Mississippi State University

Scholars Junction

Honors Theses

Undergraduate Research

4-1-2016

Microscopic analysis of Rickettsial co-infection in the Gulf Coast tick, Amblyomma maculatum

Amanda Benton Harper

Follow this and additional works at: https://scholarsjunction.msstate.edu/honorstheses

Recommended Citation

Harper, Amanda Benton, "Microscopic analysis of Rickettsial co-infection in the Gulf Coast tick, Amblyomma maculatum" (2016). *Honors Theses*. 11.
https://scholarsjunction.msstate.edu/honorstheses/11

This Honors Thesis is brought to you for free and open access by the Undergraduate Research at Scholars Junction. It has been accepted for inclusion in Honors Theses by an authorized administrator of Scholars Junction. For more information, please contact scholcomm@msstate.libanswers.com.

Microscopic analysis of Rickettsial co-infection

By

Amanda Benton Harper

An Honors Thesis

Submitted to the Faculty of

Mississippi State University

Mississippi State, Mississippi

April 2016

Copyright by

Amanda Benton Harper

2016

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Andrea Varela-Stokes for, firstly, choosing to hire me to be a part of a great lab and giving me the chance to do work that I love for the past (almost) 3 years. Thank you as well for your mentorship, guidance, and time devoted to helping me become a better scientist. Next, I would like to thank Dr. Kevin Lee for his mentorship and guidance throughout this project and all of my time working with him. I would like to also thank the other members of our lab (past and present) who provided a variety of support, both intellectual and moral, including John, Gail, Katie, Haley, Jacob, and Keiko. A special thanks to Amanda Lawrence and the Institute for Imaging and Analytical Technologies at Mississippi State University, for help beyond what was required and the use of equipment and facilities. Finally, I would like to thank my husband, Corey Harper, for support and love in spite of my occasional bad attitude, and my family, for encouraging my desire to learn from a very young age.

This research was funded by NIH R15AI099928-01A1.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1. INTRODUCTION 1
   1.1 Summary 1
   1.2 Ticks 1
      1.2.1 *Amblyomma maculatum* 2
   1.3 Rickettsiae 4
      1.3.1 Spotted Fever Group Rickettsiae 4
         1.3.1.1 *Rickettsia parkeri* 4
         1.3.1.2 *Candidatus* "Rickettsia andeanae" 5
   1.4 Microscopy for Visualizing Rickettsiae 5
      1.4.1 Fluorescence In situ Hybridization 5
      1.4.2 Fluorescence Immunohistochemistry 6
      1.4.3 Transmission Electron Microscopy 6
   1.5 Relevance 7

2. OBJECTIVES & HYPOTHESIS 8
   2.1 Objectives 8
   2.2 Hypothesis 8

3. MATERIALS & METHODS 9
   3.1 Experimental Design 9
      3.1.1 Tick Feeding 9
      3.1.2 Tissue Collection and Preparation 10
         3.1.2.1 Tick Dissection 11
         3.1.2.2 Storage of Tissues 12
   3.2 Selection of Samples 13
   3.3 Fluorescence In situ Hybridization and Immunohistochemistry 14
   3.4 Transmission Electron Microscopy 17

4. RESULTS 18

5. DISCUSSION 20

6. CONCLUSIONS & FUTURE DIRECTIONS 22

REFERENCES 23

LIST OF TABLES

Table 1: qPCR primers and probes 13

LIST OF FIGURES

Figure 1: Adult *Amblyomma maculatum* 2

Figure 2: Map of *Amblyomma maculatum* distribution within the United States 3

Figure 3: Morphology of spotted fever group rickettsiae under TEM 7

Figure 4: Line drawing of structures that may be observed during dissection 11

Figure 5: Representative fluorescence images of rickettsiae in co-infected and "*Ca.* R. andeanae"-infected samples 19

Figure 6: Representative TEM images of rickettsial aggregates in midgut, salivary gland, and ovary tissues 20

CHAPTER 1

INTRODUCTION

1.1 Summary

Ticks can pass a variety of organisms to humans, resulting in mild flu-like illnesses or more severe diseases that may be fatal if not treated. One group of tick-borne organisms of interest to us are rickettsiae, which cause an illness known as spotted fever rickettsiosis. The ticks that transmit rickettsiae to humans may also pass these organisms to animals and to other ticks, which allows for these organisms to continue to circulate in nature. In some cases, two rickettsiae will utilize the same tick host, and occasionally this presents itself as co-infection of the bacteria. Co-infection can then lead to altered distribution of the bacteria in the tick tissues and impact the modes of transmission of one or both bacteria. In this study, our goal was to locate two species of rickettsial bacteria, one that causes disease and one that is not known to, in infected ticks using two different microscopy methods. We found that, while we could tell the two species apart using fluorescence microscopy, and identify whole bacteria in specific tissues using electron microscopy, we had to rely on DNA testing to screen samples, and found inconsistent results. These techniques would likely be inefficient if pursued on a larger scale, which leads us to suggest an alternative technique for future studies.

1.2 Ticks

Ticks are classified within the Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitiformes, and Suborder Ixodida. Within Ixodida, there are three families: Ixodidae ("hard ticks"), Argasidae ("soft ticks") and Nuttalliellidae. While ticks of the family Ixodidae are characterized by a chitin-rich scutum on their dorsal surface,

Argasidae ticks have no such protection. Nuttalliellidae ticks have features of both Ixodidae and Argasidae ticks, although the family only contains one species: *Nuttalliella namaqua*. All ticks are obligate blood-sucking ectoparasites that require a blood meal to grow between their varying number of life stages. Ticks lack any body part division (no separate head, thorax and abdomen), and instead are made up of the body and a capitulum ("mouth parts"), as well as eight legs in their nymphal and adult stages. Between the families of ticks there are morphological differences, such as placement of the capitulum, and ecological differences, such as preferred climate, and whether the tick is nidicolous (nest-dwelling). Tick families also differ in life history, including the number of life stages the tick goes through before adulthood, and host.

1.2.1 *Amblyomma maculatum*

Amblyomma ticks belong to the family Ixodidae. *Amblyomma maculatum*, also known as the gulf coast tick, has a three-stage life cycle – larva, nymph, and adult – and requires a blood meal between each stage. The adult female tick takes a final blood meal, mated females and lay their eggs before dying. The adult male, however, can take multiple blood meals. The male and female of the species are easily distinguished by their ornate scuta and prominent capitulum.

Figure 1: Adult *Amblyomma maculatum*. A: Female. B: Male. Image from Sumner et al. (2007).

Amblyomma maculatum is found throughout the southeastern United States with established populations from the coast of the Gulf of Mexico to points as far north as Maryland along the east coast and west into Oklahoma, Kansas, and Texas. Figure 2 is a visual representation of *A. maculatum* distribution within the United States.

