, as observed by daily inspections under a dissection microscope. We sought to establish an *A. maculatum* cell line naturally infected and uninfected with "*Ca.* R. andeanae" from ticks purchased from the OSU and TAMU tick colonies respectively. The primary culture from the OSU source grew slowly and took two months to form a cell monolayer. After two months, we successfully passaged these cells to another flask. At that time, we were also successful in freezing and growing cells back from frozen vials. Cells from the TAMU source processed using a protocol similar to OSU tick cells did not form a confluent monolayer during three months and since they did not thrive, TAMU cells were discarded. In order to establish an uninfected cell line, two flasks of OSU tick cells were treated with tetracycline (initially 10 mg/ml and then increased to 40mg/mL, Sigma-Aldrich, St. Louis, MO) for 40 days to eliminate rickettsial infection. Two weeks into treatment, we tested for rickettsial infection by stained cytospins and PCR and QPCR assays of treated flasks once a week for a month with no evidence of "*Ca.* R. andeanae".

Regarding the isolation of "*Ca.* R. andeanae" from naturally infected GCT cells purchased from OSU, no bacteria were observed in the first biweekly cytospins that were started after two weeks of onset of experiment. We used Diff-Quik, acridine orange staining, and indirect immunofluorescence assay of

cytospins to demonstrate growth of "*Ca*. R. andeanae". We first identified bacteria five weeks after the establishment of cultures when weakly stained bacteria were observed in cytospins. After two months and a clear observation of rickettsia-like bacteria in cytospins from the *A. maculatum* cells, we challenged ISE6 and Vero cell cultures. Staining using either acridine orange (Figure 6.2) or Diff-Quik (Figure 6.3) were performed in weekly cytospins for almost three months, alternating the staining method each week for the three cell lines. Rickettsia-like bacteria were seen most commonly intracellularly as well as extracellularly, no bacterium could be identified intranuclearly. Bacteria were not consistently observed in all cytospins of cell culture flasks, and were particularly difficult to find in cytospins from ISE6 and Vero cells. Even when observed in cytospins, rickettsiae were not present in high numbers (as compared to *R. parkeri*). Immunofluorescence was performed once for OSU cells and showed characteristic fluorescence for infected cells when compared with positive control (*R. parkeri*) and just background fluorescence for negative control cells (Figure 6.1).

We challenged ISE6 and Vero cells with 0.2 ml of spent media in addition to a sample of cells that removed by pipeting or scraping from the source *"Ca.* R. andeanae"-infected *A. maculatum* flask. After the initial challenge of ISE6 and Vero, we tested all three cell lines using cytospins coupled with PCRs and QPCRs. Challenges were repeated approximately once a month for three months when cell lines were negative by PCR. PCR and QPCR assays were performed for 11 and eight time points, respectively, spread over a three month

period (Table 6.1). At least two flasks of ISE6 and Vero cells each were kept unchallenged to use as negative controls for cytospins, PCRs and QPCRs, which remained negative in the cytological and molecular analysis, tested at 11 and eight time points respectively.

We used QPCR to compare the *"Ca.* R. andeanae" growth from samples of spent media collected at eight time points from naturally infected established *A. maculatum* cell cultures, tetracycline-treated *A. maculatum* cell cultures, ISE6 cell cultures and Vero cell cultures. From Figure 6.4 (a), the naturally infected *A. maculatum* cell culture was consistently positive throughout the time points. Shortly after onset of treatment, antibiotic treated *A. maculatum* cells were positive for rickettsial DNA but became negative in later time points. Inoculated ISE6 (Figure 6.4b) and Vero cells (Figure 6.4c) differed from *A. maculatum* cells. QPCR results fluctuated throughout the three months of experiment and amplification of *"Ca.* R. andeanae" generally coincided with a time point occurring directly after challenge. Results of QPCR and PCR assays were comparable.

Tick DNA from two culture time points (March 20th and 27th) was amplified by PCR using primers for a fragment of the 16 rRNA gene as described by (Black and Piesman 1994) and subsequently sequenced. A BLAST search confirmed the 100% identity with 100% coverage of the sample sequenced with *A. maculatum* 16S rRNA sequences deposited in the NCBI Genbank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), further characterizing the establishment of a new cell line. Using "*Ca.* R. andeanae" *rompA* gene primers, we amplified DNA from a time point (February, 28th) of the rickettsiae infecting *A. maculatum*,

ISE6 and Vero cells and submitted it for sequencing as well. The rickettsial DNA present in the three cell lines was confirmed to be 100% identical to "*Ca.* R. andeanae" *rompA* fragment compared by a BLAST search of the GenBank.

