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DEVELOPMENT AND CHARACTERIZATION OF A MURINE MODEL OF
RICKETTSIA PARKERI RICKETTSIOSIS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
Through the Department of Pathobiological Sciences

by

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ABSTRACT

Rickettsia parkeri, a member of the spotted fever group of *Rickettsia*, is the agent of an emerging rickettsiosis in the southeastern United States and South America. Despite increased recognition of human cases, limited information is available regarding infection of invertebrate and vertebrate hosts for this emerging tick-borne disease. Towards development of a viable transmission model and to further characterize the pathology associated with *R. parkeri* infection, inbred mouse strains (A/J, Balb/C, C3H/HeJ, and C3H/HeN) were intravenously and intradermally inoculated with *R. parkeri*. The C3H/HeJ strain of mice were identified as the most susceptible to *R. parkeri* infection and were found to develop eschar-like lesion at the site of intradermal inoculation in the tail. These mice were further utilized to test the effect of tick feeding on the proliferation of *R. parkeri* at the intradermal inoculation site. Ticks were allowed to feed over the site of intradermal inoculation of *R. parkeri* at the nape of the neck. Rickettsial proliferation was significantly increased by tick feeding, suggesting a role for ticks as more than just fomites. Finally, the natural ecology of *R. parkeri* was investigated by screening domestic dogs in temporary housing situations for the presence of *Rickettsia* using PCR for the genus specific 17 kDa antigen gene. The more specific primers for *rompA* were utilized for PCR on the 12 positive samples identified by the screening PCR. After sequencing, the *rompA* amplicons were identified as *R. parkeri*, indicating a role for dogs in the ecology of *R. parkeri* and as a potential risk factor for development of human disease. Continued study into the pathogenesis of *R. parkeri* rickettsiosis in the murine model, the influence of tick saliva on rickettsial proliferation, and the role of dogs in the natural ecology of *R. parkeri* will lead to a better understanding of this emerging tick-borne rickettsiosis.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 General Rickettsiology

In this section general information in regards to the genus *Rickettsia* and more specifically the spotted fever group (SFG) of *Rickettsia* is provided. Within the SFG, the rickettsial outer membrane protein A (rOmpA), tick transmission, and transmission to the vertebrate host is discussed. Next, emerging rickettsioses, with special focus on those present within the United States, are reviewed. Discussion of novel transmission cycles for a well-described rickettsiosis is also presented in this subsection along with a detailed description of the emerging rickettsiosis caused by *Rickettsia parkeri*. Finally, this section concludes with an in-depth description of *R. parkeri* and a comparison to other members of the SFG of *Rickettsia*.

Members of the genus *Rickettsia* are placed in the family *Rickettsiaceae* within the order *Rickettsiales* (Dumler et al. 2001). Members of the genus *Rickettsia* are Gram-negative, obligate intracellular alphaproteobacteria which require plant, amoebae, arthropod, annelid or vertebrate hosts for survival (Noda et al. 1997; Davis et al. 1998; Kikuchi et al. 2002; Dykova et al. 2003; Ogata et al. 2006). The pathogenic members of this genus primarily infect the endothelial cells of small to medium sized blood vessels. After phagocytosis and internalization, the vacuole containing the rickettsiae is rapidly lysed and the bacteria escape into the host cytoplasm (Walker et al. 2003). These infections result in a vasculitis, which is responsible for the clinical and laboratory abnormalities typical of tick-borne rickettsioses (Parola et al. 2005).

1.1.1 Groups within the Genus *Rickettsia*

The genus *Rickettsia* is divided into four groups based on molecular and antigenic characteristics: spotted fever, typhus, ancestral, and transitional (Figure 1.1) (Gillespie et al. 2007). Members of the genus *Rickettsia* were traditionally grouped into the spotted fever,

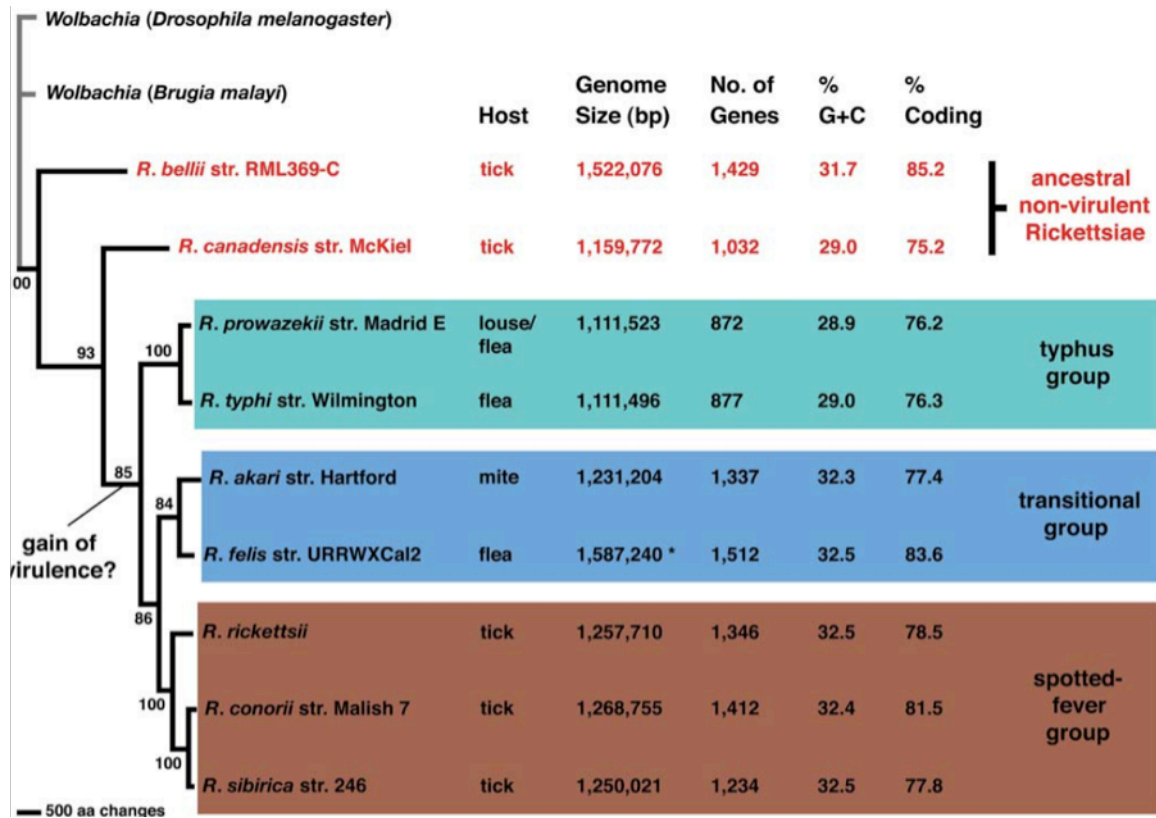


Figure 1.1. Phylogeny estimation from analysis of fifteen *Rickettsia felis* proteins. Phylogeny estimation under parsimony of fifteen *R. felis* proteins from nine rickettsial species (*Rickettsia bellii*, *R. canadensis*, *R. prowazekii*, *R. typhi*, *R. akari*, *R. felis*, *R. conorii*, *R. rickettsii*, and *R. sibirica*) and two strains of *Wolbachia*. Branch support is from one million bootstrap replicates. Genome information was compiled from the PATRIC Website.(Gillespie et al. 2007)

typhus, and ancestral groups; however, the phylogenomic and bioinformatic evaluation of the genomes of nine separate rickettsial species led to the proposal of the transitional group (Gillespie et al. 2007). The driving impetus for the reclassification of several rickettsial species into this new group was the discovery of rickettsial plasmids, which appear to have allowed transfer of genetic material among the groups of *Rickettsia* and resulted in species that bare characteristics of both the spotted fever and typhus groups (Ogata et al. 2005). For example, *Rickettsia felis*, which was originally classified as a SFG *Rickettsia*, is now placed in the transitional group. Interestingly, *R. felis* shows characteristics of both the spotted fever and typhus groups as it is primarily associated with an insect rather than a tick like typhus group *Rickettsia*, displays hemolytic activity similar to members of the typhus group, possesses the capacity for actin-based motility common to the SFG, is maintained transovarially within the vector similar to the SFG, and exhibits serological cross-reactivity with members of the SFG (Gillespie et al. 2007). Since the discovery of plasmids in *R. felis*, many other rickettsial species have been found to contain plasmids, though studies with some pathogenic *Rickettsia* such as *Rickettsia rickettsii*, *Rickettsia conorii*, and *R. parkeri* have not yet revealed the presence of plasmids (Baldrige et al. 2010).

1.1.2 Spotted Fever Group *Rickettsia*

Members of the SFG include both pathogenic and apparently non-pathogenic *Rickettsia*, which includes *Rickettsia montanensis* and *Rickettsia peacockii* (Walker and Ismail 2008). Members of this group share several defining characteristics including: expression of rOmpA, reside in tick vectors, are maintained transovarially and transstadially within the vectors, and utilize actin based motility to move within the host cell (Gillespie et al. 2007). Within the United States of America there are currently four indigenous species of SFG *Rickettsia*, which are

known to be pathogenic to humans: *R. rickettsii*, *R. parkeri*, *Rickettsia* species 364D, and *Rickettsia massiliae* (Cragun et al. 2010). As will be discussed later, however, the number of recognized pathogenic rickettsial species is likely to continue to climb.

The rOmpA protein is a member of the surface cell antigens (Sca0) expressed on the outer membrane of rickettsial species (Chan et al. 2010) and is the major characteristic antigen of the SFG as it is absent or truncated in typhus group *Rickettsia*. These Sca proteins resemble autotransporter proteins present in other Gram-negative bacteria and have modular structures (Chan et al. 2010). The proteins are secreted across the inner membrane and insert into the outer membrane to form a β -barrel-rich transmembrane pore (Chan et al. 2010). The exact function of rOmpA is not entirely clear, but some association with rickettsial adherence to host cells has been demonstrated (Li and Walker 1998).

1.1.2.a Tick Transmission of SFG *Rickettsia*

Due in large part to their capacity for transovarial and transstadial transmission of SFG *Rickettsia*, ticks are often thought to be the main reservoir for these bacteria in nature (Figure 1.2). These vertical transmission routes are very efficient for many *Rickettsia* infected ticks. Although this may hold true for some species of *Rickettsia*, ticks as primary reservoirs for all SFG *Rickettsia* have not been confirmed for the majority of pathogenic rickettsial species. The concept of tick reservoirs is probably so prevalent due to limited study on vector competence and the lack of information regarding vertebrate reservoirs and amplification hosts for rickettsial species. The transstadial transmission (from one life cycle stage to the next through the molting process) of rickettsiae by ticks is vital to ixodid (hard) ticks ability to transmit the infectious agent (Socolovschi et al. 2009). This is important because unlike argasid (soft) ticks, ixodid ticks only feed once per life cycle stage meaning that if the infection with *Rickettsia* is not

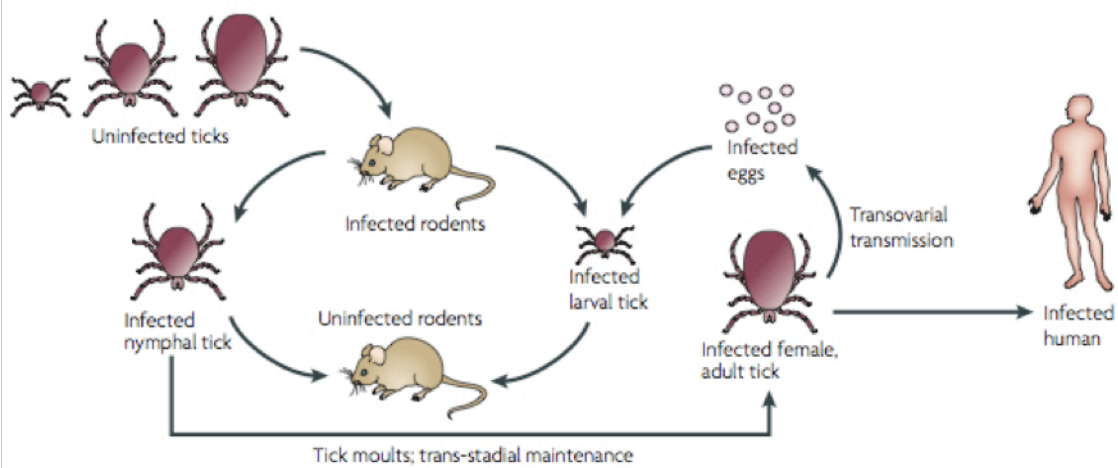


Figure 1.2. The life cycle of tick-borne rickettsiae. Spotted fever group rickettsiae are maintained in nature by transovarial and transstadial transmission in ticks and horizontal transmission to uninfected tick that feed on rickettsemic rodents and other animals (Walker and Ismail 2008).

maintained from one life stage to the next, the tick will be unable to transmit the bacteria to vertebrate hosts during subsequent feeding cycles. The mechanism of infection for ticks is via feeding on an infected animal; however, co-feeding, or feeding on the same area of an animal as an infected tick and sexual transmission have also been shown to result in infection of naive ticks (Philip 1959; Zemtsova et al. 2010). The process of blood feeding on a rickettsemic host forces *Rickettsia* to adapt to changing environmental conditions that are coupled with the tick metabolism of the blood meal. There is a strong need to characterize the relationship of ticks and SFG *Rickettsia* in regards to the mechanisms of both vertical and horizontal transmission.

1.1.2.b Transmission to the Vertebrate Host

Rickettsial entry into the endothelial cells of mammalian hosts is the result of induced phagocytosis (Figure 1.3). This process is controlled by the *Rickettsia* and requires functional host cell cytoskeletal actin (Mansueto et al. 2012). Although multiple rickettsial proteins appear to play some role in invasion of host cells, rickettsial outer membrane protein B (rOmpB) has been specifically identified as a ligand for the host cell Ku70 protein, which is a component of the DNA-dependent protein kinase that is translocated from the nucleus to the cytoplasm and plasma membrane (Martinez et al. 2005).

As previously stated, rOmpA appears to play a role in the cell adhesion process, as do other Sca proteins such as Sca1 and Sca2 (Martinez et al. 2005). It appears that the binding of rOmpB and Ku70 leads to activation of Ku70 and a subsequent signaling cascade that facilitates rickettsial entry. It cannot be overemphasized that this work is done utilizing *in vitro* models of infection; therefore, appropriate *in vivo* models are essential to confirming the validity of these findings.

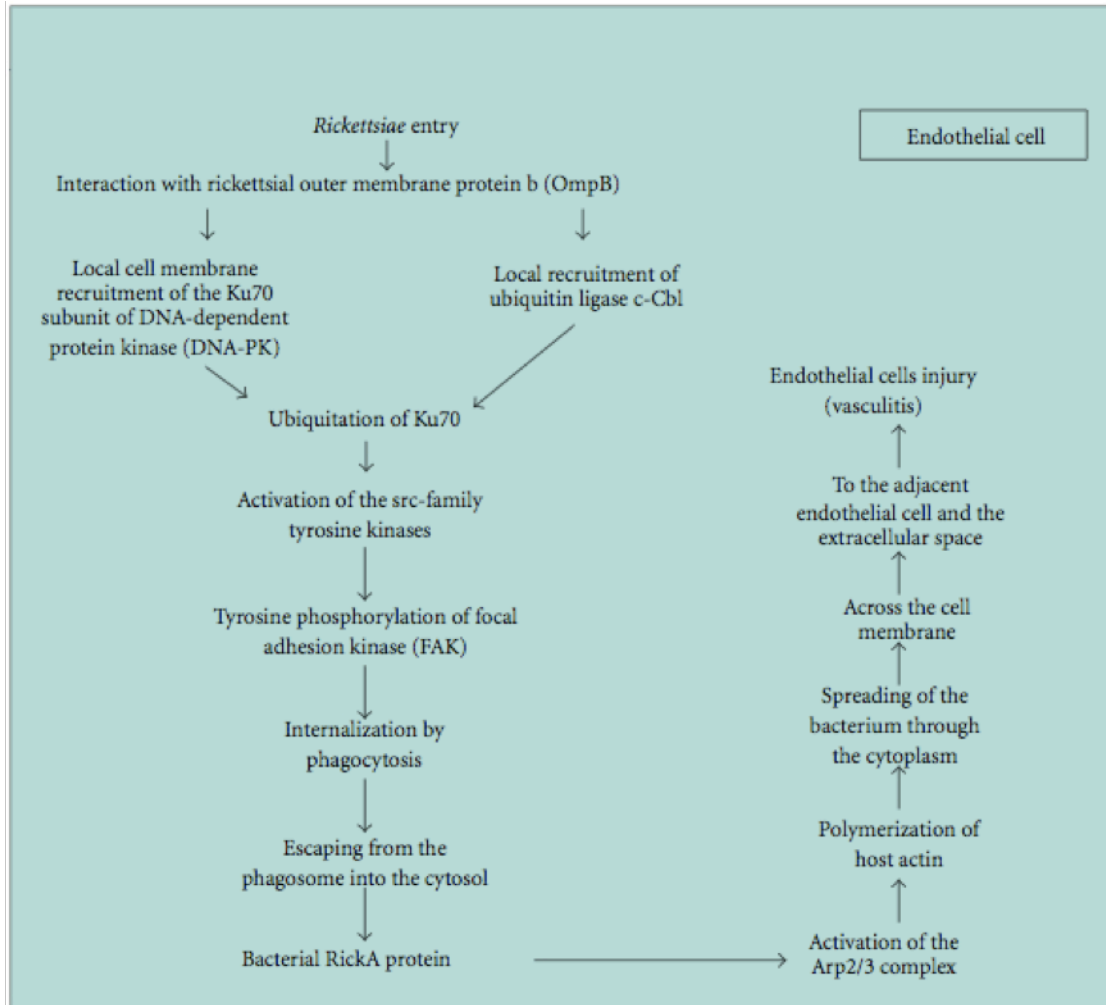


Figure 1.3. Rickettsial entry into endothelial cells (Mansueto et al. 2012).

Upon phagocytosis by the host cell, SFG *Rickettsia* escape from the phagosome prior to phagolysosomal fusion in order to avoid killing by oxidative burst, production of hydrogen peroxide and/or tryptophan degradation (Feng and Walker 2000). This escape involves the secretion of phospholipases, which are capable of disrupting the membrane of the phagosome and resulting in release of the bacteria into the cytosol. Within the cytosol, rickettsiae replicate and, in the case of the SFG *Rickettsia*, utilize the host cell actin cytoskeleton to move within the cell. The mechanisms involved have not been definitively determined, but there is evidence that the Sca2 protein of SFG *Rickettsia* is capable of recruiting and activating the host cell Arp2/3 complex, which then induces directional actin polymerization (Haglund et al. 2010). The rickettsiae utilize this propulsion to reach the inner surface of the host cell cytoplasmic membrane, which is then deformed outward into the adjacent cell forming a bridge by which the bacteria avoid reentering the extracellular space. This is an effective adaptation to avoid host immune mechanisms by not having to leave the intracellular space and is in stark contrast to the typhus group in which the rickettsiae simply multiply to the point of rupturing the host cell. The infectious process at the tick host interface has not been examined, and this topic requires additional research.

1.1.3 Emerging Rickettsioses

Over the last 30 years, at least 12 species of *Rickettsia* have been confirmed as human pathogens, including *R. parkeri* (Table 1.1), and these pathogens together encompass all continents save Antarctica (Paddock 2009). While these numbers alone are impressive, the prevalence of disease associated with other rickettsial species is staggering. For example, *R. felis* DNA was identified in greater than 4% of Senegalese patients hospitalized with fever that was not due to malaria, representing a large proportion of febrile patients that can now be attributed

Table 1.1. Interval between discovery of selected rickettsiae and confirmation of these agents as pathogens of humans (Paddock 2009).

Agent	Year of discovery (initial designation)	Year reported as a confirmed pathogen (interval from discovery)	Initial diagnosis of index patient(s)
<i>Rickettsia parkeri</i>	1937 (maculatum agent)	2004 (67)	Rickettsialpox
<i>Rickettsia honei</i>	1962 (IT-118)	1992 (30)	Queensland tick typhus
<i>Rickettsia slovaca</i>	1968 (strains B, D)	1997 (29)	Lyme borreliosis
<i>Rickettsia felis</i>	1990 (ELB agent)	1994 (4)	Murine typhus
<i>Rickettsia massiliae</i>	1992 (strains Mtu1, Mtu5)	2006 (14)	Mediterranean spotted fever
<i>Rickettsia aeschlimannii</i>	1995 (strain PoTiR8)	2002 (7)	Mediterranean spotted fever
<i>Rickettsia raoultii</i>	1999 (genotypes RpA4, DnS14, DnS28)	2006 (7)	Tick-borne lymphadenopathy
<i>Rickettsia monacensis</i>	2002 (<i>R. monacensis</i>)	2007 (5)	Mediterranean spotted fever

to one disease (Richards et al. 2010; Socolovschi et al. 2010; Parola 2011). The conundrum is that many of these emerging rickettsioses are underestimated or simply attributed to better described diseases. This poses a challenge to rickettsiologists as the field grows in that it becomes imperative to pursue avenues of research to better understand these emerging infectious agents and to prepare for the discovery of new pathologic agents in the near future.

1.1.3.a Emerging Rickettsioses within the United States

Within the United States alone, the importance of emerging rickettsioses is clear. Evidence of misdiagnosis is apparent when one considers the current reported case fatality rate of Rocky Mountain spotted fever (RMSF) of 1.4%, which is drastically below the historical fatality rate (Paddock 2009). The likely culprit in this particular incidence is the misdiagnosis of emerging diseases such those caused by *R. parkeri* and *Rickettsia* strain 364D, which both have strong cross-reactivity with *R. rickettsii* when utilizing SFG-specific serologic assays.

The overall importance of studying emerging rickettsial pathogens is three-fold (Paddock 2009). These emerging pathogens first provide a lens through which “old” rickettsioses are more accurately represented; they compete with more virulent SFG *Rickettsia* for limited niches within arthropod hosts due to the phenomenon of rickettsial interference, and; they potentially immunize vertebrate hosts involved in the circulation of highly pathogenic *Rickettsia* in nature (Paddock 2009). It is imperative to recognize these emerging tick-borne illnesses in order to define a true clinical spectrum for each particular rickettsiosis (Paddock 2005). As stated previously, it has been postulated that cases of “subclinical” or “mild” RMSF may occur rarely, if ever, and that variations in case-fatality rate have been artifactually influenced by inclusion of inaccurately classified cases (Paddock 2005). The implications are that these emerging rickettsioses are almost certainly much more prevalent than is currently recognized.

1.1.3.b Changing Epidemiology of Rocky Mountain Spotted Fever

While these emerging rickettsioses are of great importance, the changing ecology of established rickettsioses cannot be ignored. Historically, RMSF was associated with *Dermacentor andersoni* ticks and more recently *Dermacentor variabilis*. This shift in principal vector explains the accompanying shift in geography of RMSF to the south and eastern United States where *D. variabilis* is more common. The emergence of RMSF in Arizona was described in 2003 and led to epidemiologic research pointing to domestic dogs and *Rhipicephalus sanguineus* as the primary vectors. Dogs from widely separated subdivisions tested positive for *R. rickettsii* with a high prevalence, which averaged 62% across all populations (Nicholson et al. 2006). The identification of this novel transmission cycle for RMSF is alarming as it involves a very common tick, which is in frequent close contact with humans. Extrapolating to other *Rickettsia*, it becomes evident that epidemiologic surveys of possible vertebrate and arthropod hosts are essential to monitoring for risk factors to the human population and as sentinels of the movements of these diseases, and also establishes the domestic dog as a possible stepping-stone to allow the establishment of rickettsial infections in previously unrecognized arthropod hosts.

1.1.3.c Emerging Rickettsiosis Attributed to *R. parkeri*

Although Parker suggested the possibility of pathogenicity to humans when the organism was first discovered, it was not until 2004 that the first confirmed case of disease caused by *R. parkeri* was reported (Parker et al. 1939; Paddock et al. 2004). During this period, it is probable that disease due to *R. parkeri* went largely misdiagnosed as the more severe RMSF, and in fact most emerging rickettsioses circulate in human populations long before they are discovered as unique infections (Table 1.1) (Paddock 2009). It is postulated that in South America many *R. parkeri* infections are likely to be misdiagnosed as other infectious diseases as well, including

RMSF, dengue, or leptospirosis, all of which present with fever, rash and myalgias (Romer et al. 2011).