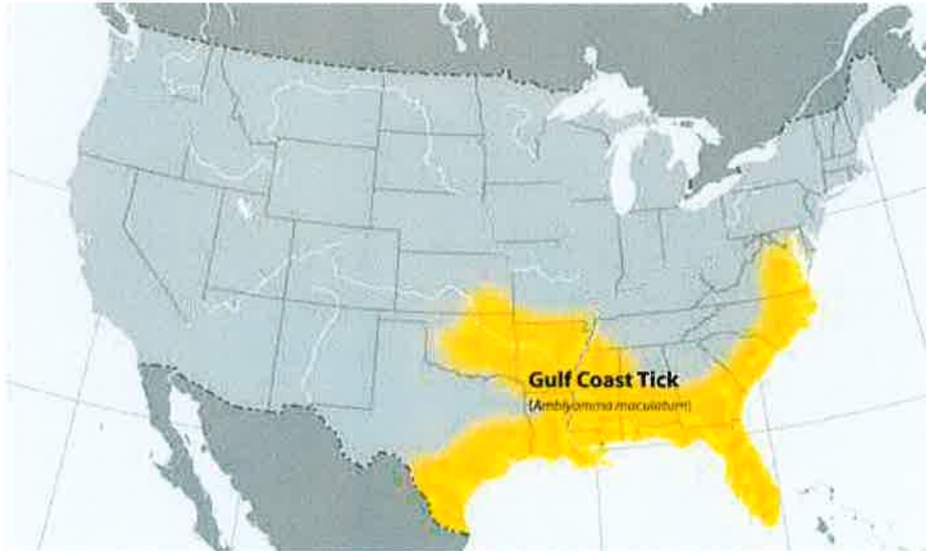

Figure 2: Map of *Amblyomma maculatum* distribution within the United States. Taken from an informational image by the Centers for Disease Control and Prevention. http://www.cdc.gov/ticks/maps/gulf_coast_tick.pdf

Because of the nature of tick feeding, ticks are competent vectors for a variety of bacteria, protozoa, and viruses. *Amblyommq maculatum* has been associated in particular with a known human pathogen, *Rickettsia parkeri,* as well as another bacterium, "*Candidatus* Rickettsia andeanae" in the wild. *Amblyomma maculatum* have been found infected with either one of these bacteria as well as with both, in instances of co-infection. With *R. parkeri*, infection rates of *A. maculatum* range from 10-56 % (Sumner et al., 2007, Paddock et al., 2010, Fornadel et al., 2011, Varela-Stokes et al., 2011, Ferrari et al., 2012, Nadolny et al., 2014). With "*Ca*. R. andeanae," infection rates of *A. maculatum* range from 0.6-2% (Sumner et al., 2007, Paddock et al., 2010, Fornadel et al., 2011, Varela-Stokes et al., 2011, Ferrari et al., 2012, Nadolny et al., 2014). Further,

instances of coinfection at a rate of 1.7% have also been reported (Ferrari et al., 2012). These percentages are not necessarily the rule, however; recently, populations of *A. maculatum* found in Oklahoma and Kansas have been found to have differing infection rates of the two bacteria, where "*Ca.* R. andeanae" (42% in Kansas and 79% in Oklahoma) was more prevalent than *R. parkeri* (0% in both states) (Paddock et al., 2015).

1.3 Rickettsiae

Rickettsiae are a group of obligate intracellular bacteria that are members of the Phylum Proteobacteria, Class Alphaproteobacteria, Subclass Rickettsiae, Order Rickettsiales, Family Rickettsiaceae. They are gram negative bacteria. Traditionally, Rickettsiae have been categorized into two main groups: the spotted fever group and the typhus group, with two other species belonging to neither group (Perlman et al., 2006). Our laboratory is focused on the spotted fever group rickettsiae, which are associated with ticks.

1.3.1 Spotted Fever Group Rickettsiae

Spotted Fever Group Rickettsiae (SFGR) are a group of rickettsiae which are closely related to *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever. Within this group, some of the bacterial species are transmitted horizontally (through the salivary glands to cause disease in vertebrates), while others seem to only be transmitted vertically (through the ovaries to infect the ticks' offspring) (Perlman et al., 2006). Both *R. parkeri* and "*Ca.* R. andeanae" belong to this group.

1.3.1.1 *Rickettsia parkeri*

Rickettsia parkeri is a member of the spotted fever group of rickettsiae and is a known human pathogen. The organism was discovered in 1937 by Ralph R. Parker (its

namesake) and others from *A. maculatum* near the Gulf Coast of Texas (Parker et al., 1939). *R. parkeri* was first described as causing disease in humans in 2002 when a case was reported from the Tidewater region of Virginia (Paddock et al., 2004). Since then, around 20 cases of *R. parkeri* infection – also called American Boutennouse Fever – have been reported within the United States (Raoult & Paddock, 2005, Whitman et al., 2007, Goddard & Varela-Stokes, 2008, Paddock et al., 2008, Cragun et al., 2010, Herrick et al., 2016).

1.3.1.2 "*Candidatus* Rickettsia andeanae"

"*Candidatus* Rickettsia andeanae" is also a member of the spotted fever group of rickettsiae, but has not been proven to be pathogenic in humans. The identification of this bacterium as a new species was performed by Blair et al. (2004), after molecular analysis of DNA samples taken from ticks in Peru. Since then, "*Ca.* R. andeanae" has also been found within the United States at relatively low infection rates and, in Mississippi, co-infected with *R. parkeri* (Sumner et al., 2007, Paddock et al., 2010, Fornadel et al., 2011, Varela-Stokes et al., 2011, Ferrari et al., 2012, Nadolny et al., 2014). The novelty of "*Ca.* R. andeanae," as well as its relationship with pathogenic *R. parkeri* necessitates further characterization of the bacterium.

1.4 Microscopy for Visualizing Rickettsiae

1.4.1 Fluorescence *in situ* Hybridization

Fluorescence *in situ* hybridization (FISH) is a method of visualizing the presence and location of an organism or other element within tissue utilizing a specific nucleic acid sequence of the organism. For our purposes, we utilized a FISH protocol targeting "*Ca.* R. andeanae" with a digoxygenin (DIG)-labelled RNA probe based on a region of the

23S ribosomal RNA (rRNA) gene of the bacteria. The DIG label can be targeted with an anti-DIG antibody (e.g. made in a sheep host, in our case), which is then targeted with a fluorescent (Cy3 or cyanine 3 dye, in our study)-conjugated antibody (Millipore, Billerica, MA, USA). This final antibody allows for visualization of "*Ca*. R. andeanae" via emission of light at 570 nm under a Tetramethyl Rhodamine Isothiocyanate (TRITC) filter.