Discussion

In this study, we successfully established a new tick cell line from *A. maculatum* embryonic cells and concurrently propagated "*Ca.* R. andeanae" in those cells. *"Candidatus* Rickettsia andeanae" demonstrated better growth within naturally infected source cells as opposed to growth of passages made to ISE6 and Vero cells. Given the source of cell line and evidence of *"Ca.* R. andeanae" that was detected in naturally infected GCT cells, as opposed to the Vero and ISE6 cells that required additional challenges based on PCR and QPCR results, our data suggest that this rickettsia is more stable in the new *A. maculatum* cell line.

Previous attempts to isolate "*Ca.* R. andeanae" in Vero E6, ISE6 and C6/36 (mosquito) cells, using *A. maculatum* naturally infected with *"Ca.* R. andeanae", were unsuccessful (Paddock et al. 2010). As with *Rickettsia peacockii,* after several failed attempts to isolate it, the first successful isolate was in *Dermacentor andersoni* tick cells (Simser et al. 2001). Recently, "*Ca.* R. andeanae" was propagated in mammalian cells (Vero and DH82) and S2 cells from *Drosophila melanogaster* (Luce-Fedrow et al. 2011). However, a stable rickettsial isolate propagated by cells from a host in which they are naturally found may provide a more realistic model for future studies. Rickettsial proteins

may be differentially expressed depending on the environment in which they are grown, particularly when grown in mammalian compared to arthropod cell lines (Rovery et al. 2005, Singu et al. 2006). In this study, "*Ca*. R. andeanae" was isolated and maintained in cells from GCTs where they were consistently detected by PCR and QPCR assays and more routinely detected by cytospins. In comparison ISE6 and Vero cells were often negative by assays shortly after challenge and were repeatedly challenged to demonstrate rickettsiae.

On the January 27th time point, a peak in the ratio of *"Ca.* R. andeanae" to tick 16S mitochondrial DNA was seen in the QPCR assay. This peak may have been due to differences in sample collection or in the growth status of the rickettsia relative to the newly established cells. For example, as this was early in the establishment of the cell line, the embryonic cells may have been more tightly adhered in the monolayer but the growth of rickettsiae may have been stable. Thus, fewer nonadherent cells would be present and the amount of tick 16S mitochondrial rDNA would be lower, allowing the ratio to be higher than in later time points where more cells have detached.

Overall, we observed low numbers of "*Ca.* R. andeanae" in cytospins and slow growth, consistent with results reported by Luce-Fedrow et al. (Luce-Fedrow et al. 2011). In that study, Vero cells maintained the highest overall quantity of "*Ca.* R. andeanae" in comparison with the growth rates in DH82 and S2, *Drosophila* hemocytes (Luce-Fedrow et al. 2011). In addition, *"Ca.* R. andeanae" was observed in the cell cytoplasm and extracellularly, with no intranuclear bacteria, which is comparable with our findings (Luce-Fedrow et al. 2011,

unpublished data). Interestingly, the use of egg masses from infected female *A. maculatum* to establish the "*Ca*. R. andeanae" isolate supports transovarial transmission of this rickettsia. Further investigation is needed to confirm vertical transmission. As of May 2012, the new GCT cell line is still growing in our laboratory and GCT, ISE6 and Vero cell lines are still infected with "*Ca*. R. andeanae" based on QPCR analysis.

We were unable to grow the naturally uninfected cells from TAMU eggs. Possible reasons for the cell line failure include incorrect timing of embryonic development or slightly different conditions in the protocol, as compared with the protocol used initially for OSU cells. For example the smaller quantities of TAMU eggs used may have yielded less embryonic tissue to seed the flat bottom tubes used, instead of flasks used for OSU tick cells. Nevertheless, infected flasks with naturally infected cell lines were successfully cleared of the infection using tetracycline, and have remained free of bacteria as shown by microscopy, PCR and QPCR assays.

In our study, the use of cells from the natural host for *"Ca.* R. andeanae" appeared to provide a more suitable environment for growth of these rickettsiae compared to other traditional cell lines used. In the process, a new embryonic tick cell line was established. The isolation of "*Ca.* R. andeanae" provides a source of organism for more in-depth studies investigating its biology, infectivity and pathogenicity to vertebrates and future genetic characterization to aid in the species status designation of the novel SFGR. In addition, the *A. maculatum* cell line provides an alternative tick cell line for cultivation of tick-associated

organisms and will be ideal for *A. maculatum* associated bacteria. As the maintenance of these cells does not differ substantially from the maintenance of other tick cell lines, it should be a convenient cell line for use in the laboratory setting already accustomed to propagating tick and other arthropod cells.

Acknowledgments

We would like to thank Dr. Ulrike Munderloh (University of Minnesota) for her essential contributions to the establishment of the *A. maculatum* cell line and isolation of "*Ca.* R. andeanae" and for reviewing this manuscript. This work was supported by intramural funding through Mississippi State University, College of Veterinary Medicine.