The index patient for infection with *R. parkeri* was a 40 year old male who presented in August 2002 from a suburban area of southeast Virginia (Paddock et al. 2004). Clinical signs at the time of presentation included fever, mild headache, malaise, diffuse myalgias and arthralgias, and multiple eschars on his lower extremities (Figure 1.4), while four days earlier small erythematous papules appeared and developed into pustules which quickly ulcerated, and the patient developed lymphadenopathy (Paddock et al. 2004). With the failure of the initial treatment with amoxicillin- clavulanic acid, the antibiotics were switched for cephalexin, which was also ineffective. Consultation with an infectious disease specialist yielded a diagnosis of rickettsialpox, and treatment with doxycycline resolved all signs within one week (Paddock et al. 2004). One of the eschars was biopsied and submitted for histopathological evaluation, polymerase chain reaction (PCR) analysis of the rickettsial 17 kDa antigen, citrate synthase, and the rOmpA (*rompA*) genes, and culture isolation. The histopathology revealed a marked lymphohistiocytic perivascular infiltrate (Figure 1.5), while the PCR was 100% identical to *R. parkeri* (Paddock et al. 2004). With the recognition of the pathogenic potential for *R. parkeri*, further studies ensued including the use of Western blot techniques, which quickly implicated *R. parkeri* as the causative agent of several previously diagnosed cases of rickettsiosis in the United States (Raoult and Paddock 2005). Additionally, a retrospective study of samples submitted between 1998 and 2007 to the Centers for Disease Control and Prevention identified 12 more either confirmed or probable cases of *R. parkeri* rickettsiosis (Paddock et al. 2008). By 2010, more than 20 cases of *R. parkeri* rickettsiosis had been identified in Alabama, Florida, Kentucky, Maryland, Mississippi, North Carolina, South Carolina, Virginia, and Texas as well as two

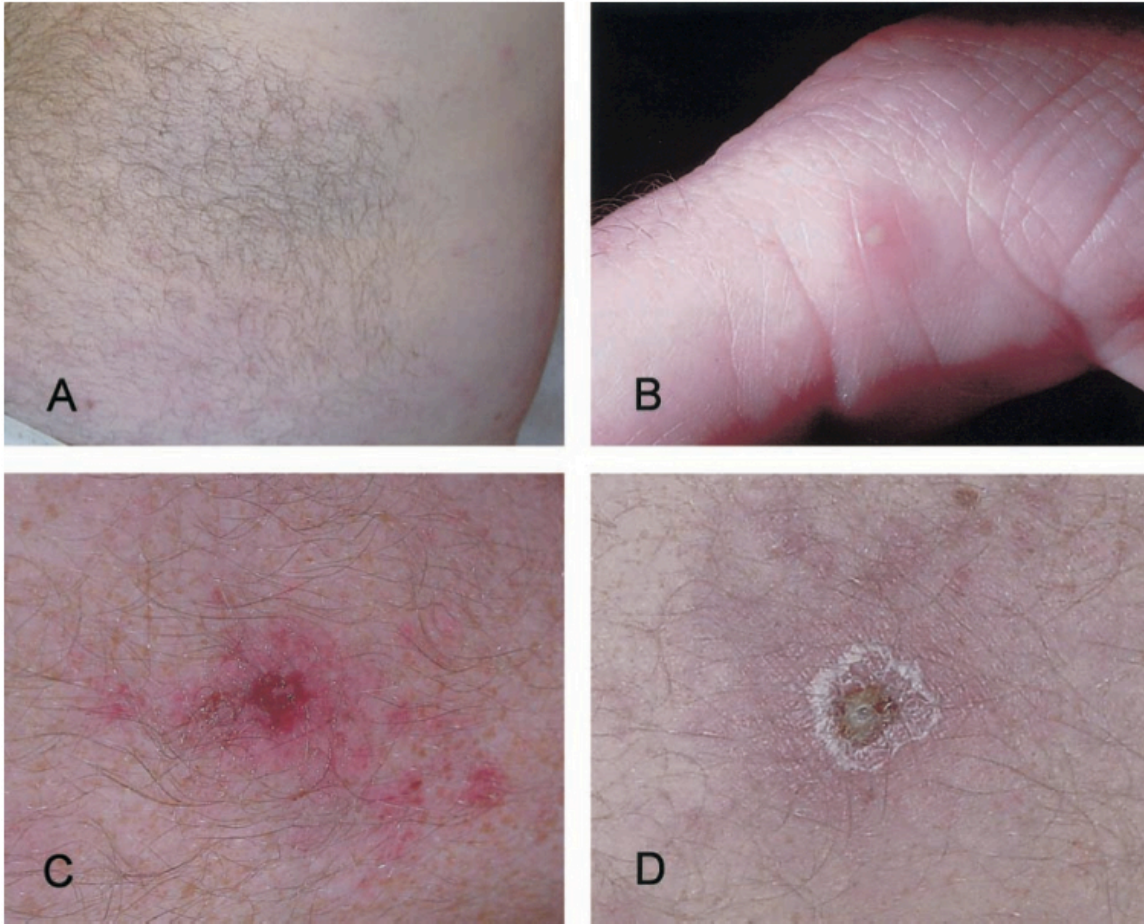


Figure 1.4. Cutaneous lesions in a patient infected with *Rickettsia parkeri*. *A*, A diffuse, pink macular rash involving the abdomen. *B*, A small pustule on the medial aspect of the first digit. *C* and *D*, Eschars located on the pretibial aspects of the right and left lower legs, respectively (Paddock et al. 2004).

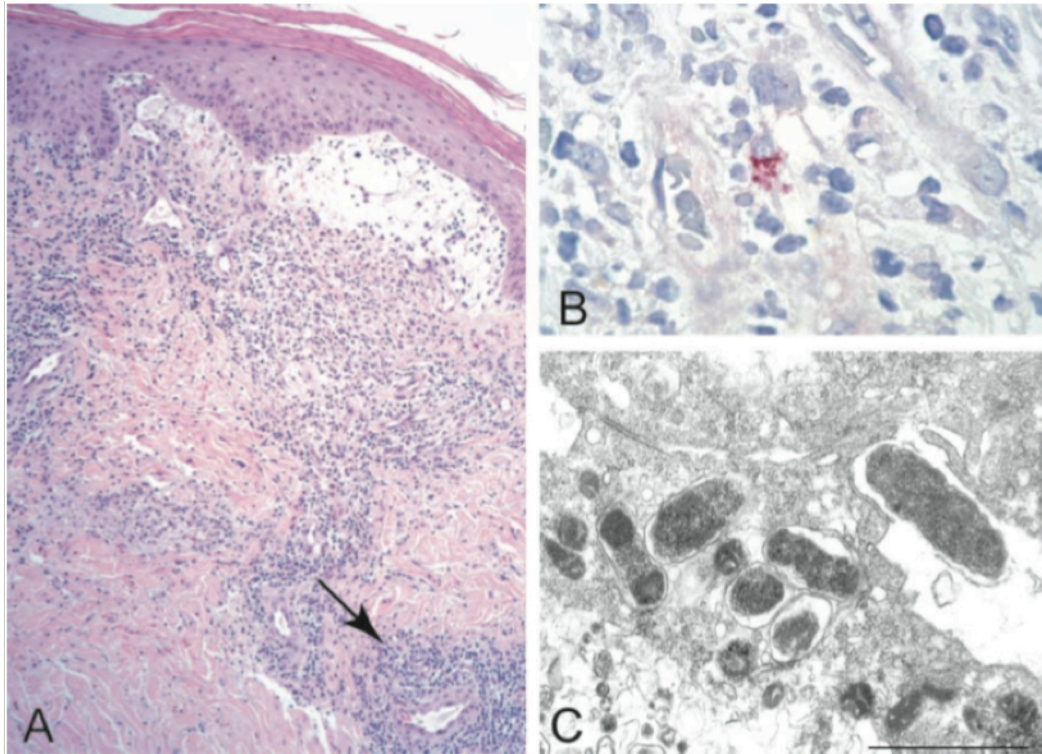


Figure 1.5. Histopathologic and immunohistochemical evaluation of a biopsy specimen from the margin of an eschar. *A*, Lymphohistiocytic perivascular infiltrates (arrow, representative focus). *B*, Immunohistochemical staining of SFG rickettsiae (red) in the cytoplasm of a cell. *C*, Ovoid and rod-shaped bacteria in the cytoplasm of a Vero E6 cell (Paddock et al. 2004).

confirmed and an additional seven suspected cases in Argentina (Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Paddock et al. 2010; Romer et al. 2011). Over the course of these various reports, the disease has been referred to as “Tidewater spotted fever,” “American boutonneuse fever,” “Maculatum rickettsiosis,” and “*R. parkeri* rickettsiosis” with the latter being the most widely accepted of the four (Jiang et al. 2012).

The disease manifestations of the first report were similar to clinical descriptions of African tick-bite fever, caused by *R. africae*. Additionally, the appearance of multiple eschars has also been reported in the rickettsiosis caused by *Rickettsia sibirica* subsp. *mongolotimonae* (Paddock et al. 2004; Parola et al. 2005). Although the index patient of *R. parkeri* rickettsiosis had no identifiable tick bite, the occurrence of multiple eschars was speculated to be the result of parasitism by *R. parkeri* infected larval *Amblyomma maculatum* (Paddock et al. 2004). The second confirmed case of *R. parkeri* rickettsiosis was unequivocally associated with a tick bite (Whitman et al. 2007). Furthermore, the patient was confident that the tick had been attached for less than eight hours, suggesting the ability of *R. parkeri* to infect a human host very rapidly after tick attachment (Whitman et al. 2007). This is in stark contrast to other tick-borne bacteria such as *Borrelia*, which have been shown to require 24 hours of tick attachment or more before the infection can be transmitted (Piesman et al. 1987). This leads to serious implications regarding prophylactic antibiotic treatment recommendations after tick bites in humans and is therefore necessary to further evaluate.

1.1.3.d Description of *R. parkeri*

Rickettsia parkeri was originally described in 1939 by Dr. Ralph Parker (Parker et al. 1939). Initially termed the “maculatum agent,” it was later named *Rickettsia parkeri* in honor of Dr. Parker in 1965 (Lackman et al. 1965). The original isolate of *R. parkeri* was from *A.*

maculatum ticks (Gulf Coast ticks) collected from cows in Liberty County, Texas (Parker et al. 1939). Early isolates were also derived from *A. maculatum* ticks from Georgia in 1938 and Mississippi in 1948 (Lackman et al. 1949). While *R. parkeri* has been molecularly identified in *Amblyomma americanum* ticks, *A. maculatum* is generally accepted as the primary vector for *R. parkeri* (Table 1.2). Additionally, *R. parkeri* has been detected in *Amblyomma triste* ticks from Uruguay and Brazil and *Amblyomma dubitatum* ticks from Brazil (Venzal et al. 2004; Silveira et al. 2007; Cohen et al. 2009; Horta et al. 2010).

Dr. Parker's initial studies involved the use of guinea pigs as experimental hosts for his newly described *Rickettsia*. The animals were intraperitoneally injected with the original tick homogenate, followed by serial passage into new guinea pigs by intraperitoneal injections of testicular washings (Parker et al. 1939). The guinea pigs displayed mild fever, edema and reddening of the scrotum, and splenomegaly of two to three times the normal size (Parker et al. 1939). These clinical signs were later recapitulated when guinea pigs were exposed to experimentally infected *A. americanum* ticks (Goddard 2003). Parker went on to describe illness of varying severity in other animals including monkeys, rabbits, white rats and the Sawatch meadow mouse (Parker 1940). It was also noted that with passage, *R. parkeri* became less virulent with many animals showing no signs of illness (Parker et al. 1939).

The morphology was originally described by Parker as more comparable to RMSF group than to typhus group, and later described in more detail by Lackman as a small, rod-shaped bacterium with an average size of $1.6 \times 0.5 \mu\text{m}$, found in the nucleus and cytoplasm of infected cells (Parker et al. 1939; Lackman et al. 1965). The organism was also noted to result in perfect reciprocal cross immunity with RMSF and boutonneuse fever and was therefore placed in the

Table 1.2. Rickettsial infections in *A. maculatum* in the United States of America

SFG <i>Rickettsia</i>	Percent infected	Region or state	Reference
<i>Rickettsia parkeri</i>	20 – 33	NC	(Varela-Stokes et al. 2011)
	40	MS, FL	(Paddock et al. 2010)
	12	FL, GA, KT, MS, OK, SC	(Sumner et al. 2007)
	1	AR	(Trout et al. 2010)
	0	TX	(Williamson et al. 2010)
	11	NC	(Smith et al. 2010)
<i>Rickettsia amblyommii</i>	28	AR	(Trout et al. 2010)
<i>Candidatus Rickettsia andeanae</i>	10	KS, OK	(Jiang et al. 2012)
	1 – 5	MS, FL	(Paddock et al. 2010)
	3	FL, GA, KT, MS, OK, SC	(Sumner et al. 2007)
	< 0.5	AR	(Trout et al. 2010)

SFG, but differences between RMSF and *R. parkeri* were noted (Parker et al. 1939; Lackman et al. 1949). The “maculatum disease” was much milder and never fatal nor did the “maculatum agent” produce *Proteus* agglutinins in rabbits (Lackman et al. 1949). The latter was hypothesized to be due to an inability of the “maculatum agent” to infect rabbits (Lackman et al. 1949).

1.1.3.e Ecology of *Rickettsia parkeri*

To date there is no definitive information describing the vertebrate hosts involved as reservoirs or amplifiers in the natural transmission cycle of *R. parkeri* (Paddock 2005). Ticks positive for *R. parkeri* were collected from bears from Florida and Georgia, while a survey of white-tailed deer, feral swine, raccoons, and Virginia opossums failed to detect *R. parkeri* by PCR but found that between 9% and 74% of the animals tested positive by serology with raccoons having the highest seroprevalence (Yabsley et al. 2009; Castellaw et al. 2011). The latter proving only that these animals are being exposed to *R. parkeri* and not necessarily developing active infections. *Rickettsia parkeri* was detected at eight days post-inoculation using real-time quantitative PCR (qPCR) in an experimentally infected South American opossum (*Didelphis aurita*) (Horta et al. 2010). In addition, rickettsial DNA was recovered from an *Amblyomma cajennense* tick, which was allowed to feed on the experimentally infected opossum (Horta et al. 2010). While this does not confirm this animal as either a reservoir or an amplification host, it does begin to provide researchers with a promising first suspect. Another study, conducted in the United States, aimed to determine any association between *R. parkeri* infection and “gotch ear” in cattle, which is a common ailment associated with parasitism of the ears of cattle by *A. maculatum* (Edwards et al. 2011). Of the six calves experimentally infected with *R. parkeri*, two yielded positive PCR results from the blood utilizing primers for the

rickettsial 17 kDa antigen gene at 11 and 14 days post-inoculation for one animal and 23 days post-inoculation for the other; however, no association was found between *R. parkeri* infection and the development of “gotch ear” in *A. maculatum* infested cattle (Edwards et al. 2011). In support of this finding, a case report of a goat with “gotch ear” due to infestation with *A. maculatum* showed no immunohistochemical evidence of SFG *Rickettsia* in the ear samples although all three *A. maculatum* ticks which were removed tested positive by PCR for *R. parkeri* (Edwards et al. 2011).

Several species of ticks have been implicated as potential vectors of *R. parkeri*. *Amblyomma maculatum* is largely regarded as the primary vector for *R. parkeri*. Along with the initial description of *R. parkeri*, infected *A. maculatum* ticks have been found in many of the states in the natural range of this tick, which suggests that this *Rickettsia* is endemic throughout a relatively large expanse of the United States (Figure 1.6) (Paddock et al. 2008). States in which *R. parkeri* infected *A. maculatum* ticks have been found include: Georgia, Florida, Kentucky, Mississippi, South Carolina, Oklahoma, Virginia and Arkansas, indicating that *R. parkeri* may be found anywhere that *A. maculatum* ticks are found (Sumner et al. 2007; Trout et al. 2010; Jiang et al. 2012). The prevalence of *R. parkeri* within the tested populations of *A. maculatum* ranges from 10% to 43%, and it has been noted that there appears to be a higher incidence of *R. parkeri* positive ticks in the spring with decreasing incidence in the later summer months (Paddock et al. 2010; Fornadel et al. 2011; Wright et al. 2011). This is an important observation as *R. parkeri* has been described as being maintained in the natural cycle almost entirely by vertical transmission within *A. maculatum*; however, a seasonal variation in the infection rates of ticks with *R. parkeri* suggests that the prevalence of infection decreases, and uninfected ticks are exposed to *R. parkeri* through a vertebrate host acting as a reservoir or amplification host.



Figure 1.6. Classic range (*dark blue*) of *A. maculatum* in the United States; a hypothetical range (*pale blue*); and the locations of confirmed (*shaded circles*) and probable (*unshaded circles*) cases of *R. parkeri* rickettsiosis (Paddock et al. 2008).

Dissection of naturally infected wild *A. maculatum* and experimentally infected *A. maculatum* ticks revealed tissue distribution to the salivary glands and ovaries as would be expected with a pathogenic *Rickettsia* that is also maintained vertically. When compiled with the prevalence data, this supports the *A. maculatum* as the primary vector of *R. parkeri* (Edwards et al. 2011).

Amblyomma americanum, which is an abundant and aggressive human biting tick commonly known as the lone star tick, has been experimentally infected with *R. parkeri*, and the infection was maintained transovarially and transstadially for at least one generation (Goddard 2003). However, this data is incomplete, as sequencing of the amplicons was not performed leading some to question the validity of these findings. The *A. americanum* tick has also been reported to be naturally infected with *R. parkeri* in Tennessee and Georgia (Figure 1.7) (Cohen et al. 2009). The possibility of *A. americanum* also serving as a vector for *R. parkeri* has serious implications as this species of tick is found in very large numbers especially in areas with large white-tailed deer populations, but the limitations of previous studies preclude definitive declaration of *A. americanum* as an important vector of *R. parkeri* (Paddock and Yabsley 2007).

In Uruguay, *A. triste* has tested positive for *R. parkeri* by PCR of *rOmpA* (Venzal et al. 2004). This tick is frequently associated with human bites and the development of rickettsioses in Uruguay, but in Brazil they are uncommon and human feeding is not reported, even though *R. parkeri* has been detected in *A. triste* from Brazil (Venzal et al. 2004; Silveira et al. 2007). Interestingly, the cases of either confirmed or suspected *R. parkeri* rickettsiosis in Argentina occurred during a time of peak activity for *A. triste*; however, no ticks were saved from these patients so confirmation of the vectors was not possible (Romer et al. 2011). *Rickettsia parkeri* has been recovered from *A. triste* in Argentina as well (Nava et al. 2008). Another species of South American *Amblyomma* tick, *A. dubitatum*, has also been reported to be infected with

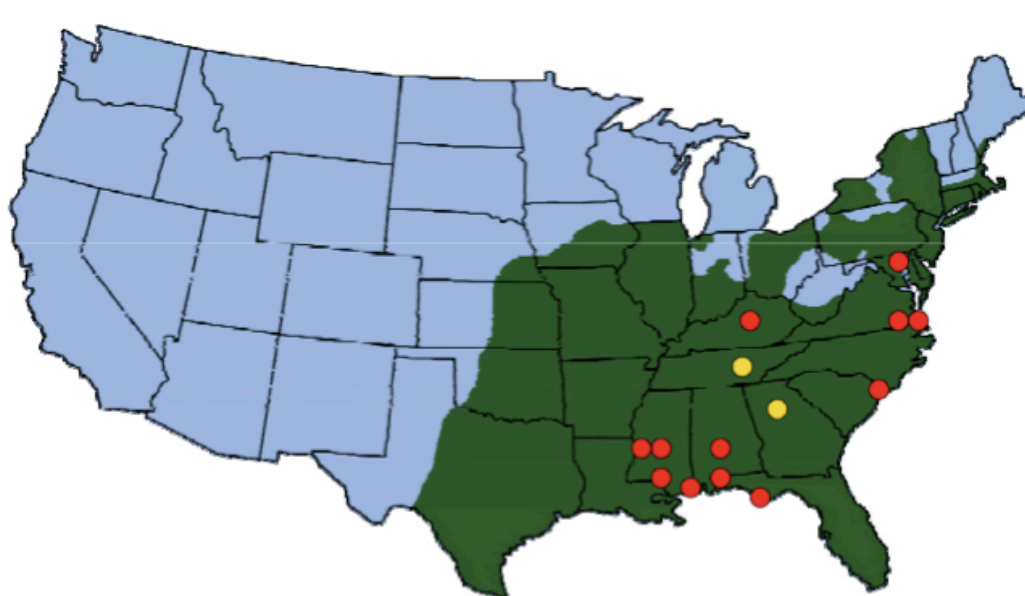


Figure 1.7. Location of ticks, *R. parkeri* in ticks, and human cases of rickettsiosis in the United States. Green shading indicates approximate distribution of *A. americanum* ticks, which completely overlaps with the known or suspected distribution of *A. maculatum*. Yellow circles indicate location where *R. parkeri* was detected in *A. americanum* ticks. Red circles indicate location of confirmed or suspected cases of *R. parkeri* infection in humans (Cohen et al. 2009).

R. parkeri and may play a role in the natural transmission cycle of *R. parkeri* in Brazil (Horta et al. 2007). The factors influencing vectorial competency of ticks for *R. parkeri* remain to be elucidated.

1.1.3.f Varying Degrees of Disease Severity in Tick-borne Rickettsioses

Rickettsia parkeri rickettsiosis is a distinct disease entity with notable differences from RMSF. A compilation of clinical cases identified by 2008 revealed patterns in the clinical course of disease with *R. parkeri* rickettsiosis (Table 1.3) (Paddock et al. 2008). Notably, all 12 patients reported mild to moderate fever (Paddock et al. 2008). Eschars were identified in 11 of the 12 patients, while 10 patients exhibited non-pruritic maculopapular rash on the trunk and/or extremities (Paddock et al. 2008). Both *R. parkeri* rickettsiosis and RMSF are characterized by fever, myalgia, malaise, headache, and a maculopapular eruption that may involve the palms or soles, and both occur approximately one week following tick bite (Paddock et al. 2008). The most striking differences between the two disease entities are the mildness of clinical signs, the eschar at the site of inoculation, and the relative absence of gastrointestinal signs in cases of *R. parkeri* rickettsiosis (Paddock et al. 2008).

As stated previously, it is possible that the most severe SFG rickettsioses are rarely encountered when compared with the occurrence of milder rickettsial infections, which is supported by their relative frequencies in human biting ticks (Paddock 2009). For example, *R. rickettsii* is detected in less than 1% of human biting ticks while *R. parkeri* is found in 10% to 43% of *A. maculatum* screened (Paddock 2009; Paddock et al. 2010; Fornadel et al. 2011; Wright et al. 2011). It has also been noted that the geographical overlap within the United States between *R. parkeri* and the two ticks from which it has been detected with that of the vectors of

Table 1.3. Comparison of selected clinical features of *R. parkeri* rickettsiosis with those of Rocky Mountain spotted fever (RMSF) and rickettsialpox, as reported in well-characterized case series (Paddock et al. 2008).

Clinical characteristic	<i>R. parkeri</i> rickettsiosis ^a (n = 12)	RMSF ^b (n = 208)	Rickettsialpox ^c (n = 197)
Fever	100 (100)	100 (100)	99 (100)
Inoculation eschar(s)			
Any	92 (100)	ND	92 (70)
Multiple eschars	17 (100)	ND	14 (18)
Rash			
Any type	83 (100)	92 (100)	100 (100)
Macules or papules	83 (100)	83 (37)	100 (100)
Petechiae	17 (100)	47 (80)	ND
Vesicles or pustules	42 (100)	ND	100 (82)
On palms or soles	45 (92)	82 (70)	2 (91)
Myalgia	92 (100)	59 (98)	39 (9)
Headache	83 (100)	72 (100)	92 (100)
Lymphadenopathy	25 (100)	20 (29)	17 (9)
Nausea or vomiting	8 (100)	60 (94)	7 (82)
Diarrhea	0 (92)	20 (94)	ND
Coma, delirium, or seizure	0 (100)	27 (86)	0 (100)
Hospitalization	33 (100)	78 (100)	18 (100)
Death	0 (100)	7 (100)	0 (100)

R. rickettsii suggests that many cases of *R. parkeri* rickettsiosis have been misidentified as RMSF (Pornwiroon et al. 2009).