1.4.2 Fluorescence Immunohistochemistry

Immunohistochemistry (IHC) is another method of visualizing an organism or other target within tissues which, while similar to FISH, does not utilize a nucleic acid probe but rather an antibody against a specific antigen. For this experiment, we targeted the green fluorescent protein (GFP) expressed by our *R. parkeri* transformant, using a mouse monoclonal anti-GFP antibody (R&D Systems, Inc., Minneapolis, MN. USA). The mouse antibody was subsequently targeted by a goat anti-mouse Cy2 (cyanine 2 dye)-conjugated antibody (Abcam, Cambridge, MA, USA). This final antibody allows for visualization of *R. parkeri* via emission of light at 510 nm under a Fluorescein Isothiocyanate (FITC) filter.

1.4.3 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) is a method of visualizing tissue at the ultrastructural level, taking advantage of electron density of proteins, lipids, and nucleic acids following staining with heavy metals. Extensive work to characterize rickettsiae under TEM has led to a characterization of the bacterial morphology, which includes two prominent features: the trilaminar cell wall and the slime layer or halo zone (Hayes & Burgdorfer, 1979, Ferrari et al., 2014). Furthermore, the bacteria tend to have a rod or

elliptical shape with a size of 1-2μm long and 0.4μm wide (Hayes and Burgdorfer 1979). Figure 3 provides a visual representation of the characteristic features, which are found throughout the spotted fever group rickettsiae (Hayes & Burgdorfer, 1979).

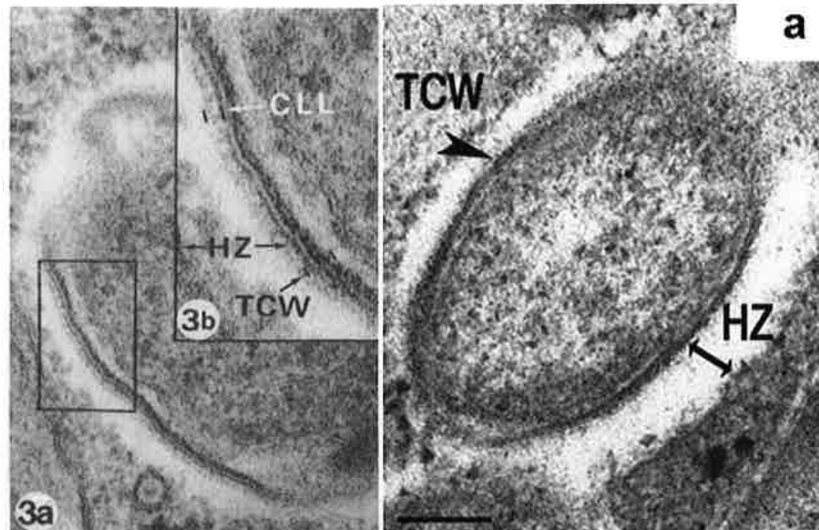

Figure 3. Morphology of spotted fever group Rickettsiae under TEM
Image modified from Hayes & Borgdorfer (1979) (left, *Rickettsia rhipicephali*) and Ferrari et al. (2014) (right, "*Ca.* R. andeanae").
Labelled features: TCW = trilaminar cell wall; HZ = halo zone; CLL = capsule-like layer

1.5 Relevance

Interest in the occurrence of these two rickettsiae in *A. maculatum* stems from a similar occurrence of rickettsial species in another tick, *Dermacentor andersoni*. *Dermacentor andersoni* acts as the primary vector of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (Burgdorfer, Hayes, & Mavros, 1981). This tick also harbors *Rickettsia peacockii* (previously known as the East Side Agent), which was found strictly in female ticks interfering with *R. rickettsii* infection of ovaries and vertical transmission (Burgdorfer, Hayes, & Mavros, 1981, Kurtti et al., 2005). There is some speculation that the relationship between *R. parkeri* and "*Ca.* R. andeanae" would cause a similar interference for *R. parkeri*. Further investigation of wild ticks revealed that the two bacteria are sometimes co-infected, and are not as closely related to each other as *R.*

rickettsii and *R. peacockii* based on common gene targets (Kurtti et al., 2005, Sumner et al., 2007). Altogether, this implies that the relationship between *R. parkeri* and "*Ca.* R. andeanae" is likely to be unique, necessitating investigation into how the single and co-infections are presented in different tick tissues.

CHAPTER 2

OBJECTIVES & HYPOTHESIS

2.1 Objectives

The objective of this study was to utilize microscopy techniques in order to visualize and distinguish our two bacteria of interest, *R. parkeri* and "*Ca.* R. andeanae," as well as the different tick tissue types of interest, midgut, salivary gland, and ovary. To do so, we set out to utilize fluorescence microscopy methods to discriminate between the two rickettsiae and transmission electron microscopy to identify rickettsiae based on ultrastructure in infected tissues.

2.2 Hypothesis

We hypothesized that one of the bacterial species would be "dominant" in specific tick tissues over the other bacteria. Specifically, we further hypothesized that *R. parkeri* would be the dominant bacterium in salivary glands compared to "*Ca.* R. andeanae." This hypothesis was based on knowledge that *R. parkeri* is a human pathogen and is therefore transmissible to tick hosts, while "*Ca.* R. andeanae" has not proven to be so.

CHAPTER 3

MATERIALS & METHODS

3.1 Experimental Design

3.1.1 Tick Feeding

Lab-reared *Amblyomma maculatum* from the Centers for Disease Control and Prevention, Oklahoma State University, and Texas A&M University were utilized in this study. These ticks were artificially infected using a capillary feeding method, where 10μL glass tubes full of rickettsiae in cell culture were fitted onto the mouth parts of each tick for 2 hours at 32-33°C. For each of the three treatment groups: *R. parkeri*, "*Ca.* R. andeanae," and co-infected with both bacteria, we filled capillary tubes with *R. parkeri* GFPuv (isolate previously transformed to express GFPuv) in Vero cell culture, "*Ca.* R. andeanae," (isolate from Ferrari et al., 2013) in Vero or tick cell culture, or a mixture of both species in cell culture, respectively. For control ticks, uninfected Vero cells were used. To ensure that the uninfected Vero cells were *Rickettsia*-free and to evaluate relative quantities of rickettsiae in cultures for treatment groups, we harvested cultures on the day of the artificial infection and tested DNA extracts from culture samples using quantitative PCR (qPCR). These samples underwent DNA extraction and qPCR, the results of which informed the extent of culture dilution prior to capillary feeding.