This chapter is under revision for manuscript submission as "Ferrari, F.A.G., Goddard J., Paddock C., Moraru G. M., Smith W. E. C., Varela-Stokes A. 2012. Establishment of Gulf Coast Tick Cell Line (Ixodida: Ixodidae) and Isolation of "*Candidatus* Rickettsia andeanae" (Rickettsiales: Rickettsiaceae). Journal of Medical Entomology".

Table 6.1 PCR results of "*Ca.* R. andeanae"-species specific *rompA* fragment for several time points of rickettsial growth in *A. maculatum* cell line (GCT cells) and in challenged ISE6 and Vero cells.

Time points	Naturally infected GCT cells	ISE6 challenged	Vero challenged
3-Jan.	Positive	Negative	N/a
23-Jan.	Positive	Positive*	Positive*
27-Jan.	Positive	Positive	Positive
30-Jan.	N/a	Negative	Negative
10-Feb.	Positive	Negative	Negative
24-Feb.	Positive	Negative*	Positive*
28-Feb.	Positive	Positive	Positive
6-Mar.	Positive	Negative	Negative
13-Mar.	Positive	Positive	Negative
20-Mar.	Positive	Negative	Negative
27-Mar.	Positive	Negative	Positive*

*indicates a PCR result following a re-challenge (re-challenges occurred on 13 Jan., 14 Feb. and 21 Feb. for ISE6 and Vero and on 23 Mar., for Vero only).

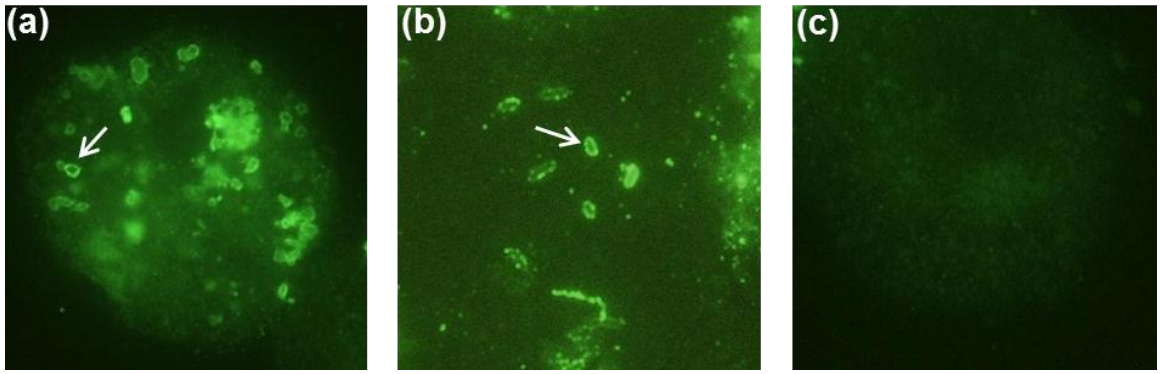


Figure 6.1 Immunofluorescence antibody assay showing extracellular rickettsiae in *Amblyomma maculatum* cells including positive and negative controls of assay

NOTE: (a) extracellular rickettsiae (tip of arrow) in GCT cells; (b) *Rickettsia parkeri* grown in ISE6 as positive control and (c) uninfected GCT cells as negative control. Images were captured using an Olympus BX41 microscope and Nikon DS-Fi1 camera at 1000x magnification.