1.1.3.g Relatedness of *R. parkeri* to Other SFG *Rickettsia*

There is a large amount of evidence that *R. parkeri* is closely related to several other members of the SFG of *Rickettsia* (Goddard 2009). Even in the initial description of *R. parkeri*, Parker observed perfect reciprocal cross-reactivity in guinea pigs with the agent of boutonneuse fever, *R. conorii*, and the inability to produce agglutinins against *Proteus* organisms later placed the agent in the same category as boutonneuse fever and Queensland tick typhus (Parker et al. 1939; Lackman et al. 1949). Toxin neutralization tests led to a sub-grouping of closely related SFG *Rickettsia* containing *R. parkeri*, *R. conorii*, and *R. sibirica* (Bell and Stoenner 1960; Lackman et al. 1965). This grouping was confirmed when microimmunofluorescence assays with mouse sera resulting from injections of triturated field-caught *A. americanum* containing *R. parkeri* revealed highest titers to *R. conorii* and *R. sibirica* (Goddard and Norment 1986). More recently, molecular analyses of the *rOmpA*, *rOmpB*, *gltA*, and “gene D” of SFG *Rickettsia* revealed clades consisting primarily of *R. parkeri*, *R. conorii*, *R. sibirica*, and *R. africae*, the agent of African tick-bite fever (Roux et al. 1997; Fournier et al. 1998; Xu and Raoult 1998; Roux and Raoult 2000; Sekeyova et al. 2001). Furthermore, the clinical disease syndromes resulting from *R. africae*, *R. conorii*, and *R. parkeri* are similar and include eschar formation, although infection with *R. conorii* often results in more severe clinical disease (Goddard 2009).

Unlike many other rickettsial species, plasmids have not been identified in *R. parkeri* (Baldrige et al. 2010). This finding is similar to other rickettsial pathogens in the SFG such as *R. conorii* and *R. rickettsii* (Baldrige et al. 2010). This is an interesting finding as typically plasmids drive horizontal transfer and the acquisition of virulence determinants and

environmental adaptive traits; however, plasmids have been noted to be lost during serial passages in culture (Baldrige et al. 2010). The loss of plasmids over time in culture is one proposed explanation for their conspicuous absence in *R. parkeri*, but this is countered by the finding that even low passage isolates have failed to test positive for plasmids.

1.1.4 Necessity of Research on *R. parkeri*

As the number of confirmed cases of *R. parkeri* rickettsiosis climb, the need for better characterization of the pathogenesis of the disease and description of the ecology increases dramatically. Specifically, the difference in clinical severity to the very closely related SFG *Rickettsia* described previously warrants the pursuit of the factors responsible for these differences. The first step in this pursuit is the careful reproduction of lesions and subsequent description of the pathology associated with infection to allow comparisons to those similar rickettsioses. Concomitant investigation into the natural ecology of *R. parkeri* is vital not only to risk assessment but also to further research into the molecular mechanisms associated with susceptibility in certain vertebrate species.

1.2 *Amblyomma maculatum*

This section discusses the Gulf Coast tick (*Amblyomma maculatum* Koch). The preferred habitats and life cycle are described and are followed by information regarding the importance of *A. maculatum* in human and animal health. Specifically, canine hepatozoonosis, tick paralysis, and livestock production losses are reviewed. Finally recent research topics focused on *A. maculatum* such as the sialotranscriptome and the discovery of a new species of *Rickettsia* are reviewed, and areas demanding future research with *A. maculatum* are discussed.

1.2.1 Original Description

The Gulf Coast tick, *A. maculatum* Koch (Acari: Ixodidae), is a relatively large, neotropical, three-host hard tick commonly found in the southeastern United States (Estrada-Pena et al. 2005). As recently reviewed, *A. maculatum* was originally described in 1844 by Carl Ludwig Koch (Teel et al. 2010). Various synonyms for *A. maculatum* Koch include: *Amblyomma rubripes* Koch, *Amblyomma ovatum* Koch, and *Amblyomma complanatum* Berlese (Cooley 1944). The *A. maculatum* Koch, 1844, tick group includes *Amblyomma neumanni* Ribaga, 1902, *Amblyomma parvitarsum* Neumann, 1901, *Amblyomma tigrinum* Koch, 1844, and *A. triste* Koch, 1844 (Estrada-Pena et al. 2005). The shared defining morphological characteristics of the adults of this group include: a 3/3 hypostome; two spurs on coxa I with the external spur being much longer than the internal; spines on tibiae II to IV; and a similar scutal ornamentation (Figure 1.8) (Estrada-Pena et al. 2005). The dental formula of the hypostome (3/3) refers to the number of rows of dental spines on either side of the hypostome, and this pattern can vary greatly among tick species and even between sexes of one species. Adults of this group have largely been misidentified, as both *A. tigrinum* and *A. triste* were considered synonyms of *A. maculatum* until they were later redefined as valid species (Kohls 1956). A major diverging characteristic of the adult female *A. maculatum* from these other species is the presence of small chitinous tubercles at the posterior body margin (Walker and Olwage 1987).

1.2.1.a Habitat Range

The preferred habitat for *A. maculatum* is coastal upland and tall-grass prairies (Hixson 1940). Original descriptions of the range of *A. maculatum* within the United States included established populations in the coastal regions of the southeastern portion of the country (within 100 miles of the coast) with fluctuating inland populations; however, more recent estimates place



Figure 1.8. Adult female (left) and male (right) *A. maculatum* ticks. Flat adult ticks illustrating the large mouthparts and ornate scutum typical of *A. maculatum*.

established populations continuing much farther inland along with populations now established in Kansas and Oklahoma (Figure 1.9) (Teel et al. 2010). Incidental collections have also been made in more northern and central states such as Maine and Iowa, but permanent populations are not believed to have been established in these areas (Smith et al. 1996). Most of these incidental collections are believed to be due to relocation of migratory birds upon which the immature stages of *A. maculatum* have fed (Smith et al. 1996).

1.2.1.b Life Cycle

The life cycle of *A. maculatum* involves three feeding stages of development: larva, nymph, and adult. *Amblyomma maculatum* have been collected from 71 different species of birds and mammals representing seven vertebrate host classes taxonomically (Teel et al. 2010). Of these vertebrate hosts, birds and rodents are preferentially parasitized by the larval and nymphal stages of the tick, while the adult ticks feed preferentially on larger hosts including domestic ungulates, wild canids and cervids (Teel et al. 2010). Cotton rats have been found to be more commonly infested with immature *A. maculatum* than other small rodents, and therefore may play a role in the ecology of infectious agents for which the Gulf Coast tick serves as a vector (Barker et al. 2004). It is also noteworthy that *A. maculatum* nymphs and adults will readily feed on humans (Felz et al. 1996; Felz and Durden 1999; Goddard 2002) and therefore have potential to serve as efficient vectors for *R. parkeri*. The immature life stages (larvae and nymphs) feed predominantly on the head and neck of mammals and birds (Hixson 1940); however, the adult ticks attach primarily in areas of sparsely haired skin of large mammals (Ketchum et al. 2005). Cattle parasitized with adult male *A. maculatum* were found to be more frequently infested with female *A. maculatum* suggesting a signaling event by the feeding males resulting in a feeding response from the female ticks (Sleebe et al. 2010). The influence of

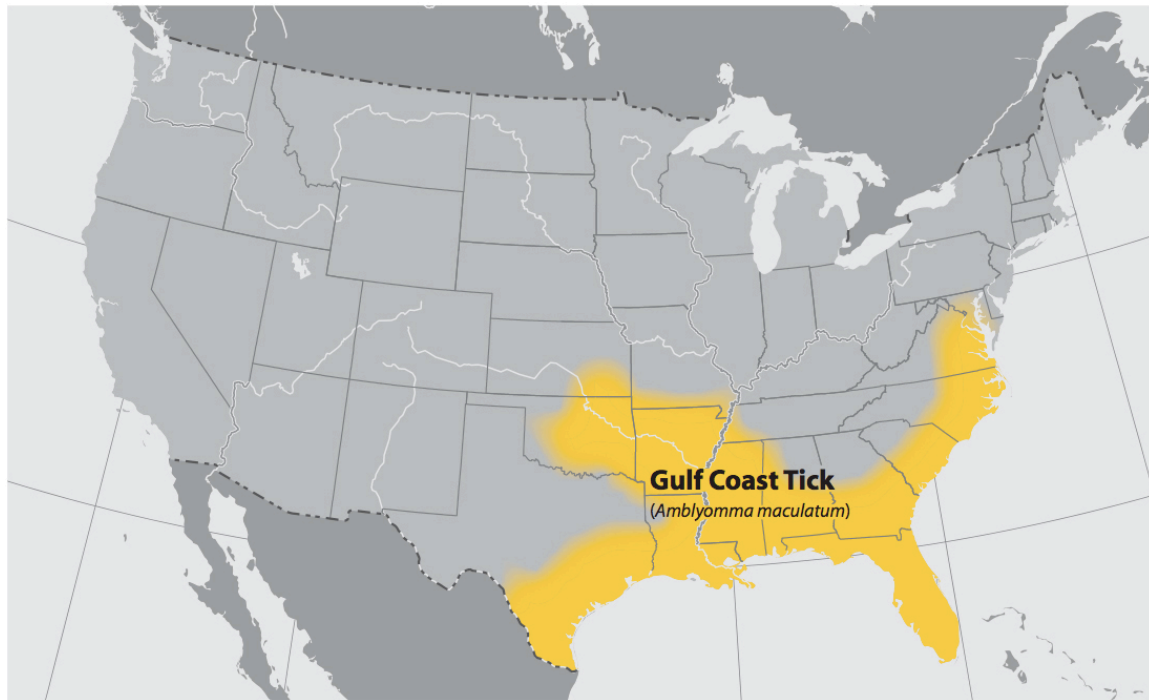


Figure 1.9. Distribution of *A. maculatum* (Courtesy of Centers for Disease Control and Prevention).

chemicals produced by feeding males on the feeding behavior of females was confirmed and demonstrated as attraction and aggregation behaviors in the presence of feeding male ticks (Rechav et al. 2000). The male pheromones in conjunction with CO₂ produce higher attraction of questing females than do either attractants alone (Teel et al. 2010).

Preoviposition times for gravid females are approximately one week on average; however, various environmental conditions may result in preoviposition times of up to 58 days (Hixson 1940). Oviposition is typical of other ixodid ticks. Egg production is highest during the first week of laying and has been reported to be as long as 75 days (Wright 1971) followed by an incubation period, which averages approximately six weeks (Teel et al. 2010). Greater than 50% of the engorged females weight is converted to eggs with an average of over 8,000 eggs laid per female (Hixson 1940; Wright 1971). Eclosion rates depend greatly on environmental conditions, including season, microclimate and habitat type with rates increasing to a peak in October coinciding with seasonal cattle infestations (Teel et al. 2010). Unfed larvae survive on average between three and four months (Hixson 1940). Larval engorgement times vary depending on host but average approximately three to seven days (Hixson 1940; Koch and Hair 1975). Molting times of engorged larvae are principally determined by temperature with increasing temperatures resulting in decreased time to molt (Koch and Hair 1975). Although photoperiod does not appear to affect the molting time of engorged larvae, a short day cycle during the larval molt results in significantly shorter time to molt for the engorged nymphs (Lohmeyer et al. 2009). Unfed nymphs have slightly longer survival times than unfed larvae with a longevity of approximately three to five months (Teel et al. 2010), while nymphal feeding to repletion requires on average five to eight days (Hixson 1940). Hixson observed that feeding was most often completed overnight when the hosts were least active, and the majority of nymphs

detached in the morning as the hosts became active (Hixson 1940). As in larvae, temperature is the primary regulator of molting time in nymphs with the longest periods associated with the coldest incubations (Koch and Hair 1975). Survival times for unfed adults are much longer than for either larvae or nymphs and range from five to ten months (Hixson 1940; Teel et al. 2010). Female engorgement usually occurs between 11 and 17 days post-attachment with an actual range of 8 to 21 days (Figure 1.10) (Hixson 1940; Drummond and Whetstone 1970).

In our laboratory colony of *A. maculatum*, prefeeding adult males for approximately 2 days prior to infesting rats, guinea pigs or rabbits with adult female ticks leads to increased percentages of attachment of adult females as well as faster attachment times. The females typically feed for 14 to 17 days, and preoviposition time is usually 7 days (Figure 1.11). Hatching then occurs after about 4 weeks with unfed larvae surviving for approximately 2 months. The majority of larvae fed on Balb/c mice engorge within 5 days of infestation with those feeding on the ears often taking as long as 9 days to feed to repletion. Molting times of the engorged larvae are between 10 and 20 days. A week after molt is allowed for scutal hardening before feeding the newly emerged. Feeding times of nymphs on mice, rats, and guinea pigs average about 8 days, while the molting times of the engorged nymphs are similar to that of the engorged larvae. As with the nymphs, a rest period for newly emerged adults allows the exoskeleton to harden prior to infestation of rats or guinea pigs.

1.2.2 Infectious Agents and Clinical Syndromes

The Gulf Coast tick has been identified or implicated as a vector for several different pathogens of medical and veterinary importance including *R. parkeri*, which was first identified and isolated from *A. maculatum* (Parker et al. 1939).



Figure 1.10. Engorged female *A. maculatum*. Females typically engorge after approximately two weeks of feeding, after which they drop from the host and lay large clutches of eggs.

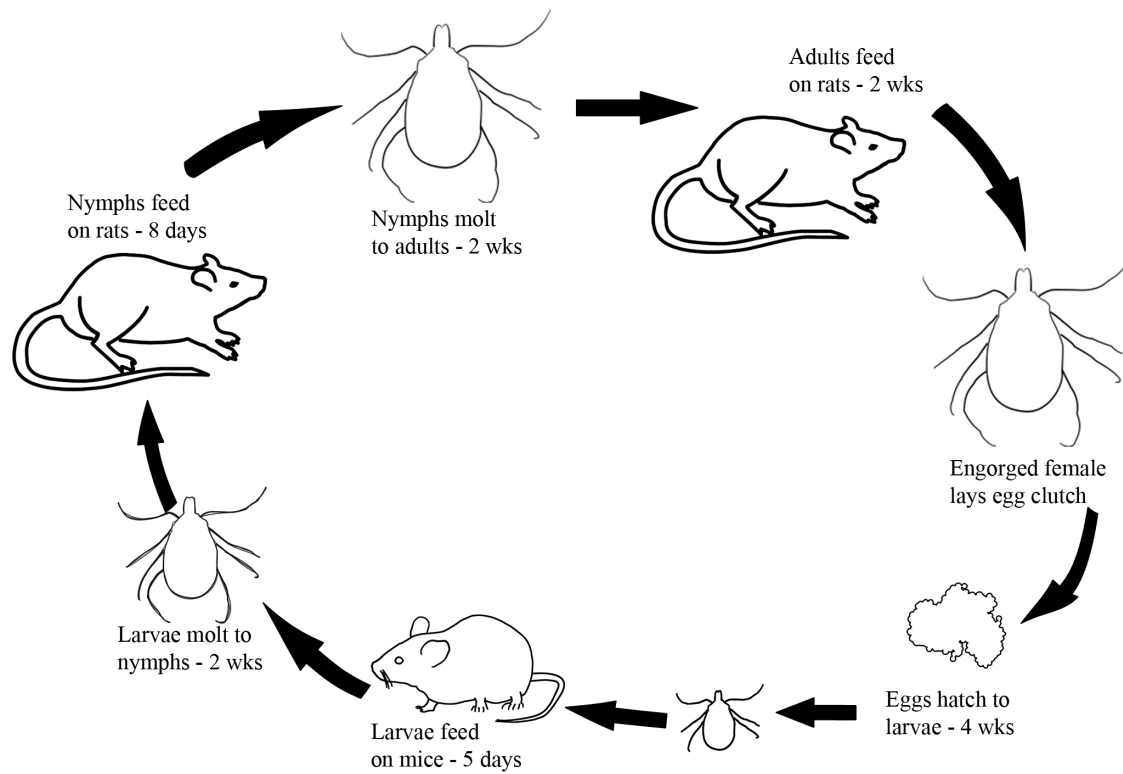


Figure 1.11. Laboratory life cycle of *A. maculatum*. At all of the molting stages, ticks were allowed a minimum of one week after molting before feeding to allow time for the exoskeleton to harden. This was done to avoid damaging the ticks during manipulation prior to feeding.

1.2.2.a *Hepatozoon americanum*

In veterinary medicine the Gulf Coast tick is important as the primary vector of *Hepatozoon americanum*, which is a protozoal pathogen of domestic dogs (Ewing et al. 2003), and also as a cause of ascending paralysis in dogs (Gothe et al. 1979). The disease process associated with *H. americanum* is termed American canine hepatozoonosis in order to differentiate it from the much milder disease associated with *Hepatozoon canis*. Unlike many other tick-borne pathogens, *H. americanum* is not transmitted through the bite of an infected tick but rather by the ingestion of an infected Gulf Coast tick by the canine host (Ewing and Panciera 2003). The clinical signs associated with American canine hepatozoonosis include pyrexia, lethargy, depression, gait abnormalities, and muscle wasting (Ewing and Panciera 2003). Patients often have highly elevated leukocyte counts characterized by a mature neutrophilia, and elevation of serum alkaline phosphatase is common (Ewing and Panciera 2003).

1.2.2.b Tick Paralysis

Amblyomma maculatum has also been associated with tick paralysis in humans, including a recent case in Mexico (Espinoza-Gomez et al. 2011). The development of tick paralysis usually requires five to seven days of tick attachment; however, preceding the onset of paralysis patients may experience general malaise, appetite loss, violent headache, and severe vomiting (Gothe et al. 1979). When paralysis does occur, it begins as slight to pronounced incoordination, ataxia and muscular weakness, which progress within a few hours until the patient is unable to move the limbs and is therefore classified as an ascending, symmetrical, flaccid tetraplegia (Gothe et al. 1979).

1.2.2.c Production Losses

The adult ticks are also of economic importance as parasitism is associated with pathology and morbidity in cattle in the coastal regions of the United States resulting in financial losses to producers (Gladney et al. 1977; Williams et al. 1977; Riley et al. 1995). Specifically, when a sufficient number of adult ticks feed on the ears of cattle, a permanent thickening and curling of the ear occurs and is commonly referred to as gotch ear (Edwards et al. 2011). The clinical definition of gotch ear consists of variable degrees of edema with crusting, alopecia, erythema, and excoriation at tick attachment sites with or without curling of the tip of the pinnae and a loss of a portion of the ear (Figure 1.12) (Edwards et al. 2011). Additionally, the irritation and blood loss associated with heavy infestations has been associated with lethargy and debilitation (Gladney et al. 1977). This results in weight loss or diminished weight gain and poor body condition due to decreased feed consumption (Gladney et al. 1977; Williams et al. 1977; Riley et al. 1995). This same effect was not found to occur in Brahman steers, suggesting zebu or zebu-cross cattle suffer less morbidity from heavy infestation of Gulf Coast ticks (Stacey et al. 1978). A secondary effect of the parasitism of beef herds is the predisposition to primary screwworm, *Cochliomyia hominivorax*, infestation (Hixson 1940), leading to further economic losses.

1.2.3 Current Research on *A. maculatum*

1.2.3.a Saliva and Host Immunomodulation

With the publication of the sialotranscriptome of *A. maculatum*, new doors of opportunity have been opened into the study of these ticks as vectors and the tick factors essential for transmission of pathogens. It is well established that hematophagous arthropods alter the hemostatic and immunological responses to blood feeding via salivary proteins (Wikel 1996).

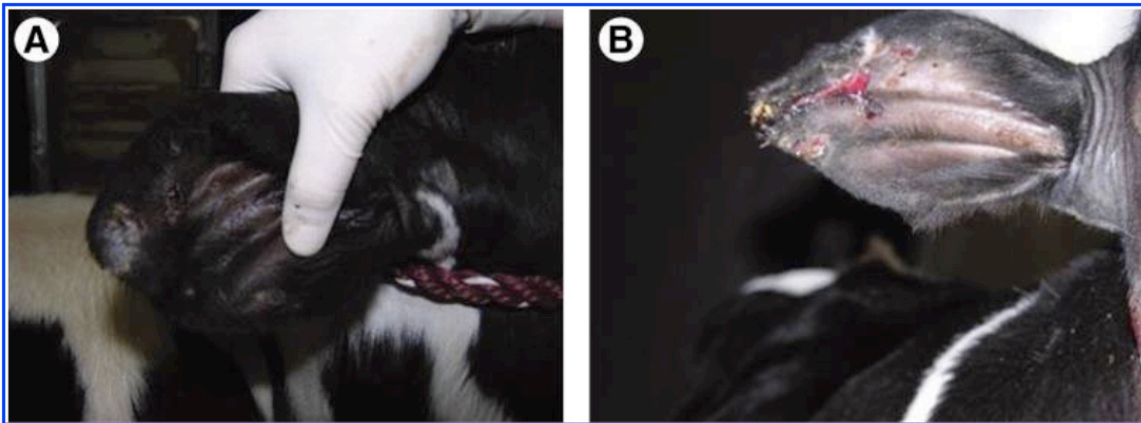


Figure 1.12. Gotch ear in calves infested with *A. maculatum* that either were (A) or were not (B) infected with *R. parkeri* (Edwards et al. 2011).

The sialotranscriptome of *A. maculatum* has elucidated numerous salivary constituents, which likely play a role in the efficiency of these ticks to act as vectors for pathogens such as *R. parkeri* (Karim et al. 2012). Important transcripts identified include: Kunitz domain-containing proteins, which may act as inhibitors of the extrinsic or tissue factor pathway of coagulation; cystatins, which are cystine protease inhibitors that may inhibit cathepsins to inhibit inflammation and suppress dendritic cell maturation; serpins, which have serine protease inhibition function and may have immunosuppressive activity; Kazal domain-containing proteins, which may inhibit thrombin and plasmin with high affinity; carboxypeptidase inhibitors, which have only been found in metastriate ticks and may affect fibrinolysis by inhibiting plasma carboxypeptidase B; proteases implicated in bradykinin degradation, fibrinolysis, fibrinogenolysis and anti-angiogenic activities; apyrases, which are common in hematophagous arthropod saliva and hydrolyze ATP and ADP to serve as antihemostatic and anti-inflammatory functions; along with many other proteins which may serve roles in the modulation of the feeding microenvironment (Karim et al. 2012). In essence these salivary gland derived proteins immunomodulate the feeding microenvironment by altering complement activation, natural killer cell function, antibody production, T-lymphocytes proliferative responses, and cytokine elaboration by antigen-presenting cells and T-lymphocytes, thus producing an environment amenable to the reproduction of pathogenic bacteria (Wikel 1996).

The modulation of host immunity to tick feeding is of great importance as acquired resistance to infestation is associated with reduced engorgement weight; increased duration of feeding; reduced numbers of ova produced; fewer viable ova; prevention of molting; and tick death (Wikel 1999). The evolutionary adaptations of ixodid ticks, which have allowed for these host evasion mechanisms, stimulate a *Rickettsia*-permissive immune response in the form of a

primarily Th2 response to tick feeding in lieu of the protective Th1 response (Wikel 1999). The development of a Th2 immune response is inhibitory to the development of a Th1 response, but the Th1 immune response is the primary pathway of resistance to *Rickettsia* (Jordan et al. 2008; Jordan et al. 2009; Quevedo-Diaz et al. 2010). After repeated infestation, natural vertebrate hosts often develop resistance to tick infestation, which correlates with a shift from the Th2 response to the Th1 response (Wikel 1999). This shift in host response may lead to a multifactorial decrease in vectorial capacity of the tick for rickettsiae due to the fitness losses suffered by the ticks and the poorly permissive microenvironment for rickettsial survival at the tick feeding site.