Seven days following this capillary feeding (day 0), approximately 36 ticks (18 males, 18 females) were placed onto each of twelve rabbits. Each rabbit was shaved and fitted with a "tick chamber" similar to those used by Embers et al. (2013) in order to ensure ticks remained on the rabbits. Three rabbits were used in each treatment and the control group. Ticks remained on rabbits for 12 days. Ticks were taken for tissue

sampling on day 0 (15 males, 15 females were subsampled that were not placed onto rabbits), day 6 (up to 9 males, 9 females were removed from rabbit hosts), and day 12 (up to 9 males, 9 females were removed from rabbit hosts). In some cases, the numbers were fewer for day 6 and day 12 collections due to loss of ticks (primarily mortality). Tick feeding trials on rabbits were replicated three times.

3.1.2 Tissue Collection and Preparation

Male ticks were dissected for collection of midgut and salivary glands. Female ticks were dissected for collection of midgut, salivary gland, and ovarian tissues. Midgut tissues were selected because the midgut is the location of initial infection of many rickettsiae, where the bacteria begin propagation and can further infect other tissues (Sonenshine and Anderson, 2014). Salivary gland tissues were selected because, for a majority of tick-borne pathogens, including rickettsiae, transmission to a host occurs through the saliva produced by salivary glands and injected into hosts during feeding (Alarcon-Chaidez, 2014). And lastly, the ovarian tissues of female ticks were selected because SFG rickettsiae, like *R. parkeri* and "*Ca.* R. andeanae," are primarily maintained in wild tick populations via transovarial transmission, from parent to offspring (Alarcon-Chaidez, 2014). A rendition of these organs within a female tick can be found in Figure 2. Male midgut and salivary glands are located similarly, while the ovary tissues would be absent.

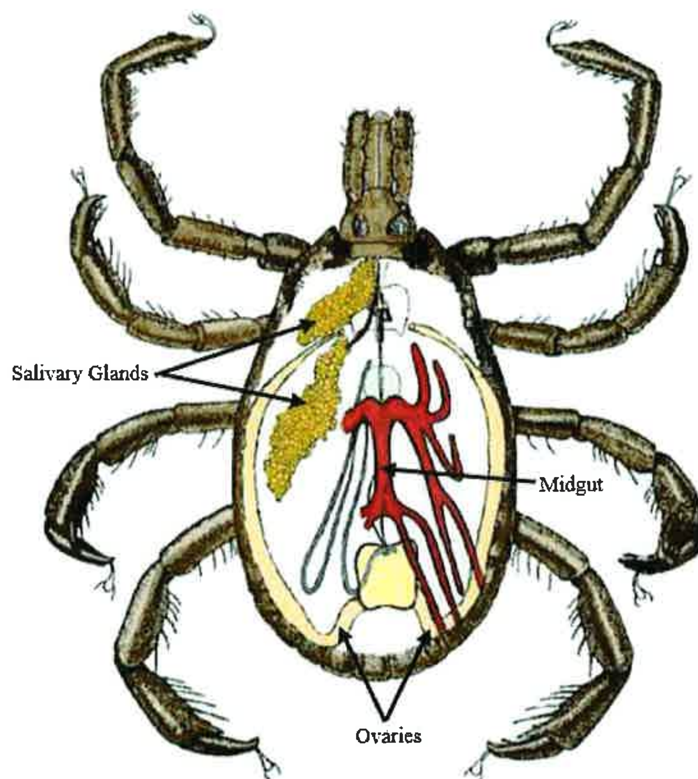

Figure 4: Line drawing of structures that may be observed during dissection. Original art work by Sylvia Burnett, Mississippi Department of Health. From Edwards et al. (2009).

3.1.2.1 Tick Dissection

Dissection methods generally followed Edwards et al. (2009). Ticks were dissected by first being immobilized, dorsal side up, on carpet tape mounted to a plain glass slide. We removed the dorsal surface of the tick, including the scutum, by applying a scalpel from one side of the capitulum to the other. Phosphate-buffered saline (PBS; pH 7.4) was applied to the inside of the tick to maintain moisture. From male ticks, salivary glands and midgut were removed and placed into separate 1.5 mL microcentrifuge tubes containing 200 μL of PBS then pooled with like organs of up to three ticks from the same treatment group, rabbit, and gender. From female ticks, salivary glands, midgut, and ovaries were removed and separated in the same manner. Thus, from day 0 there were five pools of ticks per gender, from day 6 there were up to 3 pools of ticks per gender,

and from day 12 there were up to 3 pools of ticks per gender for each of the 3 rabbits of each of the 4 treatment groups.

3.1.2.2 Storage of Tissues

The pooled tubes were vortexed briefly and split between three tubes to be utilized in either DNA extraction, paraffin embedding, or TEM. Tubes bound for DNA extraction were stored at -20°C until the time of extraction. We used the Qiagen DNEasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) according to provided instructions. The 100 μL DNA samples were stored at -20°C following extraction.

For paraffin embedding samples, PBS was removed and replaced with 200 μL of 1% agarose to form a "plug" of tissue and agarose in the tip of the tube. These plugs were removed from the tubes and collected into cassettes and stored in 10% neutral buffered formalin prior to paraffin embedding. For male tissues, 2 plugs were placed into each cassette, one from the midgut tissue and the other from the salivary gland tissue of a single pool of ticks. For female tissues, 3 plugs were placed into each cassette, one from the midgut tissue, from the salivary gland tissue, and from the ovary tissue of a single pool of ticks. These cassettes were embedded in paraffin and thin sections cut to slides by the diagnostic lab services at Mississippi State University's College of Veterinary Medicine.

For TEM samples, the PBS was removed and replaced with 1 mL Karnovsky's fixative in 0.1M sodium cacodylate buffer, pH 7.2. These samples were stored at 4°C until use. Some of these samples were later formed into plugs similar to those described for paraffin embedding and stored in 1 mL of 0.5M Karnovsky's fixative in 0.1M

cacodylate buffer, pH 7.2 diluted (1:1) with 0.2M sodium cacodylate buffer, pH 7.2 and stored at 4°C until use.

3.2 Selection of Samples

Initially, samples for visualization via microscopy were chosen at random. However, due to lack of visualization of rickettsiae when initially screening (attributed to the rarity of rickettsiae), we changed our approach. Samples were subsequently selected for high quantities of rickettsiae based on qPCR. For qPCR, we used a TaqMan Multiplex qPCR assay; primers and probes, as well as their gene targets and sequences, are unpublished, and found in Table 1. The first set of primers targeting *rompB*, rickettsial outer membrane protein B gene, targets sequences from both *Rickettsia* species, while the specific TaqMan probes differentiated the two bacteria. The other primers and probe target the macrophage migration inhibitory factor gene of *A. maculatum* to ensure the presence of tick DNA, estimate rickettsial concentrations in tick tissues, and verify all negative samples.