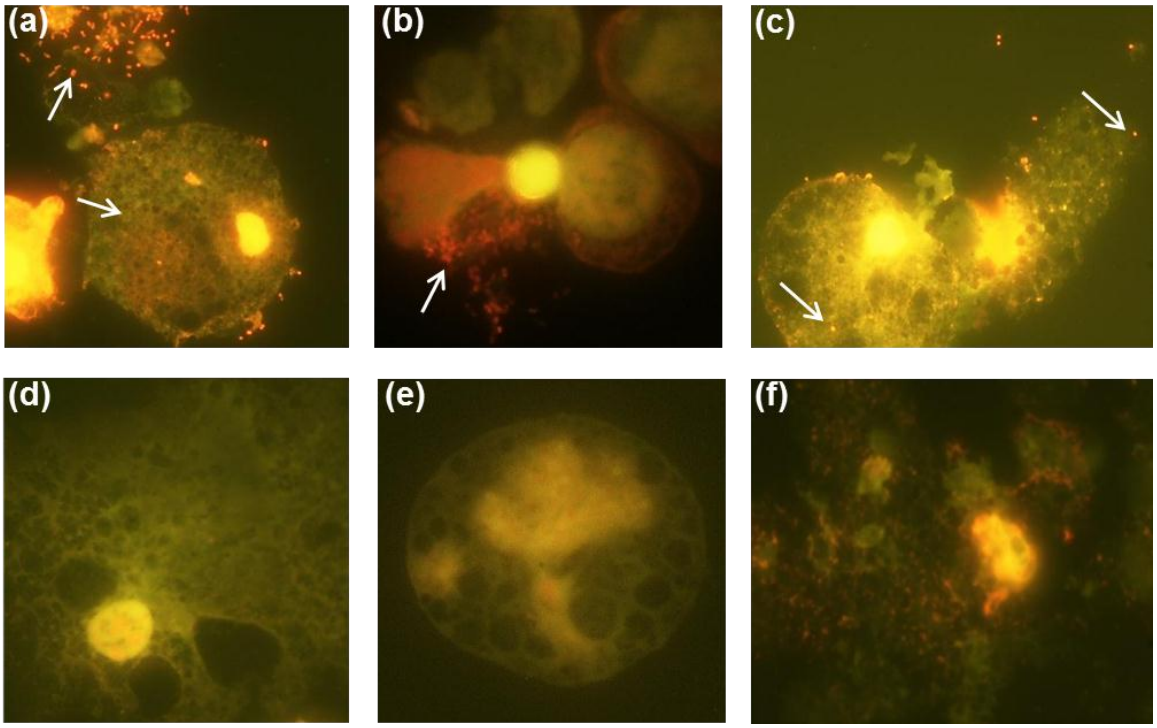


Figure 6.2 Acridine orange staining showing "*Ca.* R. andeanae"-infected *Amblyomma maculatum*, ISE6 and Vero cells including uninfected cultures

NOTE: Top row shows rickettsiae (tip of arrows) extra and intracellularly in GCT cells (a), ISE6 (b) and Vero (c) at various time points. Bottom row represents uninfected cells of GCT cells (d), ISE6 (e) and Vero (f). Images were captured using an Olympus BX41 microscope and Nikon DS-Fi1 camera at 1000x magnification.

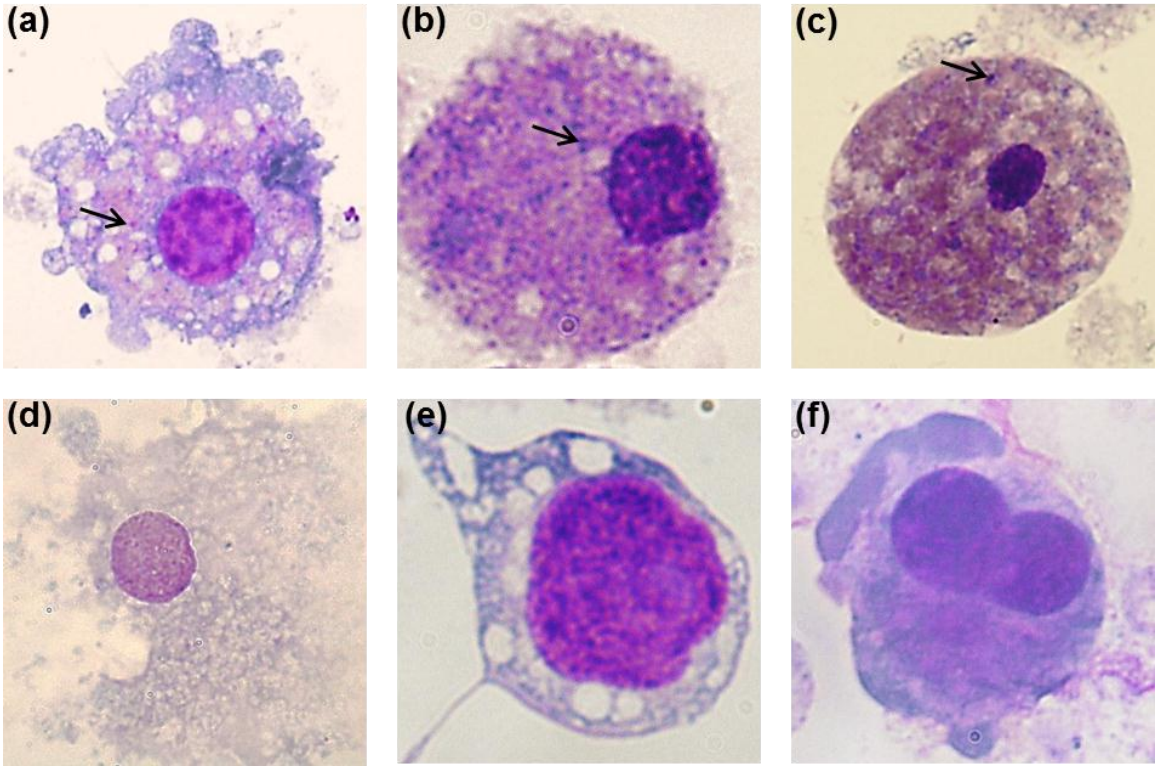
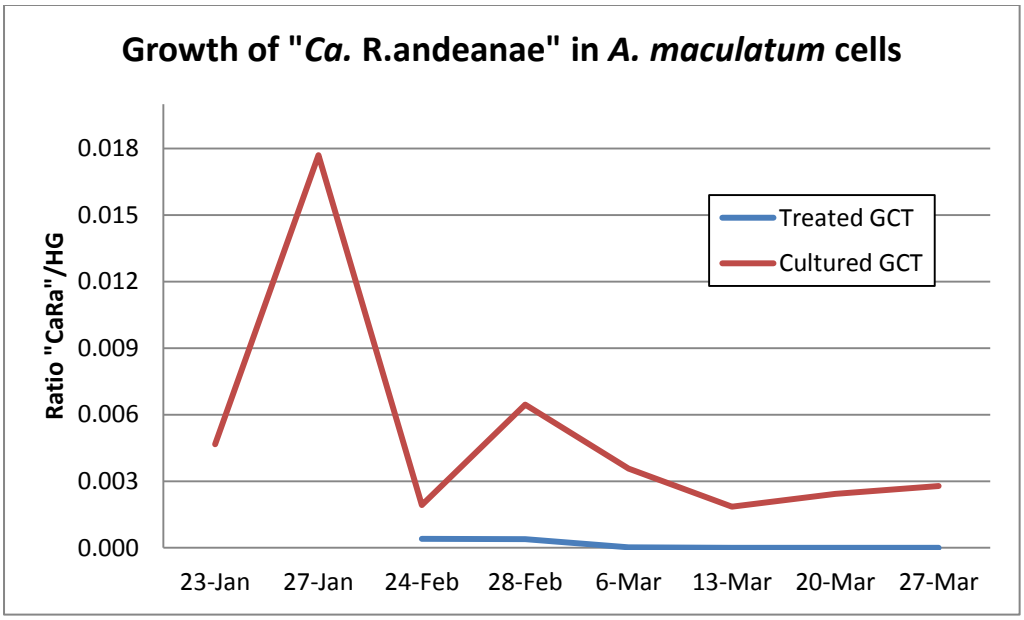