1.2.3.b Other *Rickettsia*

Recent studies have described the presence of *Candidatus Rickettsia andeanae* from multiple populations of *A. maculatum* in the United States (Fornadel et al. 2011; Jiang et al. 2012; Luce-Fedrow et al. 2012). This species was first described in *A. maculatum* and *Ixodes boliviensis* ticks collected from two horses in northern Peru (Blair et al. 2004). To date this organism has not been associated with disease in humans or animals; however, its relatively recent discovery may have precluded diagnosticians from recognizing its presence in diseased individuals. Alternatively, *Candidatus R. andeanae* may act as an endosymbiont of *A. maculatum*. The presence of a rickettsial endosymbiont in *A. maculatum* is intriguing as the phenomenon of transovarial transmission interference has been described for rickettsial species within other ticks. The best documented example is the decreased prevalence of *R. rickettsii* in *D. andersoni* ticks on the eastern side of the Bitterroot Valley (Baldrige et al. 2004; Felsheim et al. 2009). This is attributed to the presence of *R. peacockii*, a SFG rickettsial endosymbiont of *D. andersoni*. Ticks infected with the nonpathogenic *R. peacockii* are unable to maintain

transovarial transmission of *R. rickettsii* upon challenge with the Rocky Mountain spotted fever pathogen (Baldrige et al. 2004). This transovarial interference phenomenon was experimentally demonstrated utilizing *R. montana* and *R. rhipicephali*-infected cohorts of *D. variabilis* ticks as well (Macaluso et al. 2002). The presence of a similar phenomenon within the Gulf Coast tick would have major implications for the prevalence of *R. parkeri* within tick populations and associated exposure risks in humans.

1.2.4 Research Directions

The importance of *A. maculatum* as a disease vector is becoming more evident, and research into its role in the maintenance and transmission of pathogens is necessary. Specifically, the role of *A. maculatum* in the natural cycle of *R. parkeri* and its transmission to human hosts requires investigation. The capacity for transovarial and transstadial transmission of *R. parkeri* in *A. maculatum* has long been assumed but has not been definitively demonstrated. Finally the role of tick saliva in the transmission of *R. parkeri* to the vertebrate hosts poses an intriguing avenue of research, as the effect of tick saliva on the vertebrate host microenvironment is dramatic.

1.3 Animal Models of Rickettsioses

Animal models are essential to research into the pathogenesis of emerging infectious disease and the development of vaccines and therapeutic protocols to defend against these diseases. The ideal model for any pathologic process should be readily available and economical. It is also important that the model express many of the pathologic characteristics, or at least specific characteristics of interest, of the disease it is meant to represent. For tick-borne rickettsioses, the model should be able to be inoculated via the cutaneous route and display the typical endotheliotropic pattern of infection.

In this section we describe a historical progression of animal models for rickettsioses and focus on the typhus and SFG *Rickettsia* separately. We then elaborate on murine models of rickettsioses and the propensity for inbred mice with macrophage tumoricidal defects to be susceptible to rickettsial infection. This is followed by a brief review of the factors that have been identified as necessary for animal models of rickettsioses and a brief description of the immunologic response of the vertebrate host to rickettsial infection. Finally, we reiterate the importance of animal models of rickettsioses and define directions for future research utilizing these models.

1.3.1 Historical Models of Rickettsioses

While guinea pigs have been the traditional animal model for studying rickettsioses, shortcomings include being not native to the United States, limited recapitulation of pathology for many rickettsioses, the need to bypass the cutaneous route of inoculation, and the proclivity for reduced infectivity of *Rickettsia* after serial passage within guinea pigs. As early as 1942, researchers explored the possibility that more accurate animal models could be developed (Snyder and Anderson 1942). Specifically, researchers aimed to discover an animal model in which the disease could be reproduced as it occurs in man as opposed to the guinea pig in which the infection was primarily in the abdominal cavity secondary to peritoneal inoculation (Snyder and Anderson 1942). These early studies determined that the eastern cotton rat, *Sigmodon hispidus hispidus*, is a much more suitable animal than the guinea pig for the study of typhus fever as the intracardial inoculation of *Rickettsia prowazekii* into the cotton rats was fatal even after serial passage (Snyder and Anderson 1942). The *S. hispidus* model was further characterized to show that fatal infection was possible by multiple routes of inoculation although massive doses were necessary to produce these fatal infections suggesting that, for the strains

studied at least, the bacteria were only moderately virulent for the cotton rats (Anderson 1944). The investigation of this particular model also led to the observation that different strains of *Rickettsia* differed in their infectiousness between animal species (Anderson 1944). In an effort to develop a better model of murine typhus, several strains of mice, the cotton rat, guinea pig and hamster were evaluated for susceptibility, and it was determined that, in descending order, mice, cotton rats, guinea pigs, and hamsters were susceptible to intraperitoneal typhus infections (Whitmire and Downs 1957). These animals were also tested for their response to infection after cortisone treatment, which resulted in increased susceptibility to *Rickettsia* in the hamster but apparent resistance to cortisone treatment in the cotton rat and guinea pig as neither of these species showed any change in susceptibility to rickettsial infection (Whitmire and Downs 1957). These findings were interesting as they emphasize the importance of different physiologic responses of the various models to the same treatment.

1.3.1.a Typhus Group *Rickettsia*

Due to the descriptions of rickettsiae recovered from domestic livestock, the susceptibility of donkeys, goats, and calves for *R. prowazekii* was evaluated (Table 1.4) (Philip et al. 1967). Despite a high and persistent serologic response in the donkey, no overt signs of disease were observed in any of the animals, and rickettsemia could not be demonstrated suggesting these animals are poor candidates for laboratory examination of the rickettsioses (Philip et al. 1967). In contrast, a cynomolgus monkey (*Macaca fascicularis*) model for epidemic typhus infection was observed to closely resemble epidemic typhus infection in humans; however, the route of inoculation was intravenous and therefore still an incomplete model of infection (Gonder et al. 1980). Ultimately the lack of an adequate animal model was impeding the investigations of immune mechanisms and of attenuation of typhus group

Table 1.4. Susceptibility of animals to *Rickettsia*.

	<u>Animals</u>											
	Pine vole	Eastern cotton rat	Guinea pig	Hamster	Gerbil	Ferret	Rabbits	Donkey	Goat	Calf	Miniature swine	Cynomolgus monkey
<i>R. rickettsii</i>	▲	△	▲	△	△	▲	△				△	
<i>R. prowazekii</i>		▲	▲					△	△	△		▲
<i>R. typhi</i>		▲	▲	▲								

▲ = susceptible

△ = not susceptible

rickettsiae (Walker et al. 2000). The models were deficient either due to their inability to infect endothelial cells as the primary target, due to inappropriate histopathologic lesions, or due to unpredictable lethality at varying inoculation doses. As researchers focused on a viable mouse model, the C3H/HeN mouse strain was investigated and confirmed as a representative model for *R. typhi* infection because it resulted in disseminated endothelial infection with dose-dependent mortality (Walker et al. 2000). The remaining major drawbacks of this model are that it bypasses the cutaneous portal of entry and the host species differences between mice and humans (Walker et al. 2000). Further investigation into animal models for typhus group rickettsiae revealed that Balb/c mice could be persistently infected with *R. prowazekii*, and they showed lesions similar to those described in people with epidemic typhus such as mononuclear inflammation infiltrates in the liver and lungs and microhemorrhages in the brain (Bechah et al. 2007). Importantly, these studies failed to divulge a clear Th1/Th2 polarization associated with the resulting rickettsiosis (Bechah et al. 2007).

1.3.1.b Spotted Fever Group *Rickettsia*

As the field of rickettsiology matured, the need for quality animal models increased, and the need for specific criteria to be met was identified. In particular, the ideal animal model must be readily available and economical while expressing many of the pathologic characteristics of the disease in question (Sammons et al. 1977). Again, the guinea pig was the traditional model for most SFG *Rickettsia* studies; however, other than *R. rickettsii*, members of the SFG failed to exhibit the dramatic signs of clinical disease when infecting guinea pigs. While non-human primates (NHPs) often serve as accurate models for human disease, restricted responses and the cost and limited availability limit their use for the early stages of evaluation and vaccine development (Sammons et al. 1977). With an evaluation of multiple potential model species

including inbred mouse strains, guinea pigs, ferrets, gerbils, hamsters, cotton rats, miniature swine, rabbits and sheep, varying susceptibilities of SFG *Rickettsia* were observed (Sammons et al. 1977). *Rickettsia akari* was uniformly lethal for Mai:(S) and Balb/cJ mice, while Mai:(S) were also moderately susceptible to *R. sibirica* (Sammons et al. 1977). Otherwise, inbred mouse strains were relatively resistant to SFG rickettsioses. Hla:(HA) guinea pigs were confirmed to be susceptible to *R. rickettsii*, and ferrets developed intermittent febrile illness secondary to *R. rickettsii* inoculation (Sammons et al. 1977). One sheep also developed a mild fever with *R. rickettsii*, but none of the gerbils, hamsters, cotton rats, miniature swine, or rabbits showed any overt clinical signs of illness (Sammons et al. 1977). While the pine vole, *Microtus pinetorum*, was suggested as an appropriate model for studying the pathogenesis of RMSF due to the significant morbidity and mortality following intraperitoneal infection and because of its likely role as a tick host in nature, this model suffers from similar deficiencies of many of the other models in that reagents are not readily available for the study of pine voles and the route of inoculation again bypasses the natural cutaneous route (Eremeeva et al. 2003).

An attempt to develop a quality murine model of *R. australis* infection revealed Balb/c mice as a good model for the vasculopathic lesions associated with this rickettsiosis (Feng et al. 1993). This group again reemphasized the deficiencies of animal models for rickettsioses such as the utilization of mammalian species for which few immunological reagents exist, incorrect target cells and incorrect pathological lesion (Feng et al. 1993). It was also noted that infection only in peritoneal, splenic, and hepatic macrophages could not address the role of IFN- γ , TNF- α , or cytotoxic T lymphocytes in clearance of rickettsiae from the endothelium (Feng et al. 1993). Walker et al. (Walker et al. 1994) then described another murine model of endothelial-target

rickettsiosis, this time for *R. conorii* in the C3H/HeN mouse strain in which all of the animals either got sick or died and the principal target cells were clearly the vascular endothelium.

1.3.2 Defective Macrophage Function in Inbred Mice

1.3.2.a C3H/HeJ Mice

In 1978, an inbred mouse strain was described to have macrophages, which were refractory to the lethal effects of lipopolysaccharide (LPS) *in vitro* (Ruco and Meltzer 1978). The mutation in these C3H/HeJ mice was determined to have occurred between 1960 and 1965 and to be transmitted as an autosomal codominant trait (Ruco and Meltzer 1978). It was later determined that the defect was due to a mutation in a single gene located on the fourth chromosome and that the gene for control of cytotoxicity of macrophages was closely linked or identical to the LPS response gene (Ruco et al. 1978). These mice were not the only strain noted to have defective tumoricidal capacity of macrophages, and these nonresponder mice were divided into two broad categories: strains with abnormal responsiveness to the lipid A region of bacterial LPS such as C3H/HeJ and C57BL/10ScCr and strains with apparently normal lipid A responsiveness derived from the A strain (Boraschi and Meltzer 1979). The site of the tumoricidal defect in macrophages from A strain mice was found to be in the terminal stages of activation but was phenotypically comparable to the cytotoxic defect of the lipid A unresponsive mice (Boraschi and Meltzer 1979). Based on these studies of the response to LPS in the nonresponder mouse strains, macrophage activation was divided into three sequential stages as follows: recruitment, preparation, and expression with the defect in A/J mice occurring in the expression stage (Boraschi and Meltzer 1979). The defective response to LPS in C3H/HeJ mice was traced to a mutation in the *Tlr4* gene resulting in the presence of an adenine instead of a cytosine at position 2342 of the C3H/HeJ *Tlr4* cDNA sequence (Poltorak et al. 1998; Hoshino et

al. 1999; Qureshi et al. 1999). The mutation causes a proline→histidine switch at position 712 within the cytoplasmic domain of the Tlr4 protein (Poltorak et al. 1998). This same mutation is absent from the LPS responsive C3H/HeN strain and all other strains, which were examined at the time, and it was determined to have a dominant negative effect on LPS signal transduction (Poltorak et al. 1998). The macrophages from these C3H/HeJ mice fail to induce inflammatory cytokines, including TNF- α , IL-1, IL-6 (Hoshino et al. 1999).

1.3.2.b Susceptibility to Rickettsioses

Incorporating the knowledge of defective macrophage function, it was discovered that mouse strains with tumoricidal defects in macrophages were 1000- to 10,000-fold more susceptible to the lethal effects of *R. akari* (Meltzer and Nacy 1980). These susceptible strains included C3H/HeJ, C57BL/10 and A/J mice; however, P/J mice, a strain with genetic defects in macrophage activity, were resistant and NZW/N mice, a strain with normal macrophage activation, were highly susceptible to *R. akari* (Table 1.5) (Meltzer and Nacy 1980).

Concurrently, the marked resistance to *R. akari* infection of strains such as C3H/HeN and moderate resistance of several sublines of the A strain were determined to be a dominant trait, and only the C3H/HeJ strain was killed by a very small dose of *Rickettsia* (Anderson and Osterman 1980; Anderson and Osterman 1980). Further investigation revealed that the difference between susceptibility between A and B10.A mice in susceptibility to *R. akari* is due to a single autosomal dominant gene (Meltzer et al. 1982).

These studies demonstrate that host susceptibility to rickettsial infections is complex and likely is not restricted to one pathway or mechanism. Likewise, differences in rickettsial species also need to be vetted through an animal model.

Table 1.5. Susceptibility of inbred mice to *Rickettsia*.

	Mouse strains									
	A/J mice	Balb/c mice	C3H/HeDub mice	C3H/HeN mice	C3H/HeJ mice	C3H/RV mice	C57BL/10 mice	Mai:(S) mice	NZW/N mice	P/J mice
<i>R. rickettsii</i>					△					
<i>R. conorii</i>	△				▲					
<i>R. akari</i>	▲	▲		△	▲		▲	▲	▲	△
<i>R. sibirica</i>					▲			▲		
<i>R. australis</i>		▲			△					
<i>R. prowazekii</i>		▲								
<i>R. typhi</i>				▲						
<i>Orientia tsutsugamushi</i>			▲			△				
<i>Anaplasma phagocytophilum</i>					▲					
▲ = susceptible										
△ = not susceptible										

1.3.2.c The A Strain of Mice

In stark contrast to the susceptibility to *R. akari*, the A strain mice were markedly resistant to lethal infection with *R. conorii* (Eisemann et al. 1984). The A strain resistance to *R. conorii* was not mirrored within the C3H/HeJ strain, however. These TLR4-deficient mice developed overwhelming rickettsial infections from *R. conorii* at inoculation doses that are nonfatal to TLR4-competent C3H/HeN mice, while also developing greater rickettsial burdens and producing lower levels of proinflammatory cytokines (Jordan et al. 2008). The central role of LPS in the susceptibility and resistance of animals to rickettsial infection is particularly intriguing in light of the atypical LPS produced by rickettsiae, which is not the classical endotoxic LPS of other gram negative bacteria (Jordan et al. 2008). Those TLR4 deficient mice that did survive rickettsial infection, however, were resistant to reinfection with a normally lethal dose, leading to the conclusion that an anamnestic response still occurs and can provide immunity (Jordan et al. 2008).

1.3.3 Critical Factors for Models of Rickettsioses

In a continued effort to develop suitable models for SFG rickettsioses, three critical factors influencing the establishment of lethal rickettsial infection were outlined as follows: the genetic background of the mouse, the strain of rickettsial species, and the route of inoculation (Eisemann et al. 1984). The importance of the genetic background of the mouse strain is exemplified by the variability in the susceptibility of different mouse strains to *R. akari* and *R. conorii* (Eisemann et al. 1984). The effect of rickettsial strain is demonstrated by the differing lethality of the SFG *Rickettsia* for the C3H/HeJ mice, where *R. akari*, *R. conorii*, and *R. sibirica* are all highly lethal, but *R. rickettsii* and *R. australis* (later reclassified as ancestral group) were incapable of establishing lethal infections (Eisemann et al. 1984). Finally the route of

inoculation was critical to the lethality of infection with *R. conorii*, as intraperitoneal inoculation resulted in lethal infection while the mice survived after subcutaneous inoculation (Eisemann et al. 1984).

1.3.4 Immunology of Rickettsioses

Studies utilizing bone marrow derived dendritic cells from resistant B6 mice and susceptible C3H/HeN mice provided the first evidence suggesting that priming of CD4 Th1/Th2 responses and induction of T regulatory cells are the critical determinants of resistance versus susceptibility (Figure 1.13) (Fang et al. 2007). Dendritic cells from resistant mice produced significantly higher levels of IL-12p40 postinfection than the susceptible mice demonstrating the polarization towards a Th1 response in resistant mice (Fang et al. 2007). The bone marrow derived dendritic cells from the resistant mice also possessed higher phagocytic and bactericidal capabilities with fuller maturation as defined by higher levels of MHC class II and IL-12p40 production (Fang et al. 2007). Those bone marrow derived dendritic cells from susceptible mice promoted the expansion of regulatory T cells during infection with *Rickettsia*, while the IL-12p40 production from the dendritic cells of resistant mice probably accounts for the Th1-dominant response observed in the B6 mice (Fang et al. 2007).

1.3.4.a Regulatory T Cells

Regulatory T cell expression in cocultures of bone marrow derived dendritic cells was later associated with a suppression of *Rickettsia*-dependent Th1 response (Fang et al. 2009). This is reflected in the susceptible mouse model by the suppression of IFN- γ production and the increased production of IL-10 during early infection with *Rickettsia* (Fang et al. 2009). The presence of increased regulatory T cells in these infected susceptible mice suggested that lethal

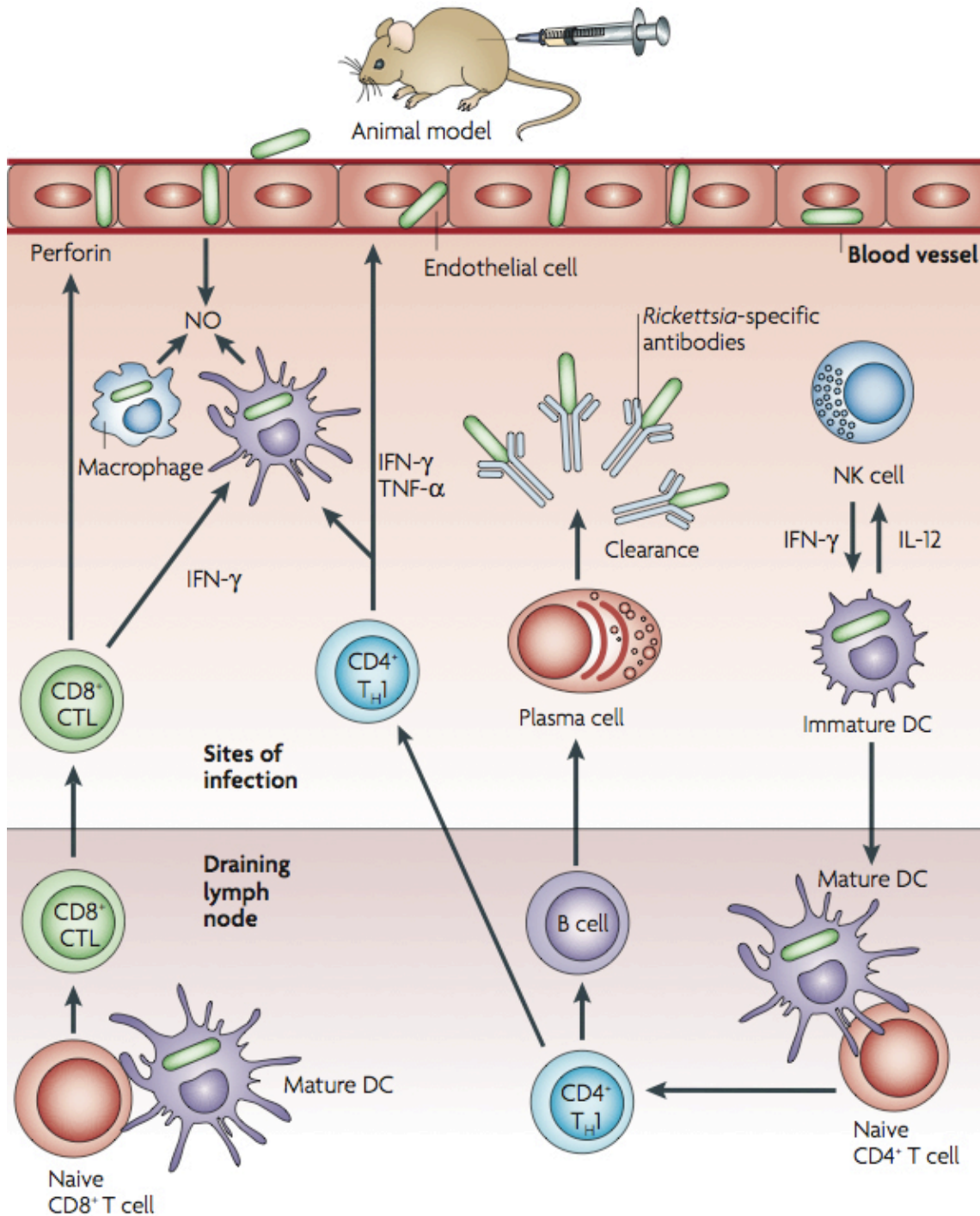


Figure 1.13. Model of protective immunity in rickettsial infection (Walker and Ismail 2008).

rickettsial disease was due to suppression of the protective CD4⁺ Th1 response rather than a bias toward the Th2 phenotype (Fang et al. 2009).

1.3.5 Importance of Models of Rickettsioses

Evolution of rickettsial studies used animal models to perpetuate and eventually isolate *Rickettsia*. Subsequently, there has been a push to develop strong models of rickettsioses, and it is vital to establish models utilizing susceptible inbred mouse strains as these are readily available with easily acquired reagents. This has met with limited success, as different rickettsial strains seem to hold different infectivity for different mice. What is realized is that for every species of SFG *Rickettsia*, an array of models must be assessed to identify the best mouse model. Based on the works cited, the A background strains, Balb/c strain, C3H/HeJ strain, and C3H/HeN strain have great potential for research into the pathogenesis of rickettsioses. Ultimately, development of a model will lead to the ability to incorporate tick-derived factors, which influence rickettsial transmission and infection of the vertebrate host.

1.3.6 Research Directions

It is clear that murine models are a necessity for the evaluation of emerging rickettsioses in addition to well-described rickettsioses. To this end, it is important to determine the adequate inbred mouse strain for the *Rickettsia* of interest. Upon discovery of the best candidate for a murine model of the particular rickettsiosis, it is then necessary to meticulously describe the pathology present within the murine model for comparison to the human rickettsiosis. Finally, the cutaneous route of inoculation and influence of the tick vector must be evaluated as a final step in determining the closeness of fit between the murine and human diseases.

1.4 Summary

The study of tick-borne *Rickettsia* has extended over 100 years now, yet we are still in the infancy for some diseases such as those caused by *R. parkeri* and *R. felis*. We know that RMSF is likely diagnosed for rickettsioses caused by other SFG *Rickettsia*, including *R. parkeri*. *Rickettsia parkeri* is of particular interest because it reflects what is most commonly described for rickettsioses in the United States (mild disease), but more is need to better understand the ecology and epidemiology of this under studied pathogen.

Towards this goal, the pathology and transmission biology within a murine model of *R. parkeri* rickettsiosis was examined, and exploration of the natural ecology of *R. parkeri* was begun. First, four strains of inbred mice were tested for susceptibility to *R. parkeri* (Portsmouth). Once a susceptible mouse strain was detected (C3H/HeJ), the length of the study utilizing only this strain was extended, and the pathology associated with infection was described. Finally, intradermal inoculation of *R. parkeri* in the C3H/HeJ strain at both the tail and the nape of the neck was attempted, which led to the development of eschar-like lesions characteristic of *R. parkeri* rickettsiosis at the tail inoculation but not the nape of the neck.