Table 1: qPCR primers and probes

Primer/Probe name	**Gene Target**	**DNA Sequence**
Primer QrompB_F	*rompB*	AAGTGGTACTTCAACATGGG
Primer QrompB_R	*rompB*	GCACCACCTTGGATTAAAG
Probe CaRa FAM	*rompB*	ATCGCGGAAGGTGCTCAAGTTAATG
Probe Rp HEX	*rompB*	ATTTTGGAAGGTGCGCAAGTTAATGC
Primer Amac MIF.18F	MIF	*Provided by K. Macaluso (LSU)*
Primer Amac MIF.99R	MIF	*Provided by K. Macaluso (LSU)*
Probe Amac MIF.63 Cy5	MIF	*Provided by K. Macaluso (LSU)*

The qPCR reactions utilized the Agilent Brilliant Multiplex qPCR Master Mix (Agilent Technologies, Santa Clara, CA). Each reaction contained 300 nM all primers, 400 nM HEX probe, 50 nM FAM probe, and 200nM Cy5 probe as well as a 1:500 dilution of ROX for normalization. In each 25 μL reaction there was also 3 μL of

template DNA. We used a Stratagene (Agilent) MX3005P qPCR Instrument, with samples run in duplicate using the following program: 95˚C for 10 min then 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Each qPCR run included positive controls - dilutions of plasmid DNA template from *R. parkeri* GFP Oktibbeha strain and "*Ca.* R andeanae," and negative, non-template (water) controls.

A sample was considered positive if both replicates had C_T values below 37 cycles and the qPCR efficiencies were between the accepted 90-110%. Some samples were confirmed using conventional PCR. Samples for TEM and FISH/IHC were chosen based on the relative amounts of one or both (if applicable) *Rickettsia* species (>600k copies total).

3.3 Fluorescence *In situ* Hybridization and Immunohistochemistry

During each set of slides processed, controls slides using uninfected or infected cell culture were included alongside sample slides. As controls, two Vero cell culture slides were run – one with both the DIG-labeled probe and the anti-GFPuv antibodies and one without either. Also, two *R. parkeri* GFPuv in Vero culture slides were included – one with both the probe and the anti-GFPuv antibodies and one with the probe only. Lastly, two "*Ca.* R. andeanae" in ISE6 culture slides were run – one with both the probe and the anti-GFPuv antibodies and one with just the anti-GFPuv antibodies only. Two slides were included for each sample as well. Depending on whether the sample was determined to be *R. parkeri* positive, "*Ca.* R. andeanae" positive, or co-infected, the pair of slides would be treated the same as the control slides with *R. parkeri* GFPuv culture slides, "*Ca.* R. andeanae" culture slides, or the Vero only culture slides respectively. All

slides received the anti-DIG antibodies as well as the antibodies conjugated with Cy2 and Cy3.

Slides were kept at 60°C in a waterbath for at least 2 hours to melt paraffin surrounding tissue. Tissue was rehydrated using 5 minute washes in xylene (2 times), 100% ethanol, 95% ethanol, 70% ethanol, and diethylpyrocarbonate (DEPC)-treated water (2 times). DEPC-treatment in the water prevents ribonuclease activity to protect the riboprobe we utilized for detection of "*Ca.* R. andeanae." Tissue was permeabilized in a 10 mM sodium citrate solution, pH 6, for 30 minutes at 100°C. Then, the slides were treated with 0.3% TritonX-100 solution for 20 minutes with gentle agitation. Slides were rinsed twice with 1X PBS made with DEPC-treated water and once more with DEPC-treated water. Next, tissue was permeabilized with 10 μg/mL of Proteinase K for 20 minutes at 37°C. Slides were rinsed once with 1X PBS made with DEPC-treated water for 5 minutes. Next, tissues were fixed in a 4% formaldehyde solution in 0.1M phosphate buffer at 4°C for 10 minutes. Slides were rinsed twice with 1X PBS made with DEPC-treated water and once more with DEPC-treated water for 5 minutes. Next the tissue was acetylated in a 0.1M Triethanolamine solution with gentle agitation for 10 minutes. Slides were rinsed twice with 1X PBS made with DEPC-treated water for 5 minutes.

Tissue was then treated with 70% formamide in 2X SSC (saline sodium citrate buffer) for 10 minutes at 70°C. Then, slides were chilled in 70% ethanol for 10 minutes at -20°C. Next, slides were bathed in 95% ethanol for 5 minutes and 100% ethanol for 5 minutes. The tissue was prehybridized using 100 μL of hybridization buffer (50% formamide, 5X SSC, 0.1 mg/mL fish sperm DNA, 0.1% Tween20, 10% dextran sulfate) at 43°C for at 30-60 minutes. During prehybridization, the DIG-labelled riboprobe was

diluted to 50 ng/μL in the hybridization buffer, denatured for 5 minutes at 95°C, plunged on ice for 5 minutes, mixed, then returned to ice until applied to proper slides. Then, all slides were hybridized overnight at 43°C.

The next morning, slides were rinsed with 4X SSC in DEPC-treated water for 10 minutes. The slides were washed in a 50% formamide in 2X SSC solution while shaking at 100rpm for 10 minutes then for 30 minutes at 44°C in a shaking water bath (Thermo Precision Reciprocal Shaking Bath 2870), with the solution refreshed between washes. The slides were rinsed twice with 2X SSC for 10 minutes then twice with PBS briefly. Next slides were blocked using a 5% BSA (bovine serum albumin) in PBS in DEPC-treated water for 20 minutes. The block was poured of and replaced with a 1:800 dilution of sheep anti-DIG antibody and 1:800 dilution of mouse anti-GFPuv antibody in the same blocking solution. This was incubated for 1 hour. Then, the slides were washed three times for 10 minutes while shaking in PBS. Next, a 1:250 dilution of Cy3 conjugated donkey anti-sheep antibody in the blocking solution was applied to the slides and incubated for 1 hour. Again, slides were washed three times for 10 minutes while shaking in PBS. And finally, a 1:500 dilution of Cy2 conjugated goat anti-mouse antibodies in the blocking solution was applied to the slides and incubated for 1 hour. And again, the slides were washed three times for 10 minutes while shaking in PBS. The slides were dried completely before a mounting medium and coverslip was applied. All slides were visualized using an Olympus BX60 fluorescence microscope under both FITC (for Cy2 detection) and TRITC (for Cy3 detection) light filters when being read. Images were captured from representative samples.