Figure 6.3 Diff-Quik staining showing "*Ca.* R. andeanae"-infected *Amblyomma maculatum*, ISE6 and Vero cells including uninfected cultures

NOTE: Top row shows rickettsiae (tip of arrows) mostly in the cytoplasm of GCT cells (a), ISE6 (b) and Vero (c) at various time points. Bottom row represents uninfected cells as shown in GCT cells (d), ISE6 (e) and Vero (f). Images were captured using an Olympus BX41 microscope and Nikon DS-Fi1 camera at 1000x magnification.

(a)



(b)

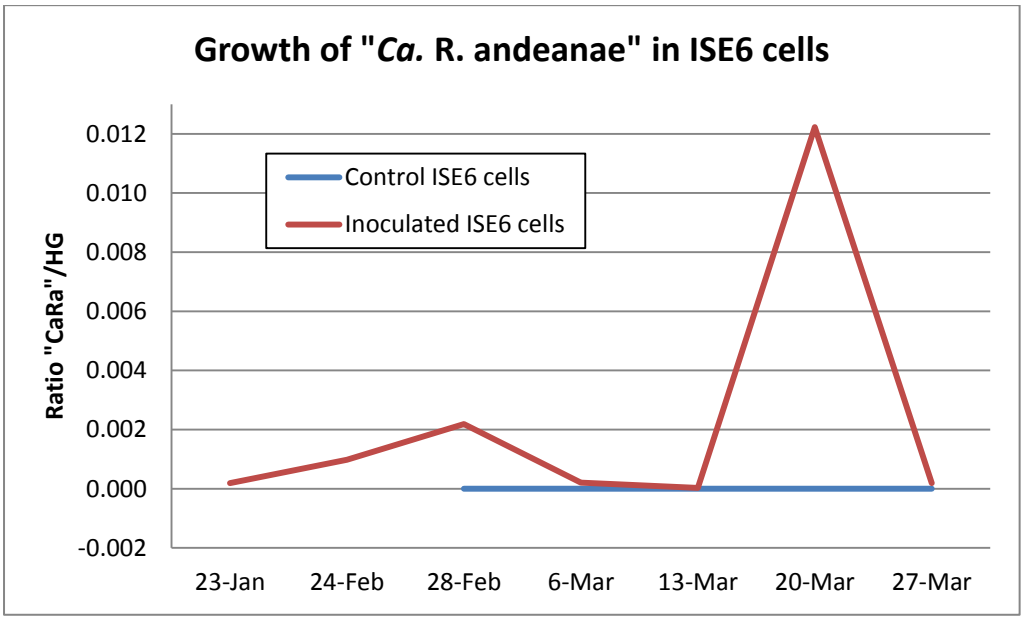


Figure 6.4 Ratio of "*Candidatus* R. andeanae" infection levels by host gene amounts based on QPCR results in three different cell lines

NOTE: (a) *A. maculatum* cell line, "GCT cells"; (b) ISE6 cells and (c) Vero cells. Re-challenges occurred on 13 Jan., 14 Feb. and 21 Feb. for ISE6 and Vero and on 23 Mar., for Vero only.