Subsequently, the susceptible mouse strain (C3H/HeJ) was used to study the impact of tick feeding on the colonization of *R. parkeri* in the skin. Intradermal inoculation of *R. parkeri* at the nape of the neck was performed, and nymphal *A. maculatum* were allowed to feed over the inoculation site. The results showed that tick feeding at the site of inoculation dramatically and, more importantly, significantly increased the number of rickettsiae recovered from the skin.

Finally, the opportunity to survey domestic dogs in southern Louisiana for the presence of *Rickettsia* to explore the role of dogs in the ecology of rickettsioses was seized. Utilizing excess blood from routine heartworm screening, screening PCR was performed followed by

more specific PCR and sequencing to test dogs from 5 different animal shelters in Louisiana for rickettsial infections. *Rickettsia parkeri* specific amplicons were identified in 13% of the dogs tested.

This work has combined laboratory and field studies to decipher some of the ecology of the emerging tick-borne rickettsial disease caused by *R. parkeri*.

CHAPTER 2

SUSCEPTIBILITY OF INBRED MICE TO *RICKETTSIA PARKERI*¹

2.1 Introduction

Over the past decade there has been a dramatic increase in human infection with tick-borne obligate intracellular bacteria belonging to the genera *Anaplasma*, *Ehrlichia*, and *Rickettsia*, collectively referred to as the tick-borne rickettsial diseases (TBRD) (Dumler 2010). Within the genus *Rickettsia*, there are numerous pathogenic species grouped together based on molecular and antigenic characteristics as the spotted fever group (SFG) *Rickettsia*. The clinical signs and pathology associated with these agents in the SFG *Rickettsia* are variable. For example, infection with *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever, is typically associated with a rash, fever, and a high mortality rate (Paddock et al. 2008); in contrast, *Rickettsia parkeri* rickettsiosis (also referred to as American Boutonneuse fever or Tidewater spotted fever) causes a milder disease with eschar, rash, regional lymphadenopathy, and low-grade fever with no reported fatalities (Paddock et al. 2008; Walker and Ismail 2008). The contributing factors responsible for the variation in disease manifestation among SFG species are currently unknown. Recognition of *R. parkeri* has increased since the first confirmed human case of disease reported in 2004 (Paddock et al. 2004), with more than 20 cases emerging around the southeastern United States (Whitman et al. 2007; Paddock et al. 2008; Paddock et al. 2010). The sympatric distribution of pathogenic SFG *Rickettsia* in the United States (Western Hemisphere) warrants differentiation of these infections to better comprehend the epidemiological landscape of TBRDs.

Animal models for rickettsial disease have been used to characterize pathology, test vaccine efficacy, and examine transmission parameters. Guinea pigs have been the prototypical

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animal model for SFG *Rickettsia*; however, these models frequently use unnatural routes of inoculation, and under certain conditions, guinea pigs can have active infection without clinical signs (Ormsbee et al. 1978). Inbred mouse strains also serve as models of disease for SFG rickettsioses, but the antigenic diversity of SFG *Rickettsia* (Philip et al. 1978) causes variability in the susceptibility of mice to rickettsial pathogens (Eisemann et al. 1984). Susceptibility to a particular species of SFG *Rickettsia* may depend on the strain of mouse used in the study. As an example, *R. akari*, the agent of rickettsialpox, is uniformly lethal for Mai: (S) and Balb/c mice, while many other strains, including DBA/1J, DBA/2J, AKR/J, and c57BL/6J are relatively nonsusceptible (Sammons et al. 1977). Compounding factors include differences in mouse strains, which play a role in their susceptibility to SFG rickettsioses. Therefore, while one strain of mice may be susceptible to an individual agent within the SFG, it is possible that the same strain of mice will exhibit a varied susceptibility to a different species of SFG *Rickettsia*. Previous studies highlight these variations in susceptibility. For example, C3H/HeJ mice appear to be the most susceptible strain for *R. conorii*, a species closely related to *R. parkeri* (Goddard 2009), while Balb/c has been reported to be the most susceptible strain of mouse for *R. australis*, and C3H/HeN mice have been found to be the most susceptible strain for *R. typhi* (Eisemann et al. 1984; Feng et al. 1993; Walker et al. 2000; Jordan et al. 2008). While the differences in clinical outcomes may be due to the mouse strain or the route of inoculation, clinical distinction between *R. rickettsii* infections and other typically nonfatal, eschar-associated disease causing agents such as *R. conorii* and *R. parkeri* has not been reported in an animal model.

Variable susceptibility of mouse strains to different SFG *Rickettsia* is also complicated by the manner in which the disease manifests in the model organism. A model that results in a fatal outcome is not needed, and may be inappropriate, for a relatively mild infection such as *R.*

parkeri. While mouse models have proven essential in the characterization of rickettsial pathogenesis, and provide insight into the pathobiology of rickettsioses, the variability in host susceptibility requires a multi-strain analysis to identify a vertebrate host that approximates most closely the pathology of the infection in human hosts. Towards development of a model for emerging eschar-associated rickettsial infection, we examined the infection dynamics of *R. parkeri* in four strains of mice. Initial susceptibility studies were expanded upon using a susceptible strain of mice to recapitulate the pathology of *R. parkeri* eschar-associated rickettsiosis.

2.2 Materials and Methods

2.2.1 Mice

Four mouse strains, A/J, Balb/c, C3H/HeJ, and C3H/HeN, were selected based on previous studies of strain susceptibility to rickettsiae (Eisemann et al. 1984; Feng et al. 1993; Walker et al. 2000). Strains A/J and C3H/HeJ were obtained from the Jackson Laboratory (Maine). C3H/HeN mice were obtained from Charles River Laboratories (Massachusetts). Balb/c mice were obtained from Louisiana State University Division of Laboratory Animal Medicine. All mice were six-week old males in good body condition. All animal research was performed under the approval of the IACUC at Louisiana State University.

2.2.2 Rickettsia Preparation

Semi-purified rickettsiae were recovered from *R. parkeri* (Portsmouth strain (Paddock et al. 2004), passage 4) infected Vero cells (5 days post-inoculation) via needle (27 gauge) lysis of host cells and low- and high-speed centrifugation (Simser et al. 2001; Sunyakumthorn et al. 2008). Absolute concentration of rickettsiae was determined utilizing the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen), a bacterial counting chamber, and fluorescent

microscope (Kurtti et al. 2005). The *Rickettsia* were resuspended in sucrose-phosphate-glutamic acid buffer (SPG) (Feng et al. 2004) to a desired inoculation dose of 5.5×10^6 rickettsiae/200 μ l. This dose was determined based upon published studies using other *Rickettsia* spp. in various mouse strains (Feng et al. 1993; Bechah et al. 2007) and our pilot studies utilizing varying concentrations of *R. parkeri*. Uninfected Vero cell culture was prepared utilizing the same techniques as above with the exception of the bacterial counting. The final lysed Vero cell suspension was diluted with the same volume of SPG as the rickettsial suspension.

2.2.3 Inoculation and Specimen Collection

Animal infection assays were completed in a series of experiments. Four strains of mice were tested for susceptibility to *R. parkeri*, followed by intravenous inoculation (i.v.) of the susceptible strain with a prolonged observation period, and finally intradermal (i.d.) inoculation of the susceptible strain. Initially, all strains of mice were challenged with *R. parkeri* Portsmouth by intravenous (tail vein) inoculation. Each strain of mouse was divided into three groups: age-matched, untouched control (N = 1), SPG injected control (N = 3), and *R. parkeri* Portsmouth injected (N = 6). Mice in the pilot group were observed for seven days post-inoculation for any overt physical signs of disease. At seven days post-inoculation (dpi), mice were sacrificed and whole blood was collected via cardiocentesis. Samples of heart, lung, liver, and spleen were snap frozen in liquid nitrogen for nucleic acid extraction, while additional samples of these same tissues were placed in 10% neutral buffered formalin for histopathology. In the long-term intravenous inoculated group of C3H/HeJ mice, cheek bleeding was performed every other day to every third day for complete blood counts and nucleic acid extraction. The mice were sacrificed at 19 dpi and tissues collected as described for the pilot group. For the intradermal inoculation group, intradermal injections of *R. parkeri* were administered in the skin at the nape

of the neck and in the proximal third of the tail and sacrificed 27 dpi. For these intradermal injections of *Rickettsia*, the volume of inoculum was reduced to 50 µl, but with the same absolute *Rickettsia* count of 5.5×10^6 rickettsiae.

2.2.4 Hematology

Blood films were made within 8 hours of collection, to avoid aging artifacts of the blood, and stained with Diff-Quik™ (Siemens). The smears were examined in a blinded manner by a board certified veterinary clinical pathologist. The complete blood counts were performed by performing total nucleated cell counts with blood diluted 1:100 in 2% acetic acid and counted in a hemacytometer. Differential leukocyte counts were performed by a veterinary clinical pathologist and were used to derive absolute differential leukocyte concentrations based upon the total nucleated cell count.

2.2.5 Histopathology and Immunohistochemistry

Tissues for histopathology were fixed overnight in 10% neutral buffered formalin. All sampled tissues were routinely processed, embedded in paraffin, and 3–4 µm sections were cut for hematoxylin and eosin (H&E) staining. The sections were examined in a randomized manner by a board certified veterinary anatomic pathologist. Selected tissues were examined by immunohistochemistry for evidence of infection with *R. parkeri* using immunoalkaline phosphate technique with a polyclonal anti-*R. rickettsii* antibody, diluted at 1/500, as described previously (Paddock et al. 2008).

2.2.6 Real-time PCR Quantitation of Rickettsial Load

Frozen tissue samples for genomic DNA extraction were processed using the Qiagen DNeasy Blood and Tissue Kit with some modifications. Approximately 10 mg of tissue were placed in a 2 ml Safe-Lock microcentrifuge tube (Eppendorf) to which two sterile 5 mm stainless steel beads (Qiagen) were added. 20 µl of proteinase K (Qiagen) and 180 µl buffer ATL (Qiagen) were then added to each tube, and samples were then placed in a TissueLyser (Qiagen) for two cycles of 30 seconds at 30 mh. Tubes were centrifuged at 7500 g for 5 minutes then incubated for approximately 16 hours in a 56°C water bath. After incubation, extraction was completed according to the manufacturer's instructions. Extracted DNA was stored at -20°C until used for qPCR. The *Rickettsia* primers and species-specific fluorescent-labeled probe for *R. parkeri* (RaRp 17.181F; 5'-GCATTACTTGGAGCAGTTCT-3', RaRp 17.289; 5'-CGCCATTCTACGTT ACTACC-3', Rp 17kDaProbe; 5'-56-FAM-TGCAGAGCTTACCTCACAGAGAGCTT-3') and the mouse primers and fluorescent-labeled probe (Cfd1461; CAGTTTCTTGCTGGCTATTGG-3', Cfd1570; 5'-CCACGTAACCACACCTTCG-3', CfdProbe; 5'-5HEX-TGTAGGGCAGGGCCACTAGTGACAT-3') were designed from the *Rickettsia* 17 kDa gene sequence and the mouse *cfd* sequence using Primer3 software. The 17 kDa antigen gene encodes a common rickettsial surface antigen protein while the mouse *cfd* encodes the complement factor D protein common to most mammals. To quantify a portion of *R. parkeri* 17 kDa gene in mouse tissues, serial dilutions of a plasmid containing single-copy portions of the *R. parkeri* 17 kDa and mouse *cfd* genes were amplified along with the sample unknowns. Briefly, qPCR components and the template that included 2 × LightCycler® 480 Probe Master (Roche); 75 nM of each primer; 200 nM of each probe; DNase/RNase-free water; and 5 µL of gDNA template (samples),

water (negative control), or serial 10-fold dilutions (3.5×10^8 to 3.5×10^3 copies) of pCR4-TOPO- *Rp17kDa +MmCfd* were premixed in 35 μ L volumes in 96-well plates and aliquoted in triplicate 10 μ L reactions on 384-well plates (Reif et al. 2011). Quantitative PCR was then performed using and LightCycler 480 system II (Roche). Analysis of amplification was conducted with LightCycler® 480 software.

2.2.7 Statistics

Data are expressed as means \pm standard errors of the means (SEM), and the significant differences were determined using one-way analysis of variance (ANOVA) with Graphpad Prism and SAS. *P* values of <0.05 were considered significant.

2.3 Results

2.3.1 C3H/HeJ Mice Display Increased Gross Pathology to *R. parkeri* Infection as Compared to A/J, Balb/c and C3H/HeN Strains

At the time of necropsy, no gross abnormalities were observed in any of the A/J or Balb/c mice regardless of age-matched control, SPG injected control, or experimental group. In contrast, 100% of the intravenously inoculated C3H/HeJ mice exhibited marked facial edema (6/6), while the control mice of this strain did not exhibit any discernible facial edema, nor did any of the other strains regardless of inoculation group. The edema was defined as marked based on swelling being severe enough to prevent the affected mice from opening their eyes. Upon necropsy 100% of the infected C3H/HeJ group also displayed marked splenomegaly of 2 to 3 times that of the untouched control, while 100% of the infected C3H/HeN group displayed mild to moderate splenomegaly of 1.5 times that of the untouched control. The degrees of splenomegaly were determined by side-by-side visual comparison of control and treatment groups. The SPG injected control groups for both C3H strains did not exhibit any enlargement of the spleen.

The liver, spleen and heart were examined from all groups. None of the A/J or C3H/HeN mice showed any inflammation within the heart regardless of group (Table 2.1); however, mild inflammation (defined as two or fewer foci of 25 or fewer inflammatory cells) was observed in the heart tissue of SPG-injected C3H/HeJ, *R. parkeri*-injected C3H/HeJ, and all Balb/c groups. The untouched control C3H/HeJ, SPG-injected C3H/HeJ, untouched control C3H/HeN, *R. parkeri*-injected C3H/HeN, SPG-injected A/J, *R. parkeri*-injected A/J and SPG-injected Balb/c groups all exhibited mild myocardial/epicardial mineralization (defined as two or fewer foci of mineralization). The *R. parkeri*-injected C3H/HeJ and *R. parkeri*-injected Balb/c groups both showed moderate myocardial/epicardial mineralization (defined as between two and six foci of mineralization within a section). The untouched control Balb/c mouse exhibited marked epicardial mineralization (greater than six foci of mineralization). Because of the inflammation and myocardial/epicardial mineralization observed in all groups of the Balb/c mice, these findings were not considered to demonstrate an abnormal process associated with the inoculation with *R. parkeri* but rather a normal strain variation typical for all of the Balb/c mice in our study. The SPG-injected C3H/HeN and the untouched control A/J groups displayed no myocardial or epicardial mineralization. Myocardial necrosis and degeneration were not observed in any of the mice except for the untouched control Balb/c mouse (mild myocardial degeneration and necrosis defined as two or fewer foci with three or fewer cells displaying necrosis and degeneration). The spleens from all groups were free from obvious inflammation histologically. The untouched control C3H/HeJ mouse, all C3H/HeN mice, SPG-injected group A/J, *R. parkeri*-injected A/J group, and all Balb/c mice had mild neutrophil loads (defined as less than 20 % of the cells consisting of neutrophils and two or fewer focal accumulations of neutrophils) present within the spleen in contrast to the SPG-injected C3H/HeJ, *R. parkeri*-injected C3H/HeJ and untouched

Table 2.1. Histopathology associated with *R. parkeri* infection (i.v.) of inbred mouse strains at 7 days post-inoculation

Group	Heart				Liver		Spleen	
	Inflammation	Mineralization	Necrosis	Degeneration	Inflammation	Sinus Neutrophilia	Inflammation	Neutrophil load
A/J	Age-matched control	0	0	0	0	0	0	2
	SPG control	0	1	0	0	0	0	1
	<i>R. parkeri</i> inoculated	0	1	0	1	0	0	1
Balb/c	Age-matched control	1	3	1	0	0	0	1
	SPG control	1	1	0	0	0	0	1
	<i>R. parkeri</i> inoculated	1	2	0	1	0	0	1
C3H/HeJ	Age-matched control	0	1	0	0	0	0	1
	SPG control	1	1	0	0	0	0	2
	<i>R. parkeri</i> inoculated	1	2	0	0	0	0	2
C3H/HeN	Age-matched control	0	1	0	1	0	0	1
	SPG control	0	0	0	0	0	0	1
	<i>R. parkeri</i> inoculated	0	1	0	2	0	0	1

0 = absence of the specified parameter, 1 = mild histologic change (rare to infrequent presence as observed by high-powered magnification),
2 = moderate histologic change (change is commonly observed in multiple high-powered fields or larger foci are present in selected areas),
3 = marked histologic change (changes frequently observed in multiple high-powered fields or severe change in focal areas).

control A/J groups, which had moderate neutrophil loads (defined as greater than 20 % of the cells consisting of neutrophils or between two and six focal accumulations of neutrophils). Mild inflammation (defined as less than five foci of fewer than 25 inflammatory cells) was observed in the livers of the untouched control C3H/HeN mouse, *R. parkeri*-injected A/J mice and *R. parkeri*-injected Balb/c mice, while the *R. parkeri*-injected C3H/HeN mice exhibited moderate hepatic inflammation (defined as five to ten foci of fewer than 25 inflammatory cells or less than five foci of greater than 25 inflammatory cells).

No overt hematologic abnormalities were observed during examination of the blood films. The volume of blood collected was insufficient for genomic DNA extraction; therefore qPCR was not performed on these samples.

2.3.2 Rickettsial Loads are Significantly Greater in the Organs of C3H/HeJ Mice

To determine the bacterial burden within the sampled organs, qPCR for the rickettsial 17 kDa antigen gene was used to quantify *R. parkeri* in the heart, lung, liver and spleen at 7 dpi. C3H/HeJ mice had significantly greater numbers of *Rickettsia* in 75% of tissues examined. *Rickettsia* was not detected in any of the tissues collected from the A/J strain of mice; however, rickettsial DNA was detected in all tissues analyzed from the *R. parkeri*-injected C3H/HeJ mice (heart mean = 11,600 copies per 5 µl extracted DNA (cped), range 0 – 32,600 cped; lung mean = 15,400 cped, range 3,530 – 23,600 cped; liver mean = 8,200 cped, range 622 – 24,500 cped; spleen mean = 1,970 cped, range 0 – 7000 cped). In contrast rickettsial DNA was detected in only 50% of the tissues of the Balb/c mice (lung mean = 173 cped, range 0 – 1630 cped; liver mean = 62,400 cped, range 7,010 – 158,000 cped) and 50% of the tissues of the C3H/HeN mice (heart mean = 1,540 cped, range 0 – 11,500 cped; lung mean = 192 cped, range 0 – 2,270 cped)

(Figure 2.1). The only tissue in which the rickettsial DNA was not greatest in the C3H/HeJ mice was the liver, where the Balb/c mice contained the highest rickettsial burden.

2.3.3 *R. parkeri* Causes Typical Rickettsial Vasculitis in C3H/HeJ Mice

To evaluate whether the lack of histopathology in the C3H/HeJ mice was due to insufficient time for progression of disease, mice were tail vein inoculated with *R. parkeri* and sacrificed at nineteen days post-inoculation. At 17 dpi, 50% (2/4) of the *R. parkeri*-inoculated mice presented with necrotic lesions on the tail (Figure 2.2A), which measured 3 mm and 5 mm in diameter. No other gross lesions were observed. The age-matched control mice and the SPG inoculated control mice exhibited no gross lesions for the duration of the experiment. A moderate to marked mononuclear vasculitis comprising lymphocytes, plasma cells, mast cells, and macrophages was present in the tails of each of the 4 *R. parkeri*-inoculated mice (Figure 2.2C). Moderate to marked fibrinoid vascular necrosis was present as well as mild to moderate perivascular edema. In contrast, no vasculitis or necrosis was observed in either the untouched control or SPG-inoculated control mice. Immunohistochemistry for SFG *Rickettsia* revealed positive staining in endothelial cells and macrophages within the tail lesions indicating the presence of intracellular *Rickettsia* (Figure 2.2B).

2.3.4 Prolonged Infection with *R. parkeri* Results in an Inflammatory Leukogram

Blood samples were collected every second to every third day during the nineteen day intravenous inoculation study. The total leukocyte concentrations in the *R. parkeri*-inoculated mice increased six dpi and exceeded the values of the uninfected mice. The leukocyte concentration then fell below the values of the uninfected mice on 14 dpi. The neutrophil count in the experimental group was significantly higher than the uninfected group at 6 and 10 dpi, whereas lymphocyte concentrations were significantly lower than the control group at all times

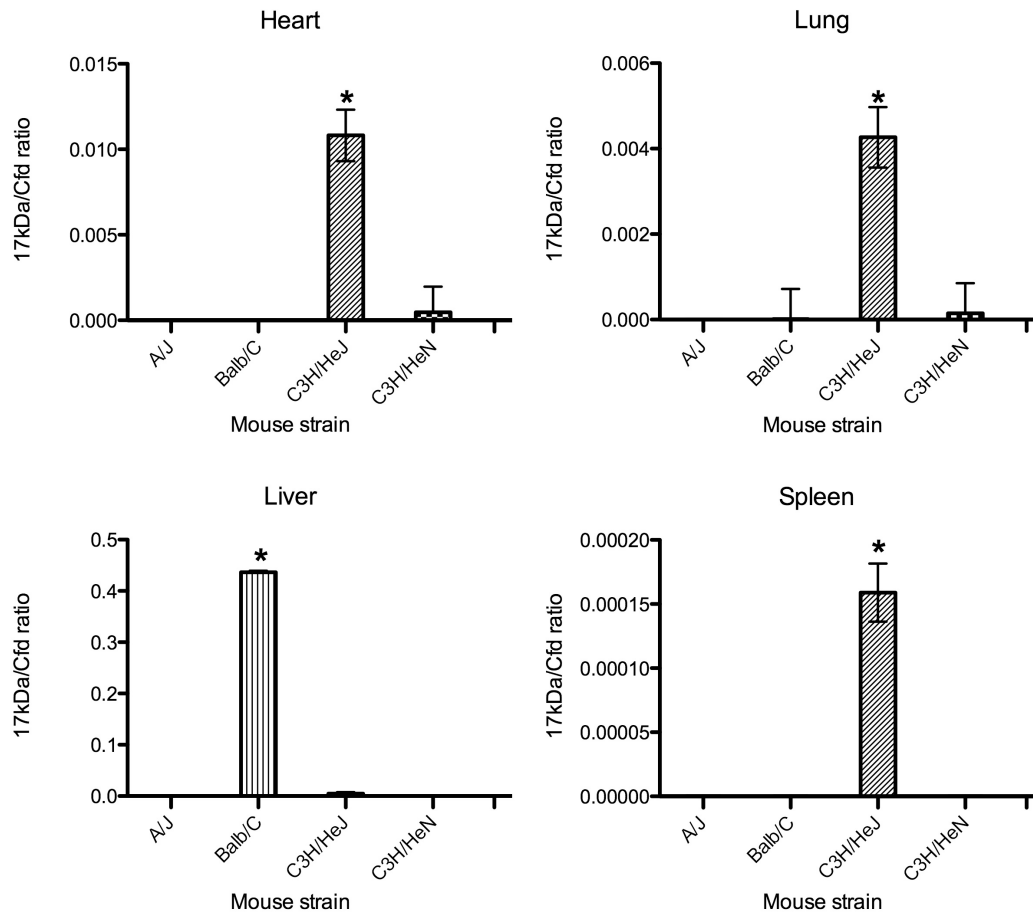


Figure 2.1. qPCR for *R. parkeri* 17 kDa antigen gene relative to mouse *cfd* in various tissues at 7 dpi. Relative quantification was utilized to account for variation in weight of tissues at the time of nucleic acid extraction. The mean *R. parkeri* numbers \pm SEM as detected in the heart and lung of both the C3H/HeN and C3H/HeJ mice, in the liver of both the C3H/HeJ and Balb/C mice, and in the spleen of the C3H/HeJ mice (* denotes significance between groups of $p \leq .05$).