3.4 Transmission Electron Microscopy

Samples for TEM analysis were processed using a slightly modified published protocol from Kocan et al. (1990). Tissue was fixed in 0.1M cacodylate buffer (pH 7.2) with 4 changes over the course of an hour, then 2% OsO_4 in 0.1M cacodylate buffer for 2 hours, 1.5 hours on ice and 30 minutes at room temperature. Then, tissue was rinsed in distilled water with 4 changes over the course of an hour. Then, the tissue was dehydrated in increasing percentage of ethanol from 35% (2 changes over 30 minutes), 50% (2 changes over 30 minutes), 70% (15 minutes), and 70% with 2% uranyl acetate (overnight, for en bloc staining). The next morning, dehydration was continued with 95% ethanol (2 changes over 30 minutes) and 100% (4 changes over an hour). Then tissue was treated with a 1:1 solution of acetone and ethanol (2 changes over 30 minutes), then with full acetone (2 changes over 30 minutes). Following, Spurr's resin was gradually introduced to tissue in acetone, beginning with 25% resin and 75% acetone for 3-4 hours on a rotator. Next, 50% resin with 50% acetone for another 3-4 hours and 75% resin with 25% acetone overnight, both on the rotator. The next morning, the tissue was treated with 100% resin for the entire day and overnight on a rotator, with several changes throughout. The next morning, the resin was replaced a few more times, then the tissue was moved to beam capsules for embedding. Embedding was performed in an oven at 68-70°C overnight.

Thick sections were cut and stained with toluidine blue to inform the cutting of thin sections. Then cut thin sections (60-90μm) were placed on 50-mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined using JEOL

1230 120kV transmission electron microscope for about 30 minutes per sample. Images were captured from representative samples.

CHAPTER 4

RESULTS

A majority of the samples chosen based on qPCR were positive for "*Ca.* R. andeanae" only and come from the day 12 time point. Samples from the other two time points – days 0 and 6 – were also "*Ca.* R. andeanae" positive. Thus, all *R. parkeri* positive samples came from the day 12 time point, as did the two co-infected samples.

From the fluorescence microscopy, we were able to visualize the separate Rickettsiae in multiple, but not all samples. Specifically, the co-infection of one of the two tested co-infected samples was captured in the representative images. This image shows the two bacteria sharing the same tissue while another image reveals tissue that is only fluorescing with Cy2, for *R. parkeri,* in some areas. In the other co-infected sample, images captured only Cy3 fluorescence, for "*Ca.* R. andeanae." Lastly, because there were a majority of "*Ca.* R. andeanae" qPCR positive samples, there were a few images captured of Cy3 fluorescence for those samples. Figure 5 shows some of these representative images.

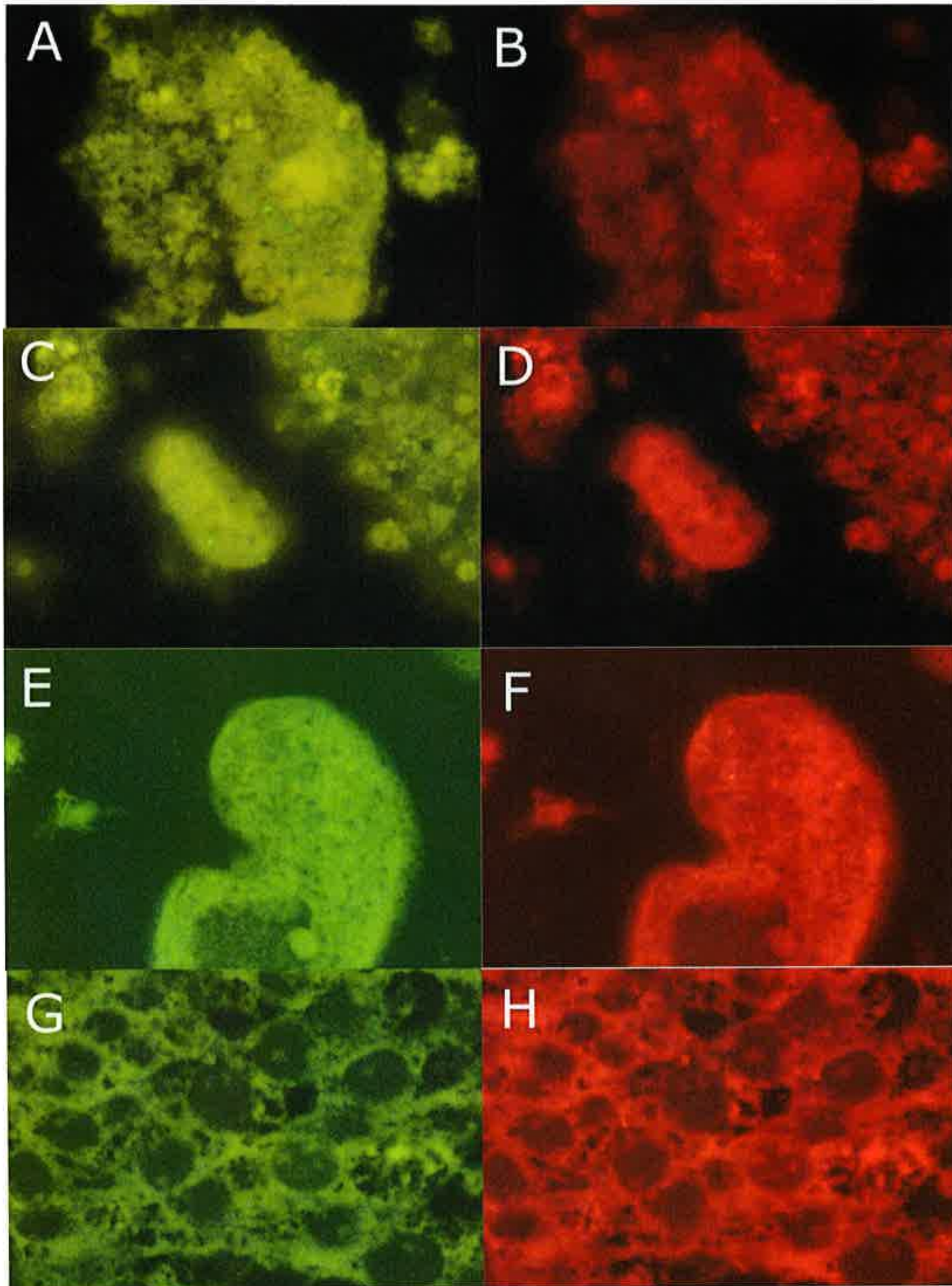

Figure 5: Representative fluorescence images of rickettsiae in co-infected and "*Ca.* R. andeanae" infected samples. (A) FITC showing Cy2 (*R. parkeri*) in co-infected male tissues from day 12 time point. (B) TRITC showing Cy3 ("*Ca.* R. andeanae) in sample from A. (C) FITC showing Cy2 (*R. parkeri*) in sample from A. (D) TRITC showing no Cy3 ("*Ca.* R. andeanae) in sample from A. (E) FITC showing no Cy2 (*R. parkeri*) in co-infected female tissues from day 12 time point. (F) TRITC showing Cy3 ("*Ca.* R. andeanae) in sample from E. (G) FITC showing no Cy2 (*R. parkeri*) in "*Ca.* R. andeanae" infected tissues from day 6 time point. (H) TRITC showing Cy3 ("*Ca.* R. andeanae) in sample from G.