(c)

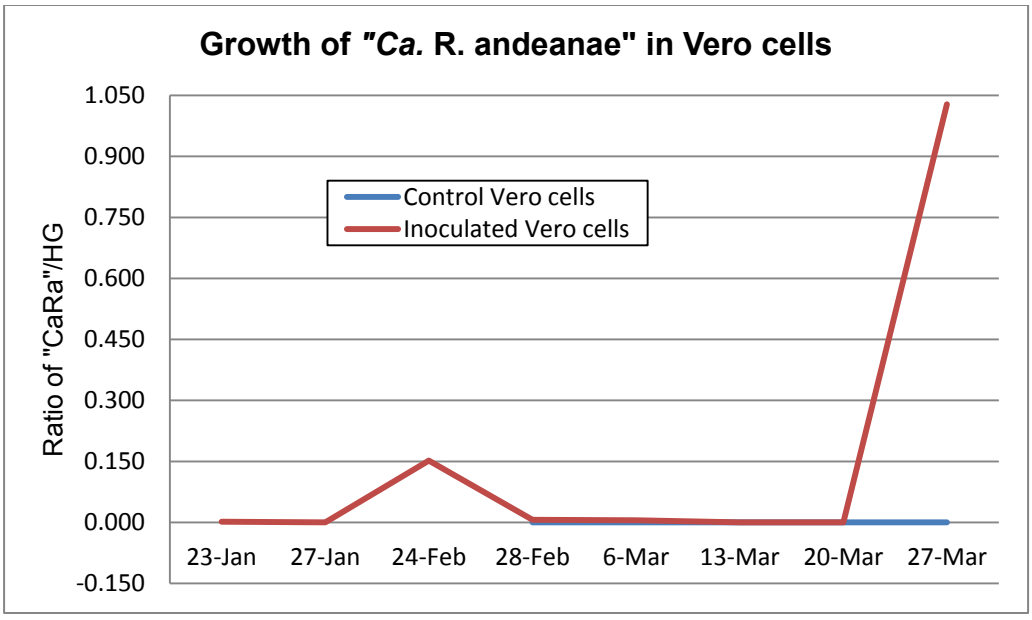


Figure 6.4 (continued)

CHAPTER VII

CONCLUSIONS AND IMPLICATIONS

We proposed to study the ecological relationships of spotted fever group rickettsiae (SFGR) in Gulf Coast ticks (GCTs) in Mississippi. All proposed aims were completed and are presented in this dissertation. In Chapter 3, we provide results from testing a large sampling of GCTs collected over a three year period from ten different sites mostly in northeastern and southern Mississippi. In this study, 698 GCTs were analyzed by PCR assays for the presence of SFGR DNA. Over 15% of GCTs were infected with a recently recognized zoonotic rickettsia, *R. parkeri.* However, to date only four cases of *R. parkeri* infection, whether confirmed or probable, have been reported in Mississippi. Based on the prevalence of *R. parkeri* detected in GCTs collected over a three year period in different regions of Mississippi, the risk for *R. parkeri* exposure in humans may be higher than for *R. rickettsii;* thus, *R. parkeri* rickettsioses may be underreported. Results from this study should raise awareness to rickettsiosis caused by *R. parkeri* infection in Mississippi. Interestingly, in Tennessee there the number of reported RMSF cases increased by 20% in 2008 from 2007, although no tick (over 1500 ticks surveyed) was found infected with *R. rickettsii* (Moncayo et al. 2010). With RMSF cases now reported as “spotted fever

rickettsiosis" to acknowledge the additional SFGR that may cause RMSF-like disease, the increase in RMSF cases reported in Tennessee in 2008 may reflect misdiagnosis of other spotted fever rickettsioses, particularly considering the high prevalence of other SFGR that were detected in resident ticks from that study (Moncayo et al. 2010). More cases of RMSF are likely to have occurred than the actual reported due to lack of specific laboratory testing (Dahlgren et al. 2012). Studies such as that presented in Chapter 3 of this dissertation are important for increasing awareness of other tick-borne rickettsiae like *R. parkeri* and for providing epidemiological data for physicians and other health care providers to assist in designing appropriate local health care policies for prevention and treatment of rickettsioses.