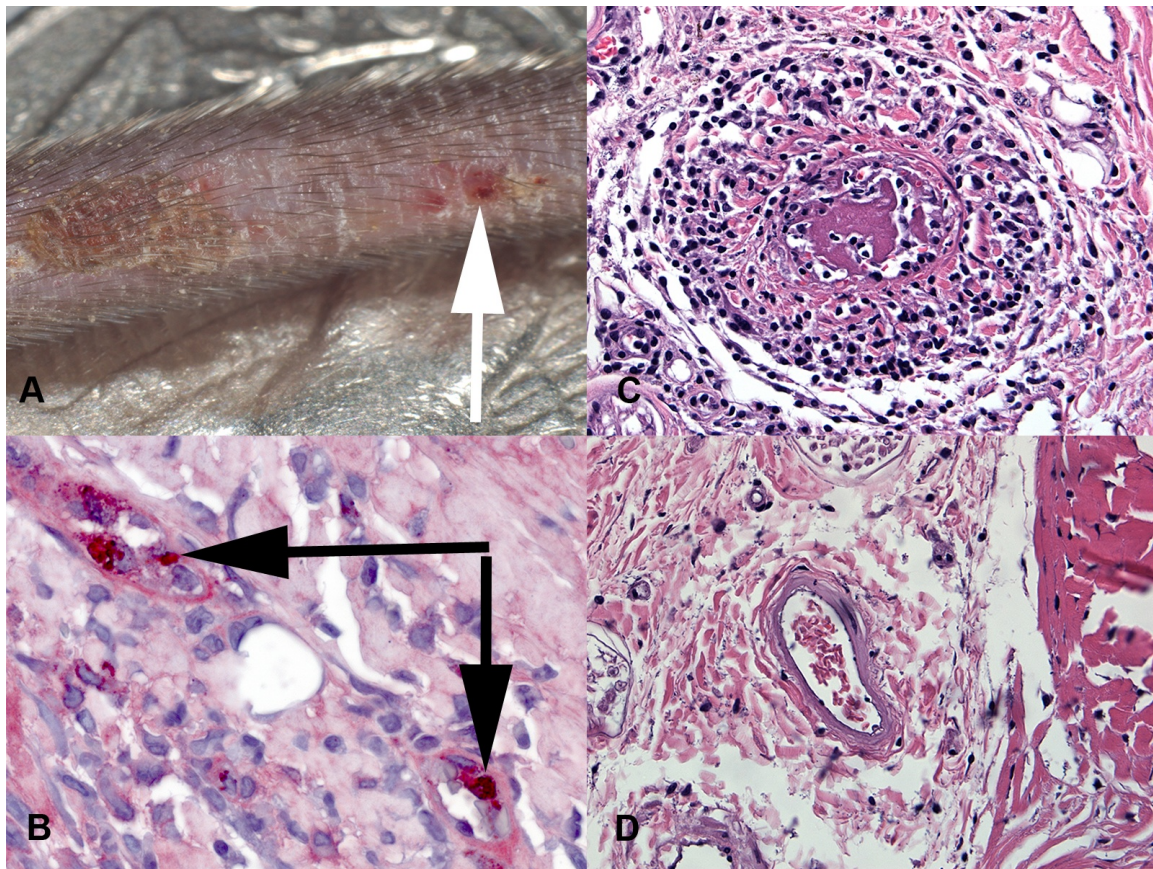


Figure 2.2. Gross and histopathology of eschar-like lesions in C3H/HeJ mice following intradermal inoculation of *R. parkeri* at 27 dpi. Gross lesions associated with inoculation site in *R. parkeri* infected C3H/HeJ mouse (white arrow), A; Immunohistochemistry displaying positive staining (black arrows) in the cytoplasm of endothelial cells and macrophages of *R. parkeri* infected C3H/HeJ mice, B; Marked vasculitis in *R. parkeri* infected C3H/HeJ mouse, C; and uninfected C3H/HeJ mouse histopathology, D, for comparison.

points except 10 dpi (Figure 2.3). Monocyte concentrations showed a similar pattern to the neutrophils with a peak concentration at 10 dpi then returning to control levels. Eosinophil concentrations were significantly lower in the experimental group; however, they did not mirror the changes over time of the other cell types. These changes were consistent among mice within the *R. parkeri*-inoculated group. When examined by qPCR, very low concentrations of rickettsial DNA were detected in the blood of 50% (2/4) of *R. parkeri*-inoculated mice, one of which also had a tail lesion, at 19 dpi. Rickettsial DNA was not detected in any of the other blood samples at any time point.

2.3.5 Eschar-like Lesions Result from Intradermal Inoculation of C3H/HeJ Mice with *R. parkeri*

Eight mice were intradermally inoculated with *R. parkeri* to determine the propensity for development of eschars in C3H/HeJ mice. Two of the eight intradermally inoculated mice developed crusting at the site of tail inoculation, which were approximately 3 mm in diameter at their maximum and began developing at between 8 and 10 dpi. The crust on one mouse resolved by 17 dpi, while the other had decreased in size by 18 dpi at which point this mouse was sacrificed. A third mouse, while not forming crusts, developed a 4 mm x 1 mm linear depigmentation at the site of tail inoculation 4 dpi. This lesion remained unchanged for the duration of the experiment. In contrast, at 7 dpi two additional mice developed circumferential swelling of the tails around the site of inoculation, which extending cranially and caudally for approximately two intervertebral spaces. The swelling was resolved in both mice by 10 to 12 dpi. Further, a linear depigmentation approximately 3 mm x 1 mm was present on the tail at 20 dpi of one of the mice that had previously had circumferential swelling. All of these mice displayed vasculitis of varying severities at the site of tail inoculation. No gross or histopathologic lesions were observed at the site of *Rickettsia* inoculation on the nape of the neck

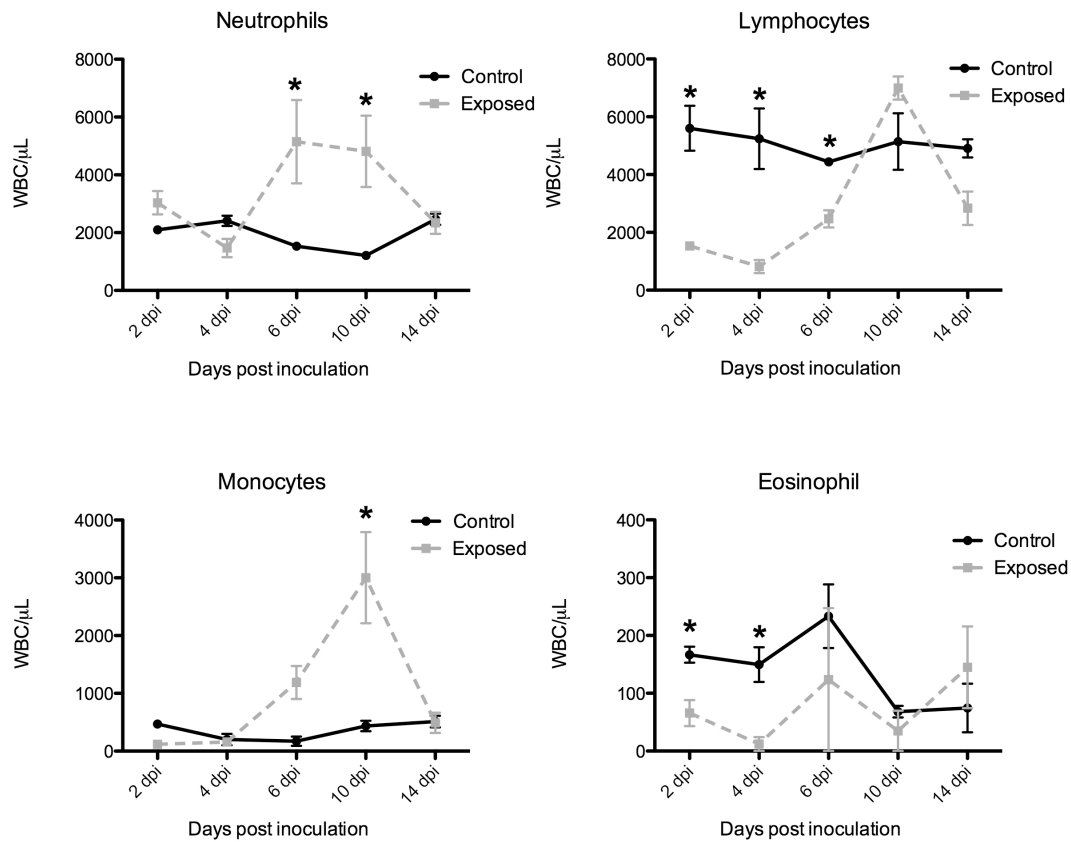


Figure 2.3. Mean nucleated cell concentration \pm SEM of the blood from *R. parkeri* infected (i.v.) C3H/HeJ mice. Neutrophils and monocytes of the infected group both showed significant increases in the total concentrations as compared to the control group, while lymphocytes showed and early decrease in concentration then a return to similar levels as the control group (* denotes significance between groups of $p \leq .05$).

and necropsy revealed no gross lesions on any of the internal organs. At 19 dpi, rickettsial DNA was recovered by PCR from multiple mice at the site of inoculation regardless of the manifestation of gross pathology, while the inoculation site in the skin at the nape of the neck was negative for all mice.

2.4 Discussion

Four strains of mice with known susceptibility to various SFG *Rickettsia* were examined for the susceptibility to infection, and pathological parameters were assessed. Of the four strains used, *R. parkeri* DNA was detectable in all tissues examined from only the C3H/HeJ mice, suggesting susceptibility to sustained infection. Additionally, although most models for rickettsioses have been based upon abnormal routes of infection (intravenous, intraperitoneal), the more natural route of intradermal inoculation of the C3H/HeJ mice resulted in several of the mice developing eschar lesions characteristic of human infection, which is a novel finding in mice utilized for SFG *Rickettsia* modeling. Therefore, although animal models of rickettsial infection appear multivariate, this study provides an experimental model of *R. parkeri* rickettsiosis, which can be utilized to elucidate the mechanisms of infection and pathogenesis.

Based on the temporality of the experiments and the quantities of *Rickettsia* recovered by qPCR, the resulting values are indicative of rickettsial replication. In the mouse strains which showed no gross pathology for example, the rickettsial concentrations were much lower than in the C3H/HeJ mice and were actually undetectable in most of the tissues. While impaired clearance may be a potential explanation for the greater concentrations of rickettsial DNA recovered from the C3H/HeJ mice, the extended time course of the consequent studies would have been expected to eliminate this factor as the rickettsial DNA should have degraded much faster in the absence of the hemostatic environment of the living pathogen. In order to further

support this, research enumerating the growth at the lesion must be undertaken, along with studies which consider the impact of tick feeding on rickettsial replication at the site of inoculation.

Animal models have proven vital to expanding our knowledge of the pathogenesis of infectious diseases. Within *Rickettsia*, murine models have elucidated the importance of TLR4 signaling and subsequent dendritic cell activation to the clearance of pathogenic *Rickettsia* (Jordan et al. 2008; Jordan et al. 2009; Quevedo-Diaz et al. 2010). Mouse models have also revealed that *R. prowazekii* lesions are independent of humoral immune response (Bechah et al. 2007). These discoveries highlight the importance of an accurate animal model, and the identification of the C3H/HeJ strain as a viable model for *R. parkeri* rickettsiosis provides a platform for future revelations.

This model animal may also prove beneficial in ways that some other model systems for rickettsial diseases do not. The C3H/HeN strain of mouse is used for modeling *R. conorii* infection; however, this system results in a fatal disease, which lacks consistency with the human illness (Jordan et al. 2008). In many ways this model remains more than adequate for studying the pathogenesis of this rickettsiosis, but the terminal nature of the infection precludes the use of long-term studies into the progression of and eventual recuperation from disease. In contrast, the C3H/HeJ model of *R. parkeri* rickettsiosis has not yet proven fatal even in prolonged studies and shows promise as a model of progression and resolution of eschars.

TLR4 has been shown to be of great importance in the immune response to rickettsial infection (Jordan et al. 2008; Jordan et al. 2009; Quevedo-Diaz et al. 2010). Mice with competent TLR4 signaling are more resistant to rickettsial infection (Jordan et al. 2008). This is primarily because of the stimulation of dendritic cells and subsequent production of interferon

(IFN)- γ , followed by the expansion of the population of activated natural killer cells (Jordan et al. 2009). This provides a probable explanation for the observed susceptibility of C3H/HeJ mice to *R. parkeri*. The lack of stimulation of the Th1 response secondary to TLR4 signaling also leads to the expansion of T regulatory cells, which may result in suppression of proinflammatory immune responses (Jordan et al. 2008). These deficiencies in the immune regulation of the C3H/HeJ mice in response to rickettsial infection warrant further study in comparison to the TLR4 competent C3H/HeN strain in order to elucidate the mechanisms of rickettsial recognition and clearance by the vertebrate host.

The facial edema, which developed in the intravenously inoculated C3H/HeJ strain, was an unexpected finding. Gross edema is not a common finding in *R. parkeri* rickettsiosis; however, edema is frequently observed histopathologically in the immediately adjacent tissues surrounding inflamed vessels. The mechanism of this edema is related to the inflammation and necrosis of the infected vessels. The distribution of the edema is intriguing as the head and neck are areas commonly associated with tick infestation. Although not tested in the current study, it is possible that a distribution pattern of heavier infection of the vessels of the head and neck increases the likelihood of uptake by and infection of the tick vectors.

Eschars are common to most SFG *Rickettsia* and form at the site of tick or mite inoculation of *Rickettsia* as a result of local dermal and epidermal necrosis with marked vasculitis (Walker et al. 1988). The eschars that developed in this murine model are especially important as this is a prominent clinical sign of *R. parkeri* rickettsiosis as well as two of its closest relatives, *R. conorii* and *R. africae* (Walker and Ismail 2008; Goddard 2009). The ability to recapitulate these lesions in a murine model provides a basis for comparison to the effects of these closely related SFG rickettsiae. Eschars from patients with Mediterranean spotted fever

express high mRNA levels of TNF, IFN γ , IL-10, RANTES, indoleamine-2,3-dioxygenase and inducible nitric oxide synthase (de Sousa et al. 2007). The lesions from *R. parkeri* infected C3H/HeJ mice can be used to determine if a similar pattern of immunological response develops for this rickettsiosis, while another potential source of future research into eschars is evaluating the differences in pathogenesis at the site of tick inoculation between eschar-associated rickettsioses and those that do not typically cause eschars. Although the non eschar-associated agent of Rocky Mountain spotted fever, *R. rickettsii*, is the most commonly implicated etiologic agent of rickettsial disease in the United States, there are at least seven eschar-associated rickettsioses encountered by clinicians in the US including: *R. parkeri* rickettsiosis, rickettsialpox, 364D rickettsiosis, cat flea-associated rickettsiosis, African tick bite fever, Mediterranean spotted fever, and *R. massiliae* rickettsiosis (Cragun et al. 2010). It would be very interesting to establish the differences in pathogenesis between *Rickettsia* associated with these distinct disease manifestations.

The histopathology of the eschars is similar to that reported in humans with epidermal necrosis and perivascular dermatitis being the prominent features (Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010). The inflammatory infiltrates associated with this murine model eschar are also analogous to the inflammation described in human patients with *R. parkeri* rickettsiosis (Paddock et al. 2008), suggesting a similar immunological response, but this will require further investigation to confirm. Rickettsiae were present within the vascular lesions as assessed by immunohistochemistry. As described in patients with *R. parkeri* rickettsiosis, the SFG *Rickettsia* were found predominantly in the cytoplasm of mononuclear cells (macrophages) and endothelial cells (Paddock et al. 2008). These similarities in necrosis at the site of inoculation, inflammatory pattern and infiltrate, and distribution of *Rickettsia* support the

concept that the infection process is similar for the murine model of infection and the human infection at least at the site of inoculation, which further supports the use of the C3H/HeJ mouse as a model for *R. parkeri* rickettsiosis. The absence of these eschar-like lesions at the intradermal-inoculation at the nape of the neck suggests that additional factors are necessary to stimulate infection at this site. It is likely that the tick vector plays a significant role in the ability of *R. parkeri* to infect its vertebrate host, and future studies are necessary to elucidate this process.

The presence of rickettsiae within the hepatic tissue of the Balb/c strain of mice is interesting when the lack of inflammation observed histologically is considered. Possible explanations for this discrepancy include: differences in sampling sites utilized for the nucleic acid extraction and the histopathology and contamination during sample acquisition, nucleic acid extraction or qPCR preparation. The latter seem unlikely for various reasons. Contamination during sample acquisition should have resulted in similar findings in other organs from the Balb/c mice, while the nucleic acid extraction and qPCR preparations were repeated with similar results suggesting that either no contamination occurred or that very similar contaminants were present at two separate time points. Sampling error appears to be the most probable cause for the discrepancy and is a commonly encountered issue in diagnostic settings. It is probable that rickettsial lesions were present in different foci of the hepatic tissue including that sampled for nucleic acid extraction, but the same was not true for the sections evaluated by histopathology. With the increase in TBRD recognized in humans, further study into the transmission of the responsible organisms and the pathogenesis of the diseases has become of paramount importance. *R. parkeri* serves as an elegant example of this as the first confirmed case of human disease was reported less than a decade ago. The relatively recent recognition of this organism

as a pathogen has deeper implications considering research into its biology remains in its infancy when compared even to other rickettsiae. To elevate the knowledge base regarding *R. parkeri* and its disease, a commonly available inbred mouse strain with strong potential for use as a model for research into the pathogenesis of *R. parkeri* rickettsiosis has been identified in the current study. The C3H/HeJ inbred mouse strain was found to be susceptible to infection with *R. parkeri*, while typical gross and histopathological features of human disease developed at the site of intradermal inoculation, and also in concert with findings in humans with *R. parkeri* rickettsiosis the infection has thus far proven nonfatal. This work identifies a new tool for investigators in subsequent studies of the pathogenesis of *R. parkeri* rickettsiosis, which can now include detailed characterization of the immune response to the bacteria along with incorporating the effects of the tick vector on establishment and maintenance of infection.

CHAPTER 3

EFFECT OF TICK FEEDING ON RICKETTSIAL PROLIFERATION IN A MOUSE MODEL

3.1 Introduction

The dramatic increase in recognition of TBRDs over the past decade is punctuated by the emergence of a rickettsiosis caused by *Rickettsia parkeri* in the southeastern United States and South America. Although originally described more than 70 years ago, *R. parkeri* was first determined to be pathogenic to humans within the last decade (Parker et al. 1939; Paddock et al. 2004). The resulting rickettsiosis has since been diagnosed at least 20 times, and it bears great resemblance to Mediterranean spotted fever, but *R. parkeri* rickettsiosis is much milder (Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Paddock et al. 2010; Romer et al. 2011). Specific findings shared amongst these eschar-associated rickettsioses are fever, myalgia, malaise, headache, and a maculopapular or vesicular eruption (Cragun et al. 2010). Although the geographic distributions of these *Rickettsia* are vastly different, the range of *R. parkeri* and its tick vector, *Amblyomma maculatum*, overlap greatly with the range of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, in the United States (Sumner et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Trout et al. 2010; Jiang et al. 2012). The paucity of information and sympatry with other SFG *Rickettsia* for this eschar-associated disease necessitate comprehensive exploration of the mechanisms vital to infection establishment.

Investigation of the pathogenesis of rickettsioses depends heavily on the utilization of animal models; however, the cutaneous route of inoculation is often circumlocuted as is the influence of arthropod feeding. A murine model has recently been proposed for *R. parkeri* rickettsiosis, in which the C3H/HeJ strain of inbred mouse was determined to be the most susceptible (Graspege et al. 2012). These mice lack competent TLR4 signaling due to a mutation, which causes an amino acid switch in the cytoplasmic domain of the TLR4 protein

(Poltorak et al. 1998; Hoshino et al. 1999; Qureshi et al. 1999). Interestingly, the eschars associated with *R. parkeri* rickettsiosis were inducible by intradermal inoculation of *R. parkeri* into the tail but the same did not hold true for the skin over the nape of the neck (Grasperge et al. 2012). The reason for this difference is unclear but may relate to temperature differences at the inoculation sites or differences in immunological response of the tissues. Explanation of the mechanisms preventing infection at the inoculation site at the nape of the neck are central to understanding the pathogenesis of TBRDs as this is a common site for tick feeding (Teel et al. 2010), and therefore a probable site for introduction of pathogenic rickettsiae. Mild, eschar-associated rickettsioses such as those caused by *R. parkeri* and *Rickettsia africae* demand an infection model in which the infection is initiated cutaneously.

As a result of a prolonged feeding period, ticks have developed mechanisms to modify the host microenvironment to allow blood meal acquisition. Typical mechanisms include modulation of: complement activation, natural killer cell function, antibody production, T-lymphocyte proliferative responses, and cytokine elaboration by antigen-presenting cells and T-lymphocytes (Wikel 1996). The influence of tick feeding on bacterial transmission to and infection of vertebrate hosts has been described for other systems. The supplementation of cytokines normally down-regulated by tick feeding resulted in decreased infection rates in mice exposed to *Borrelia burgdorferi* infected ticks (Zeidner et al. 1996). Animals with acquired resistance to ticks have been shown to be more resistant to infection with pathogens transmitted by those ticks (Bell et al. 1979; Wikel et al. 1997; Nazario et al. 1998; Narasimhan et al. 2007; Dai et al. 2009). Some pathogens also undergo developmental transitions within the tick vector, which result in a form of the pathogen that is more infectious for the vertebrate host (Mastrorunzio et al. 2012). The dramatic impact of tick saliva on the immunology of the

microenvironment at the feeding site may play a critical role in the inception of infection with tick-borne pathogens, but surprisingly little is known with respect to the influence of tick feeding on rickettsial infection. Here the recently described model for *R. parkeri* rickettsiosis was used to evaluate the role of the tick in rickettsial infection of the vertebrate host. It was hypothesized that tick feeding enhances rickettsial population of the cutaneous feeding site prior to dissemination of the infection. The results indicate that tick feeding at the site of rickettsial inoculation enhances local rickettsial proliferation dramatically.

3.2 Materials and Methods

3.2.1 Mice

C3H/HeJ mice were selected based on previous susceptibility studies (Graspege et al. 2012). Seven week old, male mice were obtained from the Jackson Laboratory (Maine). All mice were in good body condition and were ear punched for identification purposes. The animals were monitored daily during the course of the experiment for overt clinical signs of disease and body temperature fluctuations. The research was performed under the approval of the IACUC at Louisiana State University.

3.2.2 Tick Preparation

A colony of *A. maculatum* were developed from wild caught adults and maintained as previously described (Troughton and Levin 2007). Briefly, nymphal and adult ticks were fed on adult Sprague-Dawley rats (Louisiana State University Division of Laboratory Animal Medicine) within capsules fashioned from plastic 50 ml conical tubes and attached with a 3:1 tree rosin to bee wax mixture. Engorged females were kept in vials at 27° C and approximately 90% relative humidity. Larvae were fed on adult Balb/c mice housed on wire grates over fresh water, and

engorged larvae were collected twice daily as the water was changed. For this experiment 120 nymphs, which originated from the same egg clutch, were utilized.

3.2.3 Rickettsia Preparation

Semi-purified rickettsiae were recovered from *R. parkeri* Portsmouth strain (Paddock, Sumner et al. 2004), passage 4 infected Vero cells (5 days post-inoculation) via needle (27 gauge) lysis of host cells and low- and high-speed centrifugation (Simser, Palmer et al. 2001; Sunyakumthorn, Bourchookarn et al. 2008). Absolute concentration of rickettsiae was determined utilizing the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen), a bacterial counting chamber, and fluorescent microscope (Kurtti, Simser et al. 2005). The rickettsiae were resuspended in sucrose-phosphate-glutamic acid buffer (SPG) (Feng, Whitworth et al. 2004) to a desired inoculation dose of 5.5×10^6 rickettsiae/200 μ L. Uninfected Vero cell culture was prepared utilizing the same techniques as above with the exception of the bacterial counting. The final lysed Vero cell suspension was diluted with the same volume of SPG as the rickettsial suspension.