From the TEM, we were able to visualize suspect rickettsiae in each tissue type and from each time point. A majority of these were in the form of a few single bacteria scattered throughout multiple sections on a single grid. For midgut tissues, the most promising sample was from the day 0 time point ("*Ca.* R. andeanae" qPCR positive), in which a small aggregate of bacteria was seen in representative images. For salivary gland tissues, the most promising sample is from the day 12 time point (*R. parkeri* qPCR positive), in which a small aggregate of bacteria was visible in representative images. Finally, for ovary tissues, the most promising samples are from the day 12 time point ("*Ca.* R. andeanae" qPCR positive), in which multiple small aggregates of bacteria were visible in representative images. Figure 6 shows some of these representative images.

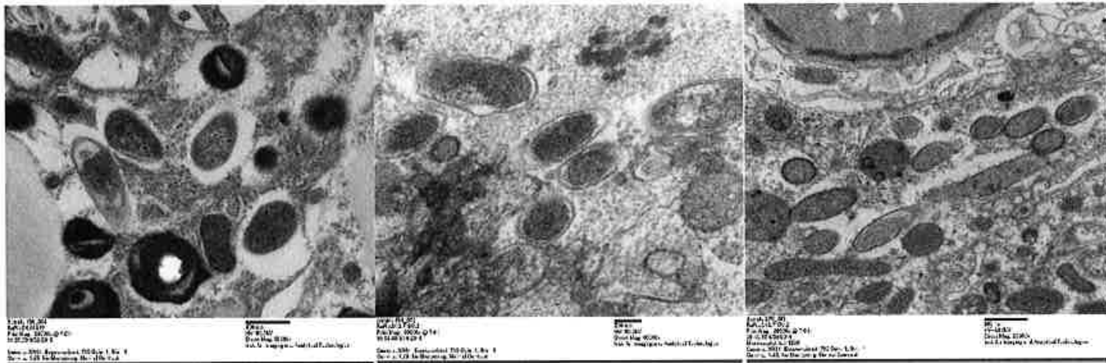

Figure 6: Representative TEM images of rickettsiae aggregates in midgut, salivary gland, and ovary tissues. "*Ca.* R. andeanae" qPCR positive midgut from day 0, scale bar = 400nm (left), *R. parkeri* qPCR positive salivary glands from day 12, scale bar = 200nm (middle), and "*Ca.* R. andeanae" qPCR positive ovaries from day 12 scale bar = 800nm (right)

CHAPTER 5

DISCUSSION

Utilization of fluorescence microscopy methods allowed us to discriminate between the two rickettsiae, while electron microscopy revealed the presence of organisms within the different tissues. Both visualization methods showed, for the most part, sparse bacteria, even with those samples that were most likely to have rickettsiae

based on qPCR. However, TEM revealed multiple "*Ca.* R. andeanae" positive samples with more substantial infections in the ovary tissues, which may indicate a similarity between this bacterium and *R. peacockii*, which is only found in ovary tissues. Furthermore, a single sample of *R. parkeri* infected salivary glands at day 12 with multiple aggregates of bacteria compared to the sparse infections of "*Ca.* R. andeanae" in the two day 12 salivary gland samples seems to support our hypothesis that *R. parkeri* would have a stronger presence in salivary glands. Lastly, as might be expected, midgut samples had heaviest rickettsial infection at the day 0 time point, albeit represented only by a single sample. Because the midgut is the starting point of the rickettsial infection when rickettsiae enter via an infected blood meal (or artificial capillary feeding in our study), this may explain the decreased bacteria in this tissue over time.

It is important to note that, due to the nature of both of these methods, we are limited to seeing a small portion of the tissue. Furthermore, because these ticks were pooled prior to splitting samples between DNA extraction, TEM, and paraffin embedding, there is a chance that only one tick in the pool may have been infected, which would leave less infected tissue to be shared when samples were split.

This project had other challenges. Specifically, in tissue selection, due to lack of microscopic evidence of rickettsiae in unbiased samples, qPCR results were used to inform sample selection. However, qPCR is imperfect because of the assay's inability to distinguish whether there is a live infection in the tissue, as it measures only DNA. In spite of this biased approach to sample selection, rickettsiae were still being visualized inconsistently. Lastly, the preparation of samples for visualization for both methods is time-consuming – with TEM taking around 2 weeks and the fluorescence methods almost

2 days before samples can be viewed – while also being insensitive, due to the small picture each presents of large tissues.

CHAPTER 6

CONCLUSIONS & FUTURE DIRECTIONS

Through this study we were able to microscopically confirm intact rickettsiae in samples with high levels of rickettsial DNA. In doing so, we demonstrated rickettsial presence in samples after up to 12 days of tick feeding in tick organs, especially in ovary tissues. Due to the challenges of both methods and the necessity to use two different methods to gain a whole picture of the co-infection and tropism of these bacteria, we would suggest an alternative method to confirm presence of intact rickettsiae, especially if it were to be used at a larger scale. For this purpose, future studies include exploring biomarkers to target specific tick tissues and specific rickettsiae while still being a high throughput system.

REFERENCES

Alarcon-Chaidez, FJ. "7. Salivary Glands: Structure, Physiology, and Molecular Biology" Biology of Ticks. 2nd ed. Vol. 1. Ed. DE Sonenshine and RM Roe. New York: Oxford University Press, 2014. 163-205.

Blair PJ, J Jiang, GB Schoeler, C Moron, E Anaya, M Cespedes, C Cruz, V Felices, C Guevara, L Mendoza, P Villaseca, JW Sumner, AL Richards, JG Olson (2004). Characterization of Spotted Fever Group Rickettsiae in Flea and Tick Specimens from Northern Peru. Journal of Clinical Microbiology 42: 4861-4967

Burgdorfer W, SF Hayes, AJ Mavros (1981). Nonpathogenic rickettsiae in Dermacentor andersoni: A limiting factor for the distribution of Rickettsia rickettsii. In W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, New York, NY. p. 585-594

Cragun WC, BL Bartlett, MW Ellis, AZ Hoover, SK Tyring, N Mendoza, TJ Vento, WL Nicholson, ME Eremeeva, JP Olano, RP Rapini, CD Paddock (2010). The Expanding Spectrum of Eschar-Associated Rickettsioses in the United States. Archives of Dermatology 146: 641-648.