We also found that 1.4% of GCTs were singly infected with "*Ca.* R. andeanae" and other 1.7% were co-infected with *R. parkeri* and "*Ca.* R. andeanae". A low infection rate of ticks singly infected with "*Ca.* R. andeanae" supports data from previous studies in the U.S., although, to our knowledge, no other study of "*Ca.* R. andeanae" used as large a sample size as was used in our study. Interestingly, to our knowledge we are the first group to report co-infection of *R. parkeri* and "Ca. R. andeanae". Recently, one GCT male from North Carolina was reported co-infected with the novel SFGR and *R. parkeri* (Varela-Stokes et al. 2011) and 12 GCTs in this study (Chapter 3). Co-infections between many rickettsiae are not a common event due to transovarial interference, such as that reported with *R. peacockii* and *R. rickettsii* (Azad and Beard 1998). Further study is needed to determine the relationship of "*Ca.* R. andeanae" with

R. parkeri in GCTs. These efforts are currently being undertaken in our group as a result of data presented in Chapter 3.

From the GCTs collected for the prevalence study, we amplified DNA of the tick mitochondrial rRNA gene to examine genetic variation among populations of GCTs from the North and South of Mississippi at that locus using Single Strand conformational polymorphism analysis (SSCP; Chapter 4). At least four genetically different haplotypes or GCT populations exist in Mississippi and were resolved based on the SSCP analysis coupled with sequencing. Statistical analyses of haplotype frequencies showed no significant differences between the four sites in southern Mississippi compared to the four sites in northern Mississippi. However, haplotype distribution was significantly different if locally analyzed, i.e. by sites. As supported by a calculus of migration, there appears to be significant movement between local populations of GCTs. In a similar SSCP study of GCTs from Oklahoma, Kansas and Texas (Ketchum et al. 2009), it was suggested that Kansas ticks may have founded the coastal population (Teel et al. 2010). We propose an alternative event, that different introductions from elsewhere in Central and South America may have founded Kansas and the coastal population and formed distinct populations in these regions. This alternative explanation is based on the high variability in Mississippi haplotypes and the unique and ancestral haplotype identified in our samples. This is comparable to the scenario observed in Kansas (Ketchum et al. 2009). A more complete sampling of GCTs from throughout its distribution in the U.S. and from

other potential source populations in South and Central America may answer questions raised by this work.

In addition to tick population analysis, we studied the population of *R. parkeri* infecting those ticks based on SSCP. Our results showed that different populations of ticks are infected with the same population of *R. parkeri*, at least for the six loci examined here. That a coding gene, *rompA*, would show no genetic variability among our samples was expected, however finding no variation using five different intergenic spacer regions was surprising. In the future, the use of a larger selection of intergenic regions may produce a better marker for genetic variation among *R. parkeri* strains. Additionally, it is reasonable ecologically that the variability seen with ticks was larger than that within the rickettsiae as ticks are capable of reproducing sexually. Additionally, selection pressure is larger for arthropods adapted to a variety of habitats while the rickettsia is more "protected" in its niche inside the tick, where contact with other bacteria, that could add variability, is limited (Roux et al. 2002). As stated previously, "the ecologic separation and reduced selective pressure due to these associations (rickettsiae in blood-sucking arthropods) may explain rickettsial genetic conservation" (Azad and Beard 1998).

Most of the knowledge of "*Ca.* R. andeanae" was previously limited to molecular evidence of the rickettsia in infected ticks. Blair *et al*. studied the phylogeny of "*Ca.* R. andeanae" and was able to group it with other SFGR based on the rickettsial outer membrane protein B (Blair 2004). In Chapter 5, we demonstrated that "*Ca.* R. andeanae" possess a similar ultrastructural

morphology to other previously described SFGR, which corroborates with the results from the earlier phylogenetic study (Blair et al. 2004). Additionally, we described its occurrence in salivary glands and digestive system of female adult GCTs. In female ticks, it was observed in the Malpighian tubules and reproductive tissues. Ultimately, a long-term goal for the study of *"Ca.* R. andeanae" is to determine interactions between this SFGR and *R. parkeri,* as well as the infectivity and pathogenicity of the novel SFGR itself. The results presented in this dissertation will contribute to the generation of hypotheses and design of future studies to pursue these answers. For example, our demonstration of rickettsia-like organisms in the salivary glands of the ticks analyzed helped support studies to evaluate horizontal transmission of the novel SFGR. Horizontal transmission is the ability of some bacteria to infect and replicate in vertebrates producing a bacteremia at which point another tick vector bites this host becoming infected (Sonenshine 1993); one avenue for horizontal transmission is through saliva contaminated with the organism.