3.2.4 Inoculation and Tick Infestation

Mice were divided into five groups containing six animals each: age-matched controls, SPG injected control, *R. parkeri* injected, SPG injected + tick feeding, and *R. parkeri* injected + tick feeding. The hair over the nape of the neck was clipped for all mice except the age-matched controls. For each injection group, 200 μ L of the appropriate inoculum was injected intradermal in the clipped area of skin over the nape of the neck. The bleb formed by the injection was marked with felt tip pen immediately after inoculation, and the mark was retraced daily in the groups without tick feeding to overcome fading due to grooming and hair regrowth. The tick feeding groups were fitted with capsules fashioned from plastic 15 mL conical tubes directly

over the inoculation site within 20 minutes of the injection. After allowing approximately 30 minutes for the rosin/wax mixture to harden, 10 nymphal *A. maculatum* were added to each capsule. The capsules were monitored daily for damage due to grooming or activity of the mice and repaired as needed. Ticks were allowed to feed to repletion and removed from the capsules at the time of detachment. The study was concluded as the final tick detached at eight days post-infestation.

3.2.5 Sample Collection

Mice were sacrificed at eight days post-inoculation (dpi). Samples of heart, lung, liver, spleen and skin from the inoculation site were snap frozen in liquid nitrogen for nucleic acid extraction, while additional samples of the skin were placed in RNAlater (Ambion, Austin, Texas) until RNA extraction could be performed. Samples of the skin were also placed in 10% neutral buffered formal for histopathology.

3.2.6 Histopathology and Immunohistochemistry

Tissues for histopathology were fixed overnight in 10% neutral buffered formalin. All sampled tissues were routinely processed, embedded in paraffin, and 3–4 µm sections were cut for hematoxylin and eosin (H&E) staining. The sections were examined in a randomized manner by a board certified veterinary anatomic pathologist. Vasculitis was scored on a scale of 0 – 3 as follows: 0 – no evidence of inflammation, 1 – endothelial cell swelling with or without rolling of leukocytes along the surface of the endothelium, 2 – inflammatory cells dissecting through the endothelial cells with endothelial cell swelling, and 3 – large numbers of leukocytes through all layers of the vessel with loss of the tunica media. Selected tissues were examined by immunohistochemistry for evidence of infection with *R. parkeri* using immunoalkaline

phosphate technique with a polyclonal anti-*R. rickettsii* antibody, diluted at 1/500, as described previously (Paddock, Finley et al. 2008).

3.2.7 Real-time PCR Quantitation of Rickettsial Load

Frozen tissue samples for genomic DNA extraction were processed using the Qiagen DNeasy Blood and Tissue Kit with some modifications. Approximately 10 mg of tissue were placed in a 2 ml Safe-Lock microcentrifuge tube (Eppendorf) to which two sterile 5 mm stainless steel beads (Qiagen) were added. 20 μ l of proteinase K (Qiagen) and 180 μ l buffer ATL (Qiagen) were then added to each tube, and samples were then placed in a TissueLyser (Qiagen) for two cycles of 30 seconds at 30 cycles per second. Tubes were centrifuged at 7500 g for 5 minutes then incubated for approximately 16 hours in a 56°C water bath. After incubation, extraction was completed according to the manufacturer's instructions. Extracted DNA was stored at -20°C until used for qPCR. *Rickettsia* primers and probe for the 17 kDa antigen gene and mouse primers and probe for mouse *cfb* were utilized as previously described (Graspege et al. 2012). The 17 kDa antigen gene encodes a common rickettsial surface antigen protein while the mouse *cfb* encodes the complement factor D protein common to most mammals. To quantify a portion of *R. parkeri* 17 kDa gene in mouse tissues, serial dilutions of a plasmid containing single-copy portions of the *R. parkeri* 17 kDa and mouse *cfb* genes were amplified along with the sample unknowns. Briefly, qPCR components and the template that included 2 \times LightCycler® 480 Probe Master (Roche); 75 nM of each primer; 200 nM of each probe; DNase/RNase-free water; and 5 μ l of gDNA template (samples), water (negative control), or serial 10-fold dilutions (3.5×10^8 to 3.5×10^3 copies) of pCR4-TOPO- *Rp17kDa* +*MmCfb* were premixed in 35 μ l volumes in 96-well plates and aliquoted in triplicate 10 μ l reactions on 384-

well plates (Reif et al. 2011). Quantitative PCR was then performed using and LightCycler 480 system II (Roche). Analysis of amplification was conducted with LightCycler® 480 software.

3.2.8 RNA Extraction and Reverse Transcriptase PCR Quantitation

RNA was purified from the samples stored in RNAlater using the Quick-RNA MiniPrep kit (Zymo) according to manufacturers instructions. Extracted samples were stored at -80°C. RNA was treated with DNaseI for 30 minutes and re-purified using an RNA clean-up kit (Zymo Research). cDNA was generated from RNA samples using an iScript reverse transcription kit (Bio-Rad) following manufacturer's instructions. cDNA was diluted 5 fold in RNase-free water prior to use in real-time PCR. All primers used for real-time PCR analysis were designed using Primer3 software [36]. Primer sequences were blasted against the National Center for Biotechnology Information (NCBI) database to confirm that all primer pairs were specific for the gene of interest and that no homology to other genes was present. PCR reactions were prepared using SYBR green mix with Rox (Bio-Rad) in a 10 µl volume with approximately 10 ng of cDNA and 1.8 µM forward and reverse primers. Samples were run in triplicate on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Analysis of dissociation curves was used to confirm the amplification of a single product for each primer pair per sample. Confirmation of a lack of DNA contamination was achieved by analyzing samples that had not undergone reverse transcription. Untranscribed controls had at least a 1,000 fold lower expression level than analyzed samples or were negative for all genes after 40 cycles. Gene expression was quantified by the cycle number at which each sample reached a fixed fluorescence threshold (CT). To control for variations in RNA amounts among samples, data were calculated as the difference in CT values (log2) between the housekeeping gene, *Gapdh*, and the gene of interest for each sample ($\Delta CT = CT_{Gapdh} - CT_{gene\ of\ interest}$). Data were

calculated as a percentage of *Gapdh* expression for each gene of interest per sample. These data were then calculated as fold expression relative to the average of mock samples for each gene and each group.

3.2.9 PCR of Ticks Fed on Infected and Uninfected Mice

All of the engorged nymphal ticks from both tick feeding groups were maintained in separate vials at 27°C and 90% relative humidity. After molt, the adult ticks were processed for DNA extraction and standard PCR. Briefly, ticks were halved and pooled into groups of two ticks, which were always from the same mouse. The samples were process for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit as previously described with a few modifications. Initially, 180 µl of buffer ATL and 20 µl of proteinase K were added to each sample. The samples were then incubated overnight in a 56°C water bath. Extractions were then completed according to manufacturers instructions with a final elution volume of 100 µl. Standard PCR was performed using 190.70p and 190.602n and 190.70p and 190.701 primer pairs for *rompA* as previously described (Regnery et al. 1991; Fournier et al. 1998).

3.2.10 PCR of Skin from Tick Infested Mice

To determine if *Candidatus* Rickettsia andeanae was introduced into the skin of the mice during tick feeding, standard PCR was performed on the previously extracted DNA of the skin samples from the mice of both tick infested groups. Amplification was performed using primers 190.70p and 190.701 for *rompA* as previously described (Regnery et al. 1991; Fournier et al. 1998).

3.2.11 Statistics

Mouse temperature data were evaluated using the mixed procedure in SAS. Real-time quantitative PCR and reverse transcriptase PCR data were evaluated by paired t-tests using Prism 5.0 (GraphPad). *P* values of <0.05 were considered significant.

3.3 Results

3.3.1 Tick Feeding Results in Enhanced Rickettsial Proliferation at the Intradermal Inoculation Site

Quantification of the genus-specific 17 kDa antigen gene revealed a significant increase in rickettsial proliferation when nymphal *A. maculatum* were allowed to feed at the site of *R. parkeri* inoculation. While no amplification was observed in the *Rickettsia* inoculated group that did not include tick feeding, the skin of the mice from the *Rickettsia* inoculated with tick feeding all had detectable levels of rickettsial DNA, which totaled larger numbers of rickettsiae than were originally inoculated into the skin (average of 640,500 copies per 5 µL extracted DNA) (Figure 3.1). Rickettsial DNA was not recovered from the skin of any of the mice from the remaining groups, SPG inoculated control; *R. parkeri* inoculated without tick feeding; and SPG inoculated with tick feeding.

Immunohistochemistry revealed a marked increase in cells staining positive for SFG *Rickettsia* in the *R. parkeri* inoculated with tick feeding group when compared to the *R. parkeri* inoculated without tick feeding group (Figure 3.2). Rare to low numbers of SFG *Rickettsia* positive cells could also be found in the *R. parkeri* inoculated without tick feeding group and the SPG inoculated with tick feeding group, but not in the SPG inoculated without tick feeding group.

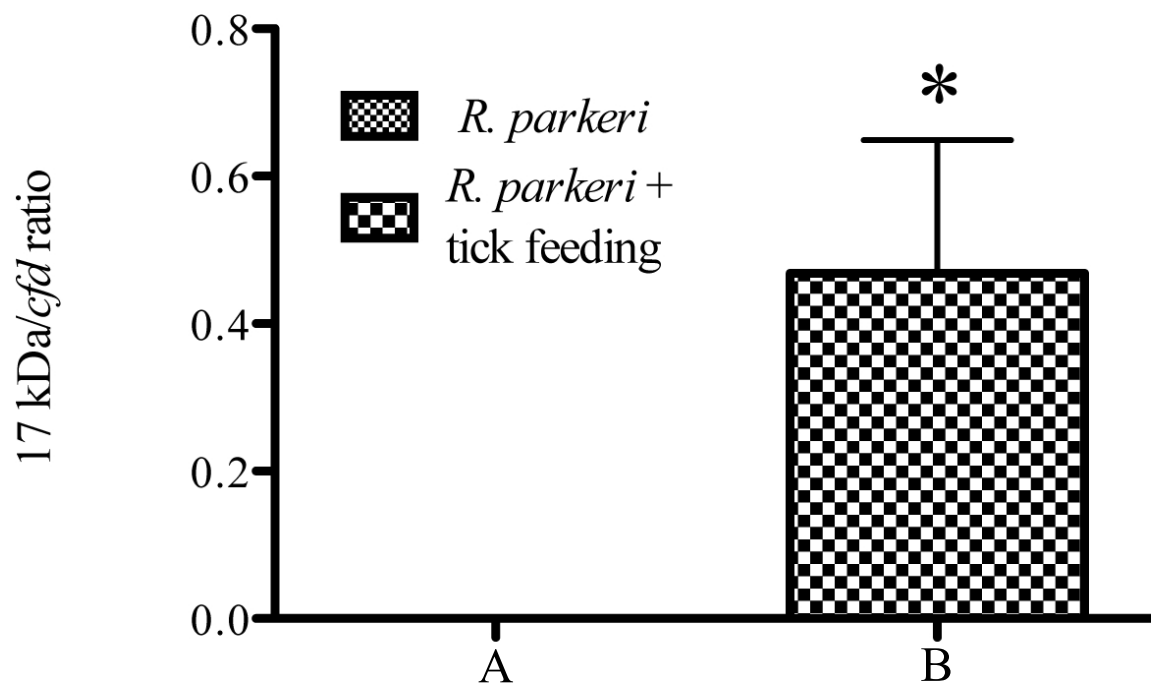


Figure 3.1. qPCR for *R. parkeri* 17 kDa antigen gene relative to mouse *cfd* in the skin at 8 dpi. Relative quantification was utilized to account for variation in weight of tissues at the time of nucleic acid extraction. The mean *R. parkeri* numbers \pm SEM as detected in mice that either A) had no nymphal *A. maculatum* infestation or B) had nymphal *A. maculatum* feeding at the inoculation site (* denotes significance between groups of $p \leq .05$).

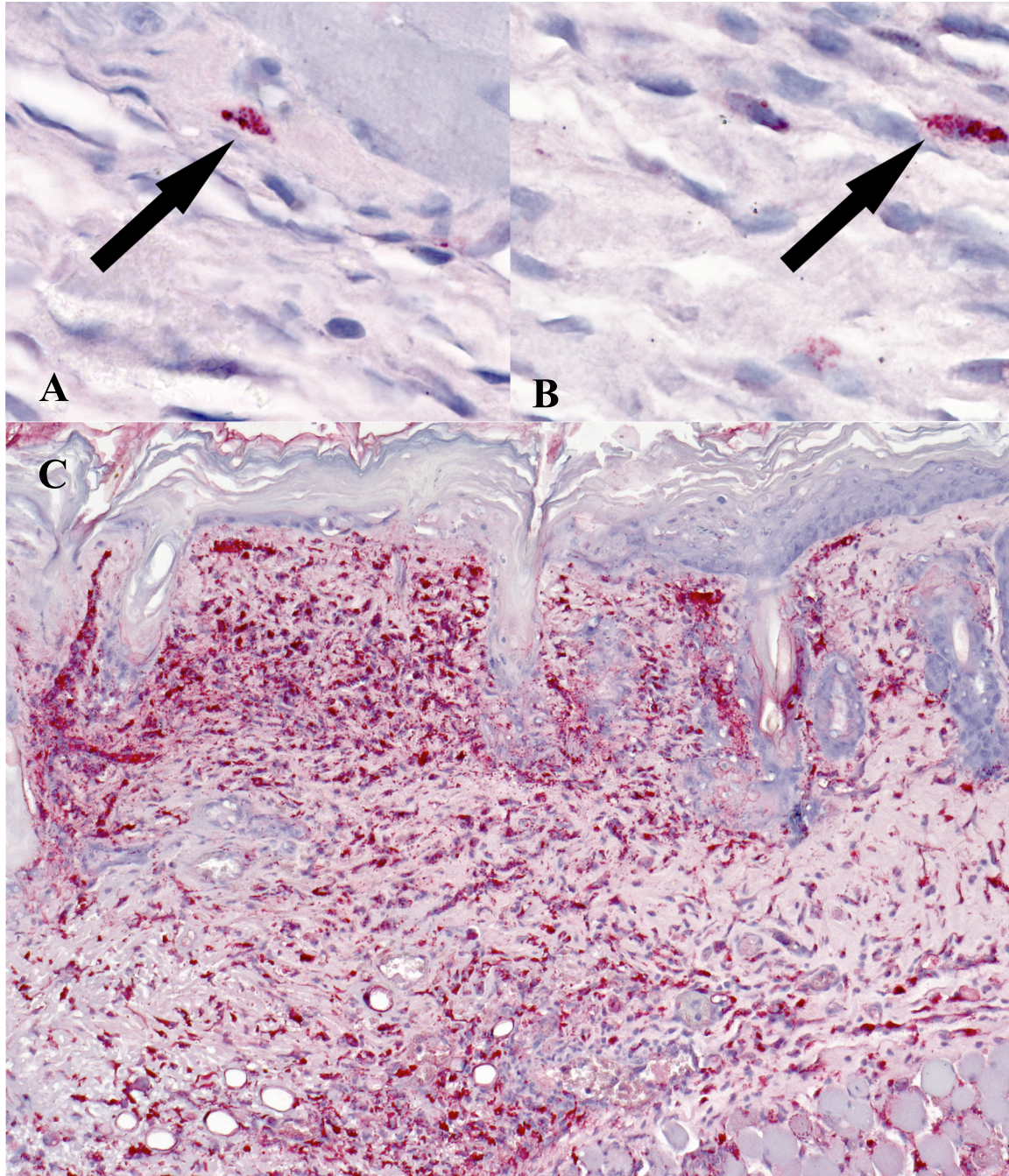


Figure 3.2. Immunohistochemistry of skin at tick feeding sites. A and B represent cutaneous tissues of the SPG inoculated mice with tick feeding group. Arrows indicate rare positive staining for SFG *Rickettsia*. Frame C displays positive staining (red staining) in the cytoplasm of histiocytic cells and endothelial cells in the skin of *R. parkeri* inoculated mice with tick feeding at 8 dpi.

3.3.2 Tick Feeding Leads to Inflammation and Necrosis at the Feeding Site

Both tick-feeding groups displayed mixed lymphoplasmacytic/histiocytic inflammation and necrosis when examined histopathologically; however, the *R. parkeri* inoculated with tick feeding group presented with marked inflammation and necrosis (Table 3.1). In contrast, only one mouse displayed mild inflammation from the *R. parkeri* without tick feeding group, while the remaining mice failed to present with any signs of inflammation or tissue damage, paralleling the minimal rickettsial proliferation observed by qPCR and immunohistochemistry.

3.3.3 Localized *R. parkeri* Infection During Tick Feeding Fails to Induce a Demonstrable Systemic Temperature Response

As measured by rectal temperature changes, no difference was demonstrated among groups at any time point during the study (Figure 3.3), indicating minimal systemic response to the early localized rickettsial infection.

3.3.4 Ticks Acquire *R. parkeri* from Feeding on Skin Intradermally Inoculated with *R. parkeri*

All pooled tick samples from the *R. parkeri* inoculated with tick feeding group showed appropriately sized amplicons using the *rompA* 190.70p and 190.602n primers, while all ticks from the SPG inoculated with tick feeding group failed to amplify. This demonstrates that the nymphal ticks acquired *R. parkeri* during the blood feeding over the *R. parkeri* inoculation site. As the laboratory colony of *A. maculatum* is persistently infected with *candidatus* Rickettsia andeanae, these findings also indicate that the 190.70p and 190.602n primer pair fails to amplify *candidatus* Rickettsia andeanae. The 190.70p and 190.701 primer pair successfully amplified rickettsial DNA from all ticks indicating this primer pair can be used to amplify *candidatus* Rickettsia andeanae.

Interestingly, only 11 of 60 ticks from the *R. parkeri* inoculated with tick feeding group successfully molted, and all of these were dead by the time of collection for DNA extraction. In

Table 3.1. Histopathology associated with *R. parkeri* infection at 8 dpi

Group	Vessel continuity disrupted	Inflammation	Edema	Necrosis	Fibrinoid change	Myodegeneration/ regeneration	Endothelial necrosis	Endothelial swelling
Control	-	0	0	0	-	-	-	-
SPG	-	0	0	0	-	-	-	-
<i>R. parkeri</i>	-	0	0	0	-	-	-	-
SPG + tick feeding	-	2	1	1	+	+	-	+
<i>R. parkeri</i> + tick feeding	+	3	2	2	+	+	+	+

0 = absence of the specified parameter, 1 = mild histologic change (rare to infrequent presence as observed by high-powered magnification),

2 = moderate histologic change (change is commonly observed in multiple high-powered fields or larger foci are present in selected areas),

3 = marked histologic change (changes frequently observed in multiple high-powered fields or severe change in focal areas).

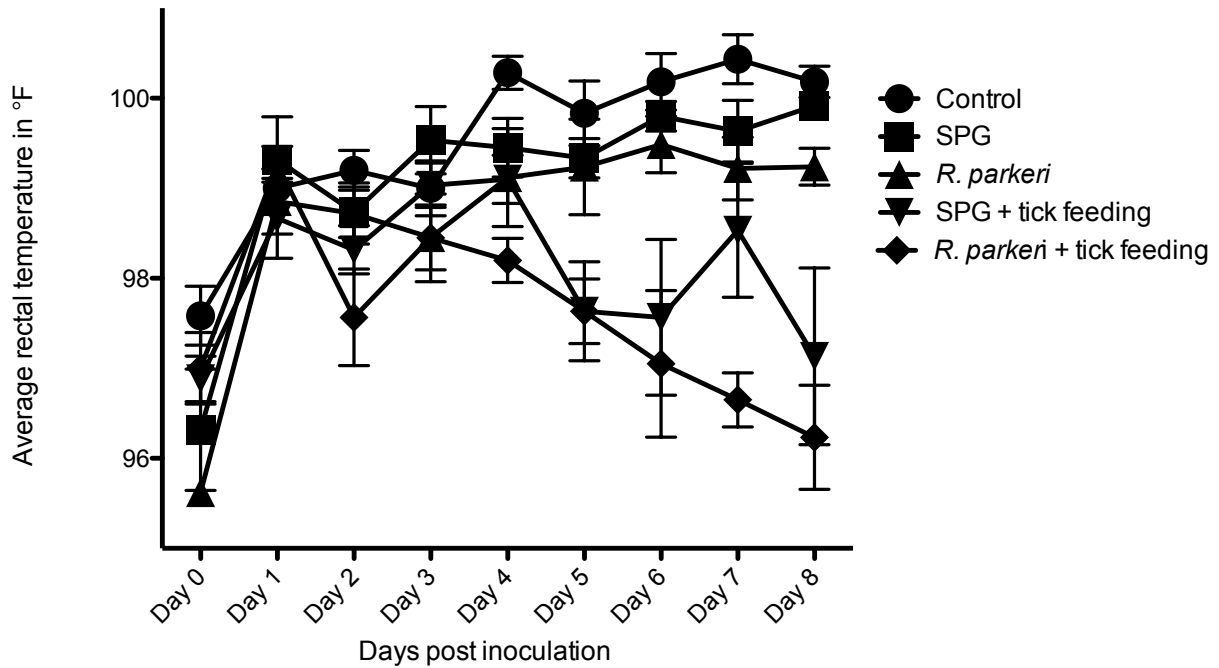


Figure 3.3. Daily rectal temperatures of mice inoculated with *R. parkeri* with or without concurrent nymphal *A. maculatum* tick feeding. The values were assessed using the mixed procedure in SAS, which revealed that the two tick feeding groups approached significance ($p = 0.1$) beginning at day 5 post-inoculation. The tick feeding groups had decreased temperatures relative to the other groups. Data expressed as mean \pm SEM.

contrast, all of the nymphs from the SPG inoculated with tick feeding group successfully molted and were still alive and mobile at the time of collection for DNA extraction. This suggests a possible fitness loss associated with *R. parkeri* infection in *A. maculatum* in the current model

3.3.5 *Candidatus* Rickettsia andeanae is not Efficiently Transmitted to the Skin during Nymphal Tick Feeding

PCR for *rompA* using the 190.70p and 190.701 primer pair amplified rickettsial DNA in the *R. parkeri* inoculated with tick feeding group of mice but failed to amplify in the SPG inoculated with tick feeding group. The 190.70p and 190.602n primer pair also successfully amplified rickettsial DNA within the skin samples from the *R. parkeri* inoculated with tick feeding group but again failed to amplify from the SPG inoculated with tick feeding group. The failure of the more general primer pair to amplify rickettsial DNA from the SPG inoculated with tick feeding group suggests that *candidatus* *Rickettsia andeanae* is not transmitted to the mouse during feeding.

3.3.6 No Obvious Immunological Alterations Presented as Differential RNA Expression

Reverse transcriptase quantitative PCR for *Itgax* (CD11c), *IL1b*, and *MCP1* showed no significant difference amongst the groups (Figure 3.4). *CD11c* is a marker for dendritic cells while *IL1b* is a cytokine produced by activated macrophages. *MCP1* (monocyte chemoattractant protein-1 or *CCL2*) is a chemokine secreted by monocytes, macrophages, and dendritic cells, and it primarily recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury. The lack of response in these proteins suggests that histiocyte cells fail to be activated by infection with *R. parkeri*.