Edwards KT, J Goddard, AS Varela-Stokes (2009). Examination of the Internal Morphology of the Ixodid Tick, Amblyomma maculatum Koch, (Acari: Ixodidae); a "How-to" Pictorial Dissection Guide. Midsouth Entomologist 2: 28-39.

Embers ME, BJ Grasperge, MB Jacobs, MT Phillipp (2013). Feeding of Ticks on Animals for Transmission and Xenodiagnosis in Lyme Disease Research. Journal of Visualized Experiments 78: e50617.

Ferrari FAG, J Goddard, CD Paddock, AS Varela-Stokes (2012). Rickettsia parkeri and Candidatus Rickettsia andeanae in Gulf Coast Ticks, Mississippi, USA. Emerging Infectious Diseases 18: 1705-1707.

Ferrari FAG, J Goddard, GM Moraru, WEC Smith, AS Varela-Stokes (2013). Isolation of "Candidatus Rickettsia andeanae" (Rickettsiales: Rickettsiaceae) in Embryonic Cells of Naturally Infected Amblyomma maculatum (Ixodida: Ixodidae). Journal of Medical Entomology 50: 1118-1125.

Ferrari FAG, J Goddard, CD Paddock, AS Varela-Stokes (2014). Ultrastructure of Presumed "Candidatus Rickettsia andeanae" in Amblyomma maculatum (Acari: Ixodidae). Journal of Medical Entomology 51: 1317-1321.

Fornadel CM, X Zhang, JD Smith, CD Paddock, JR Arias, DE Norris (2011). High Rates of Rickettsia parkeri infection in Gulf Coast Ticks (Amblyomma maculatum) and Identification of "Candidatus Rickettsia andeanae" from Fairfax County, Virginia. Vector-borne and Zoonotic Diseases 11: 1535-1539.

Goddard J and A Varela-Stokes (2008). The Discovery and Pursuit of American Boutonneuse Fever: A New Spotted Fever Group Rickettsiosis. Midsouth Entomologist 2: 47-52

Hayes SF and W Burgdorfer (1979). Ultrastructure of Rickettsia rhipicephali, a New Member of the Spotted Fever Group Rickettsiae in Tissues of the Host Vector Rhipicephalus sanguineus. Journal of Bacteriology 137: 605-613.

Herrick KL, SA Pena HD Yaglom, BJ Layton, A Moors, AD Loftis, ME Condit, J Singleton, CY Kato, AM Denison, D Ng, JW Mertins, CD Paddock (2016).

Rickettsia parkeri Rickettsiosis, Arizona, USA. *Emerging Infectious Diseases* 22: 780-785.

Kocan KM, TB Crawford, PM Dilbeck, JF Evermann, TC McGuire (1990) Development of a Rickettsia Isolated from an Aborted Bovine Fetus. Journal of Bacteriology 172: 5949-5955.

Kurtti TJ, JA Simser, GD Baldridge, AT Palmer, UG Munderloh (2005). Factors influencing in vitro infectivity and growth of Rickettsia peacockii (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, Dermacentor andersoni (Acari, Ixodidae). Journal of Invertebrate Pathology 90: 177-186.

Nadolny RM., CL Wright, DE Sonenshine, WL Hynes, HD Gaff (2014). Ticks and spotted fever group rickettsiae of southeastern Virginia. Ticks and Tick-borne Diseases 5: 53-57.

Paddock CD, JW Sumner, JA Comer, SR Zaki, CS Goldsmith, J Goddard, SLF McLellan, CL Tamminga, CA Ohl (2004). Rickettsia parkeri: A Newly Recognized Cause of Spotted Fever Rickettsiosis in the United States. Clinical Infectious Diseases 38: 805-811.

Paddock CD, RG Finley, CS Wright, HN Ribonson, BJ Schrodt, CC Lane, O Ekenna, MA Blass, CL Tamminga, CA Ohl, SLF McLellan, J Goddard, RC Holman, JJ Openshaw, JW Sumner, SR Zaki, ME Eremeeva (2008). Rickettsia parkeri Rickettsiosis and Its Clinical Distinction from Rocky Mountain Spotted Fever. Clinical Infectious Diseases 47: 1188-1196.

Paddock CD, P-E Fournier, JW Sumner, J Goddard, Y Elshenawy, MG Metcalfe, AD Loftis, A Varela-Stokes (2010). Isolation of Rickettsia parkeri and Identification

of Novel Spotted Fever Group Rickettsia sp. from Gulf Coast Ticks (Amblyomma maculatum) in the United States. Applied and Environmental Microbiology 76: 2689-2696.

Paddock CD, AM Denison, MW Dryden, BH Noden, RR Lash, SS Abdelghani, AE Evans, AR Kelly, JA Hecht, SE Karpathy, RR Ganta, SE Little (2015). High prevalence of "*Candidatus* Rickettsia andeanae" and apparent exclusion of *Rickettsia parkeri* in adult *Amblyomma maculatum* (Acari: Ixodidae) form Kansas and Oklahoma. *Ticks and Tick-borne Diseases* 6: 297-302.

Parker RR, GM Kohls, GW Cox, GE Davis (1939). Observations on an infectious agent from Amblyomma maculatum. *Public Health Reports* 54: 1482-1484.

Perlman SJ, MS Hunter, E Zchori-Fein (2006). The emerging diversity of Rickettsia. Proceedings of the Royal Society B 273: 2097-2106.

Raoult D and CD Paddock (2005) Rickettsia parkeri Infection and Other Spotted Fevers in the United States. The New England Journal of Medicine 353: 626-627.

Sonenshine DE and JM Anderson. "6. Mouthparts and Digestive System: Anatomy and Molecular Biology of Feeding and Digestion" Biology of Ticks. 2nd ed. Vol. 1. Ed. DE Sonenshine and RM Roe. New York: Oxford University Press, 2014. 122-162.

Sumner JW, LA Durden, J Goddard, AY Stromdahl, KL Clark, WK Reeves, CD Paddock (2007). Gulf Coast Ticks (Amblyomma maculatum) and Rickettsia parkeri, United States. Emerging Infectious Diseases 13: 751-753.

Varela-Stokes AS, CD Paddock, B Engber, M Toliver (2011) Rickettsia parkeri in Amblyomma maculatum ticks, North Carolina, USA, 2009-2010. Emerging Infectious Diseases 17: 2350-2353.

Whitman TJ, AL Richards, CD Paddock, CL Tamminga, PJ Sniezek, J Jiang, DK Byers, JW Sanders (2007). Rickettsia parkeri Infection after Tick Bite, Virginia. Emerging Infectious Diseases 13: 334-336.