Tick embryonic cells are useful in propagating fastidious organisms, especially those that naturally replicate in arthropod cells, like tick-borne arboviruses, bacteria, and protozoa (Bell-Sakyi et al. 2007). Chapter 6 presented the successful establishment of new cell line from GCT embryonic cells. The GCT cell line may prove useful for isolation of other organisms that have been fastidious in currently available cell lines. In addition, its maintenance does not differ substantially from other currently available tick cell lines. Additionally, as it has been shown for *R. conorii* and *Ehrlichia chaffeensis,* expression of

transcripts or proteins (respectively) was different in bacteria grown in vertebrate cells in comparison with arthropod cells (Rovery et al. 2005, Singu et al. 2006). Therefore, this cell line may provide a better model for proteomic and genetic modification studies for organisms naturally occurring in GCTs (e.g. *R. parkeri* and *Hepatoozon americanum*), and may aid in further studies on the potential bioterrorism agent, *Ehrlichia ruminantium*.

Additionally, in chapter 6 we reported successfully isolating "*Ca.* R. andeanae" in the *A. maculatum* embryonic cell line, which we believe to be a better and more stable model for studying of "*Ca.* R. andeanae". From that isolate, we were also able to propagate the novel SFGR in Vero and ISE6 cells although momentarily. An important implication of acquiring an isolate of "*Ca.* R. andeanae" is that the ability now exists to evaluate its pathogenicity. As has been shown previously with other rickettsiae, most remarkably with *R. parkeri*, there is a potential for poorly characterized, newly recognized rickettsiae to be pathogenic. An established isolate for *"Ca.* R. andeanae" will allow for more in-depth studies for evaluating disease potential in vertebrates as well as studies on the interactions between this SFGR and *R. parkeri* in individual ticks and vertebrate hosts. Additionally, a stable isolate will allow for a better understanding of the basic biology and a more complete genetic characterization of *"Ca.* R. andeanae", which paves the way for formal designation of species status. This isolate of *"Ca.* R. andeanae" provides a foundation for further studies in our lab in elucidating infectivity and pathogenicity of *"Ca.* R. andeanae", further

characterization of the novel SFGR and interactions between this SFGR and *R. parkeri* in *A. maculatum.*

In summary:

1. GCTs are commonly found infected with a human pathogen, *R. parkeri*, in different regions of Mississippi, including the northern part of the state where the occurrence of the tick was not historically accepted as established.
2. At least four genetically different populations of GCT exist in Mississippi based on analysis of the tick mitochondrial 16S rDNA gene.
3. Different populations of ticks are infected with the same population of *R. parkeri*, at least for the six loci examined here.
4. A recently described SFGR, "*Ca.* R. andeanae", is found in a small percentage of GCTs in Mississippi. Interestingly, another small percentage of ticks are co-infected with "*Ca.* R. andeanae" and *R. parkeri*.
5. "*Candidatus* R. andeanae" possesses similar ultrastructural morphology to other SFGR described previously.
6. Transmission electron microscopy identified "*Ca.* R. andeanae" in salivary glands, gut, Malpighian tubules and reproductive organs of female adult GCTs.
7. A successful cell line from GCT embryonic cells was developed.
8. "*Candidatus* R. andeanae" was successfully isolated in the cells of its natural host, GCT, as well as propagated in Vero and ISE6 cells.

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APPENDIX A

SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS

Single strand conformation polymorphism analysis

1. Start ORIGINS™ machine (Elchrom Scientific AG, Cham, Switzerland) about 40 minutes to cool the running buffer to 7-9°C before loading the samples to the gel.
2. Place in a new tube, 8.4µl of PCR products and add to it, 3.6µl of formamide-NaOH solution:
   a) Mix just before use: 150µl of formamide, 1.5µl of 1M NaOH and 3.9µl of reverse and forward primers.
   b) Add a few grains of bromphenolblue for better visualization, the amount of powder of dipping a tip in it is enough. Mix well by vortex.
3. Heat at 95°C for 5.5 minutes. Immediately place the hot tubes on ice for 3 minutes. Load 9 µl of ladder mix to the gel with pump off (3µl PhiX ladder plus 6µl of loading buffer provided with the gels).
4. With pump off, load all 12 µl of the samples as quick as possible. Run the gel at 6 V/cm (Elchrom suggests 72V) at 7 to 9°C. Running time depends on the size of PCR fragments, the company's suggestions are: fragments of 250-300bp run for 15h and 350-450bp for 17h.
5. After run, release the gel from its plastic backing against a 1L glass bottle using a nylon string and stain it with SYBR Green II (10µL in 100ml 1X TAE) for 40 min or EtBr (5µL in 100ml 1X TAE). If needed, destain in double distilled water or destaining solution for at least 30 min.

6. Photograph with Alpha Innotech 302 nm, EtBr filter, ~10 second exposure or other photodocumentation machine.

Adapted from:

http://www.elchrom.com/fileadmin/pdf/Short%20Technical%20Manual%20ORIGINS%209C-1.pdf