3.4 Discussion

The ability of *R. parkeri* to initialize colonization of the intradermal inoculation site depends greatly on the influence of tick feeding on the vertebrate host microenvironment. The

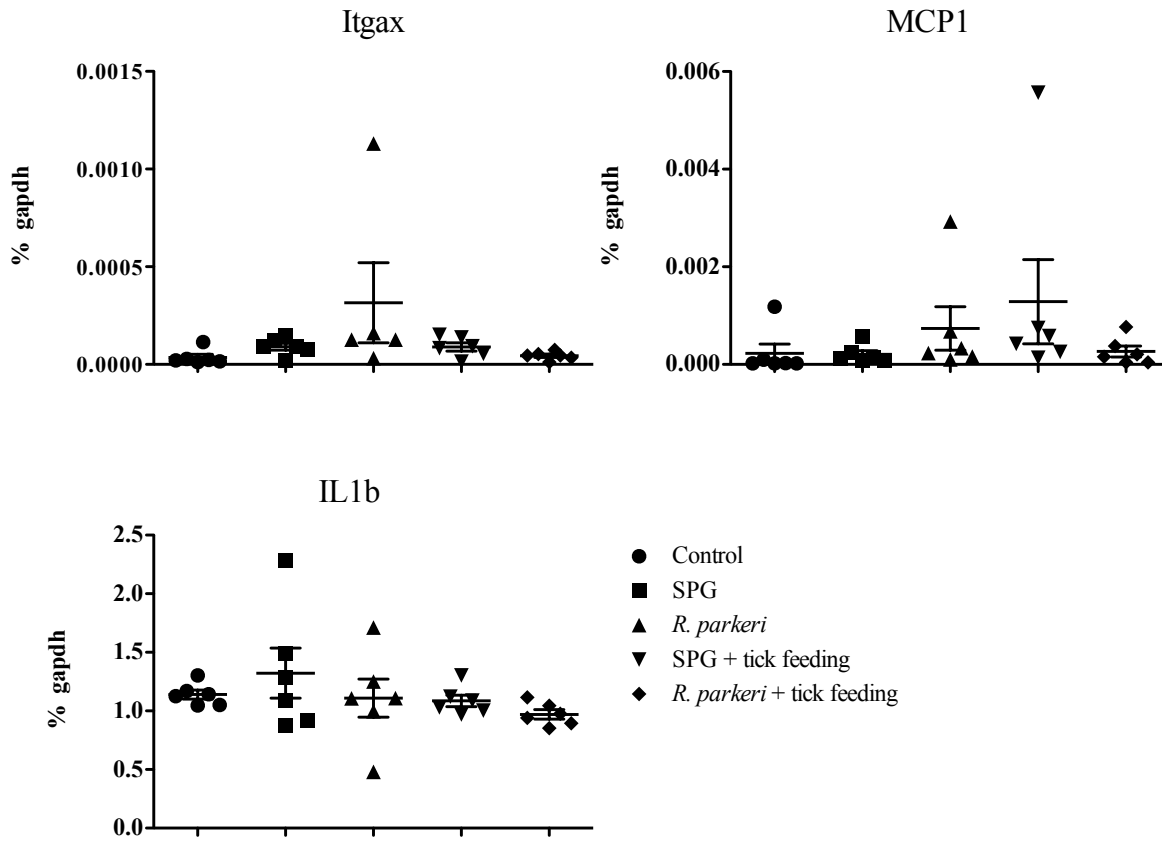


Figure 3.4. Cytokine profile from the *R. parkeri* intradermal inoculation site as determined by reverse transcriptase qPCR. Itgax (CD11c, a histiocytic cell marker), MCP1 (monocyte chemotactic protein 1, a chemokine for histiocytic cells), and IL1b (produced by activated macrophages) were assessed to determine histiocytic cell activation, and results showed no significant difference among groups. Data presented as mean \pm SEM.

finding of relatively large numbers of rickettsiae present secondary to tick feeding is compelling evidence supporting the importance of the tick vector as more than a simple vessel or fomite in the transmission of rickettsial pathogens.

The apparent localization of rickettsiae primarily to histiocytic-type cells within the cutaneous tissues is an intriguing finding. Dendritic cells serve as the initial line of defense against rickettsial infection and produce IL-12p40 upon stimulation of TLR4 by *Rickettsia* (Fang et al. 2009). As a model for *Rickettsia conorii*, the TLR4 deficiency of C3H/HeJ mice failed to cause the normal lag phase of growth normally observed early in rickettsial infection and, instead, allowed exponential growth during early infection (Jordan et al. 2009). Our current findings suggest that not only do the defective dendritic cells fail to induce protective immune mechanisms, but they may also serve as a primary host cell for *R. parkeri* during the initiation of infection of the vertebrate host. This is an interesting possibility when considering that rickettsial infection of endothelial cells is often described for the systemic infection, but the local infection early in the disease process does not always present with primarily endothelial cell infection.

There are conflicting results in the presence of *Rickettsia* identified by immunohistochemistry in the skin of the SPG with tick feeding group as compared to the lack of PCR amplification in these samples. This probably represents a difference in the sensitivities of these two tests. The rare *Rickettsia* identified in the skin of the SPG with tick feeding group most likely represent *candidatus Rickettsia andeanae*. The presence of this *Rickettsia* within the laboratory colony of *A. maculatum* represents one limitation of the current study. Although currently not believed to be pathogenic, the presence of another *Rickettsia* poses two scenarios for the interpretation of our data. The first is that *candidatus Rickettsia andeanae* was the

Rickettsia amplified by our qPCR, and we found that our qPCR primers do indeed amplify *candidatus Rickettsia andeanae*, our probe fails to label the amplicon when used to test DNA extracts from the infected ticks. The second potential issue is the possibility that the presence of another *Rickettsia* is having a synergistic effect on the growth of *R. parkeri* at the intradermal inoculation site. While this cannot be definitively ruled out, the dramatic difference in qPCR and immunohistochemistry in the *R. parkeri* inoculated groups and the rare presence of *Rickettsia* in the SPG with tick feeding group suggest that the factors responsible for the increased rickettsial proliferation are not representative of the presence of *candidatus Rickettsia andeanae* in the skin.

The absence of rickettsial DNA in the *R. parkeri* inoculated group without tick feeding may be due to one or more of several possibilities. It is likely that had the regional lymph nodes been examined, they would have revealed some rickettsial DNA as it would be expected for some migration of *Rickettsia* laden leukocytes to occur. It is also possible that although the skin was sampled, the *Rickettsia* had spread beyond the initial inoculation area and was therefore missed during sampling.

The death of the ticks feeding on *R. parkeri* inoculation sites may represent some pathogenicity towards the *A. maculatum* nymphs, but the relatively large inoculum introduced into the skin is likely above that which would be encountered naturally. The probability of *R. parkeri* being pathogenic within the tick, however, is worthy of further investigation.

The impact of tick feeding was very pronounced as indicated by the histopathology. With the recent description of the sialotranscriptome of *A. maculatum* (Karim et al. 2012) and the development of this murine model for *R. parkeri* rickettsiosis, detailed evaluation of the role of tick saliva in rickettsial infection of the vertebrate host is the next logical iteration of this line of research. While it is possible to introduce only one saliva protein at a time at the site of

intradermal inoculation of *R. parkeri*, it would probably prove as useful if not more so to add whole tick saliva with specific inhibitors for tick proteins. This line of research opens the door to extensive expansion of our understanding of not only tick-borne rickettsioses but arthropod-borne diseases in general as similar findings have been made in studies utilizing *Leishmania* and sand flies (Rogers et al. 2010).

Additional studies with hematophagous arthropods have revealed specific molecules present in the saliva of these blood feeders as promoting local infection of an infectious agent in the skin of a murine model (Volfova et al. 2008). This provides a framework for future studies into the mechanisms involved in the increased proliferation of *Rickettsia* secondary to tick feeding and emphasizes the importance and redundancy of these processes as many hematophagous arthropods share similar salivary protein profiles. Specifically in ticks, the role of cystatins is intriguing. Cystatins are a family of cysteine protease inhibitors, and they have been described in tick saliva including that of *A. maculatum* (Karim et al. 2012). These molecules may play a role in impairing the influx and efflux of inflammatory cells at the site of tick feeding, thereby limiting rickettsial recognition and clearance by the vertebrate host.

The lack of difference in RNA expression is probably due to different processes within the varying groups. Within the control and SPG inoculated groups, no or minimal alterations would be expected, while in the *R. parkeri* inoculated group the *Rickettsia* was cleared by the time of sampling at eight days. In both of the tick feeding groups, it is probable that tick salivary components suppressed the inflammatory response even in the face of rickettsial infection.

Our study provides compelling evidence that the initiation of rickettsial infection of the vertebrate host via the cutaneous route of inoculation is largely dependent on the alteration of the vertebrate host microenvironment by tick feeding. This drastically alters our concepts of the

factors necessary for acceptable animal models of rickettsioses as these have typically excluded the role of the tick vector even though the cutaneous route of inoculation is often included (Eisemann et al. 1984). It now becomes necessary to include discussion of the effect of tick saliva on establishment and pathogenesis of SFG *Rickettsia* infections.

With the emergence of new rickettsioses, the necessity for adequate animal models with which to study the pathogenesis of these diseases is greatly increasing. The recent identification of C3H/HeJ mice as a potential model for *R. parkeri* rickettsiosis has allowed further characterization of this disease process, and specifically, permitted us to demonstrate a role for the tick vector as more than just an inert vessel for the transmission of the pathogen. This work serves as a strong advancement in the field of SFG *Rickettsia* and refines an excellent tool for the investigation of the pathogenesis and ecology of *R. parkeri* rickettsiosis.

CHAPTER 4
RICKETTSIA PARKERI INFECTION IN DOMESTIC DOGS, SOUTHERN LOUISIANA,
USA, 2011²

4.1 Introduction

Tick-borne spotted fever group (SFG) rickettsioses are maintained in tick populations through vertical transmission of the rickettsial agent and horizontal transmission among vectors by a vertebrate host. Companion animals, specifically dogs, can serve as vertebrate hosts for arthropod vectors and SFG *Rickettsia* (McQuiston et al. 2011), as shown by a report of a *Rickettsia parkeri*-infected dog in South America (Tomassone et al. 2010). Likewise, cases of rickettsioses in humans have been associated with cases in companion animals (Breitschwerdt et al. 1985). Because of a substantial increase in tick-borne rickettsial diseases in the last decade, much effort has been directed to identifying the rickettsial agents present in ticks (Dumler 2010). Based on findings from field surveys of rickettsial infections in ticks and characterization of rickettsioses in humans, most cases of what is considered Rocky Mountain spotted fever, a disease caused by *Rickettsia rickettsii*, are likely caused by infections with rickettsial species other than *R. rickettsii* (Stromdahl et al. 2011).

One of the better documented emerging rickettsial pathogens is *R. parkeri*, a spotted fever group tick-borne rickettsial disease associated with Gulf Coast ticks (*Amblyomma maculatum*) (Paddock et al. 2004) and commonly identified in the coastal states of the southeastern United States. We investigated the potential role domestic dogs play in the ecology of *R. parkeri* transmission to better understand the epidemiologic landscape of this emerging rickettsiosis.

² Reprinted by permission of “Emerging Infectious Disease” Grasperge, B. J., W. Wolfson and K. R. Macaluso (2012). "*Rickettsia parkeri* Infection in Domestic Dogs, Southern Louisiana, USA, 2011." Emerg Infect Dis **18**(6).

4.2 The Study

We obtained blood from dogs at 5 animal control centers in 5 parishes in southern Louisiana during June and July 2011. The blood for the study was provided from excess samples collected for routine heartworm screening. In total, 93 dogs were included in the study. Within 12 hours of collection, whole blood samples (~50–100 mL) were processed individually for DNA extraction by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). DNA was stored at –20°C until PCR analysis.

DNA extracts from the collected blood, environmental DNA extraction controls, or water (negative controls) were used as template for PCR. PCR products were amplified by using genus-specific 17-kDa antigen gene primers and previously described thermocycling conditions (Pornwiroon et al. 2006). Amplicons were visualized on a 2% agarose gel. Positive samples were excised from the gels, and the amplicons were purified by using the PCR Clean-Up System (Promega, Madison, WI, USA). Positive samples were sequenced, and sequences were aligned by using MEGA 5.05 (<http://megasoftware.net/mega.php>), and nucleotide similarities were assessed by using the GenBank BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

DNA samples positive for *Rickettsia* spp. by the genus-specific 17-kDa antigen gene primers were also assessed for the SFG-common rickettsial outer membrane protein A (*rompA*) gene by using a heminested PCR with primers 190.70p and 190.701 followed by primers 190.70p and 190.602n. Primers and thermocycling conditions for the heminested PCR were as described (Pornwiroon et al. 2006), and subsequent purification and sequencing were performed as described above.

Of the 93 DNA samples, 12 (13%) produced positive amplicons for the genus-specific 17-kDa antigen gene. Based on the sequence data, the positive samples were determined to be

most closely related to SFG *Rickettsia*. The resulting 315 bp sequence showed 100% identity to *Rickettsia montanensis* (GenBank accession no. DQ402377.1) and 99% identity to several other members of the SFG including *R. rickettsii*, *R. parkeri*, *Candidatus Rickettsia andeanae*, and *Rickettsia sibirica* (GenBank accession nos. CP000766.2, EF689732.1, GU395295.1, and AF445384.1, respectively). The heminested PCR for *rompA* yielded a 491-bp product with identical sequences for each of the 12 *Rickettsia*-positive samples. Sequence analysis of the *rompA* amplicon identified a 99% similarity with several different strains of *R. parkeri* (GenBank accession nos. U43802.1, EU715288.1, EF102238.1, FJ172358.1, and HM587252.1). These *Rickettsia*-positive samples were obtained from 3 of the 5 sites surveyed, and 2 of the 3 sites were in parishes that directly adjoined each other (Table 4.1). Within the dog populations tested in the 3 sites, 22% (2/9), 16% (9/55), and 8% (1/12), respectively, of the dogs were infected with *R. parkeri* (Table 4.1).

None of the 12 dogs with PCR-positive tests were infested with ticks at the time of sampling. Six female dogs and 6 male dogs had detectable levels of *R. parkeri* DNA in their blood. Nine of the 12 dogs were adults; 3 were <6 months of age. Many of the dogs in the study were classified as mixed breed because breed could not be objectively determined for most of the animals. All animals appeared to be in good health; no overt pathology was noted at the time of blood collection.

Although molecular detection of rickettsial DNA within the blood of vertebrates indicates infection, rickettsial cultures from the positive samples would confirm patent rickettsemia. Most of the samples in our study were insufficient in volume to attempt culture after heartworm testing and DNA extraction. Of the 12 samples with PCR results positive for rickettsial DNA, only 3 were of sufficient volume to attempt culture, and all of those attempts proved unsuccessful. It

Table 4.1. *R. parkeri* infection in domestic dogs in 5 animal shelters from 5 parishes in southern Louisiana, USA, June and July 2011*

Location	No. (%) canines positive for <i>R. parkeri</i>
Ascension Parish	0% (0/13)
Livingston Parish†	22% (2/9)
Iberville Parish	0% (0/4)
East Feliciana Parish	16% (9/55)
Tangipahoa Parish†	8% (1/12)

*The presence of *R. parkeri* was determined by PCR for *rompA*. *R. parkeri*, *Rickettsia parkeri*; *rompA*, rickettsial outer membrane protein A.

†Parishes directly adjoin one another.

would also have been beneficial to determine if dogs that were positive for *R. parkeri* carried ticks that were also positive for *R. parkeri*. However, it is common practice for animal control centers to treat dogs for ectoparasites on admission; thus, no ticks were present on the dogs in our study at the time of sampling. The presence of rickettsial DNA in the blood of dogs, in the absence of ectoparasites, supports the hypothesis that domestic dogs may serve as reservoirs of rickettsial diseases, now specifically including the emerging pathogen *R. parkeri*.

4.3 Conclusions

We examined the potential role of domestic dogs in transmission of SFG *Rickettsia*. *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, is known to cause clinical disease in dogs. It is also associated with findings similar to cases of Rocky Mountain spotted fever in humans, including cutaneous petechiae and ecchymoses, anorexia, depression, weight loss, and dehydration (Breitschwerdt et al. 1988). The role of dogs as vehicles for *Rickettsia*-infected ticks to encounter susceptible humans has also been proposed (McQuiston et al. 2011). The possibility of dogs as reservoirs of rickettsial disease has previously been investigated in studies evaluating *R. felis* rickettsemia and seropositivity for *R. parkeri* (Hii et al. 2011; Toledo et al. 2011); however, strong cross-reaction among antibodies precludes finding of definitive results from serologic testing. The current study suggests that domestic dogs may become rickettsemic with *R. parkeri* infection, but further investigation of the duration of rickettsemia and monitoring for clinical disease that may be associated with infection is required.

It is also vital to determine the potential for dogs to serve as infectious sources of *R. parkeri* for feeding ticks. Dogs infected with *R. rickettsii*, for example, have proven relatively inefficient at transmitting rickettsiae to naive ticks and therefore may not play a large role in maintenance or amplification of the *R. rickettsii* transmission cycle (Norment and Burgdorfer

1984). Conversely, domestic dogs have recently been shown to be competent reservoirs for the causative agent of Mediterranean spotted fever, *R. conorii*, a species closely related to *R. parkeri* (Levin et al. 2012). The prevalence identified in this study establishes an important first step in the examination of the domestic dog for reservoir competency of *R. parkeri*.

Since the first reported case of *R. parkeri* rickettsiosis in 2004, >20 additional cases have been identified in humans (Paddock et al. 2010), and to date no viable vertebrate reservoirs for the pathogen have been identified. Although the current study consists of a relatively small survey, the results are considerable because of the recognized importance of domestic dogs as potential reservoirs for transmissible pathogens (Chomel 2011). In addition, the presence of *R. parkeri* has not previously been described in Louisiana; thus, this report expands the known distribution of *R. parkeri*. The results of the current study clearly establish dog infection by *R. parkeri*; however, a role for dogs in the natural cycle of this pathogen, and the arthropod vectors involved in transmission, requires further investigation.

CHAPTER 5

DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

5.1 Discussion of Results and Future Directions

Since the first published report of *R. parkeri* rickettsiosis in 2004 (Paddock et al. 2004), more than 20 cases of the disease have been identified (Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Paddock et al. 2010; Romer et al. 2011). As our recognition of this emerging rickettsiosis grows, so too does our need to study the pathogenesis and ecology. Although *R. parkeri* was first described nearly 75 years ago (Parker et al. 1939), there is a relative paucity of information on this SFG *Rickettsia*. With its recognition as a human pathogen, however, there is a renewed interest in this organism and enormous potential for prospective research. Many studies have already begun to emerge focusing on the identification of human cases and the prevalence of *R. parkeri* within populations of *A. maculatum* (Sumner et al. 2007; Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Paddock et al. 2010; Trout et al. 2010; Fornadel et al. 2011; Romer et al. 2011; Wright et al. 2011; Jiang et al. 2012), but bench top laboratory research lags behind. Of the few studies into the pathogenesis and ecology of *R. parkeri* rickettsiosis, the vector studies using *A. americanum* are not definitive (Goddard 2003); cattle have not proven to be efficient hosts for the infection of naive ticks (Edwards et al. 2011); and the South American opossum failed to maintain heavy rickettsial burden after experimental infection (Horta et al. 2010). Based on the emergence of this rickettsiosis, there is a clear need for enhanced appreciation of the 1) pathology; 2) transmission biology; and, 3) ecology of this emerging disease.

With such a limited number of confirmed cases of disease, the requirement for an animal model to better characterize the disease is an absolute, and mice are an ideal candidate for animal models of infectious disease because these animals are readily available, have easily acquired

reagents, and have been shown to express many of the pathologic features comparable to human rickettsioses (Sammons et al. 1977). The drawbacks, however, to the murine models developed to date are that they bypass the cutaneous route of inoculation and fail to incorporate the effect of tick feeding on the establishment of rickettsial infection.

To fill the void of information regarding the pathogenesis of *R. parkeri* rickettsiosis, the potential of four inbred mouse strains to serve as animal models of this disease was evaluated. The intravenous inoculation of *R. parkeri* showed a very clear susceptibility to infection in the C3H/HeJ mice, while all other mouse strains appeared relatively resistant. These susceptible C3H/HeJ mice developed facial edema and marked splenomegaly, and qPCR for *R. parkeri* revealed dissemination to the heart, lungs, liver, and spleen. As previously stated, most animal models for rickettsioses fail to account for the cutaneous route of inoculation. Therefore, we inoculated *R. parkeri* intradermally into the skin at the nape of the neck and the tail in the susceptible C3H/HeJ mice. The tail inoculation sites developed eschar-like lesions from which rickettsial DNA was recoverable, typical vasculitis was observed histopathologically, and immunohistochemistry revealed intracellular SFG *Rickettsia*. These results suggest that the C3H/HeJ mouse strain is a useful model for the examination of the localized pathology associated with eschar formation in *R. parkeri* rickettsiosis.

A confounding result in this study was the recovery of larger amounts of rickettsial DNA from the liver samples of Balb/c mice as compared to other tissues of the same mice and hepatic tissues of the other strains of mice. To further investigate this phenomenon, it is necessary to evaluate the Kupffer cells of the liver for infection and also the peripheral blood for rickettsial infection as both of these may have served as the source of the positive qPCR results from the hepatic samples of the Balb/c mice.

The role of *A. maculatum* in the ecology of *R. parkeri* and its transmission to vertebrate hosts is an area of vast possibilities for research. The prolonged feeding times of hard ticks have caused them to evolve mechanisms to modify the vertebrate host microenvironment, most of which are aimed at decreasing or altering the immune response to tick feeding (Wikel 1996). Studies in other tick-borne diseases have shown that tick feeding plays an integral role in the establishment of infection in the vertebrate host (Bell et al. 1979; Zeidner et al. 1996; Wikel et al. 1997; Nazario et al. 1998; Narasimhan et al. 2007; Dai et al. 2009), but investigation into this possibility in SFG *Rickettsia* is deficient. Similar mechanisms are likely in play in the establishment of rickettsial infections in vertebrate hosts, but these processes remain to be elucidated.

To establish the role of tick feeding in the rickettsial proliferation at the site of inoculation, the murine model for *R. parkeri* rickettsiosis was utilized. Armed with the knowledge that rickettsial replication did not occur at the injection site at the nape of the neck, we hypothesized that tick feeding over this intradermal inoculation site would result in rickettsial proliferation. We inoculated the C3H/HeJ mice with *R. parkeri* intradermally, and encapsulated 10 nymphal *A. maculatum* over the infection site of each mouse. Ticks were allowed to feed to repletion. Our findings confirmed our hypothesis, as large numbers of rickettsiae were identified by qPCR on the tick feeding group, while no rickettsiae were identified in the non-tick feeding group. These results confirm a substantial role for the tick as a modulator of the infection capacity of *R. parkeri* in the vertebrate host. Also, more intricate research into the role of tick saliva in the colonization of the vertebrate host skin can now be pursued.

It was also interesting to find that the ingestion of *R. parkeri* by the *A. maculatum* nymphs during feeding was uniformly lethal. This may be a dose dependent finding, but it is an intriguing avenue for further research.

Finally, the ecology of *R. parkeri* remains a mystery. The belief has long been held that *R. parkeri* is maintained in nature almost entirely by vertical transmission in the tick vectors, but this would require very efficient transovarial transmission, which has not been documented for *R. parkeri*. It is probable that a vertebrate reservoir or amplification host exists for *R. parkeri* and serves as a source of reinfection of *A. maculatum* populations.

It has been observed that dogs can become infected with SFG *Rickettsia* (Tomassone et al. 2010; McQuiston et al. 2011), and they are common hosts for the adults of many species of ticks. In turn, associations have been shown between outbreaks of SFG rickettsioses in humans and companion animals (Breitschwerdt et al. 1985). The potential role for dogs in the ecology of *R. parkeri* and as transport hosts carrying ticks into close contact with humans requires investigation. These domestic animals may serve as either a reservoir or amplification host for *R. parkeri*, and the dogs close proximity to humans represents a powerful risk factor for development of human disease.

Based on the literature it is probable that rickettsial infections would be present in dogs in temporary housing situations in southern Louisiana. Falling well within the historical habitat range of *A. maculatum*, it was hypothesized that *R. parkeri* was likely to be present within the dog populations sampled. The results indicated that approximately 13% of shelter dogs in southern Louisiana were infected with *R. parkeri*, with three of the five sites sampled showing the presence of *R. parkeri*. Additionally, two of these *R. parkeri* positive sites were from directly adjoining parishes. Although no ticks were recovered from any of the animals, the samples were

collected in the summer months of June and July, which are times of high activity for *A. maculatum*. It is likely that these animals were parasitized by infected ticks prior to entry into the shelters, but it is common practice to treat animals for ectoparasites upon admission to the animal shelters. Our results show a possible prominent role for dogs in the ecology of *R. parkeri*, and further research is necessary to determine if dogs can serve as efficient sources of infection for naive *A. maculatum*. It is also likely that wild canids, such as coyotes, wolves, and foxes, are involved in the maintenance of *R. parkeri* in more rural settings, and research to investigate this possibility is necessary.

Although a role for domestic dogs in the ecology of *R. parkeri* has been proposed by these findings, the prevalence of the cotton rat as a vertebrate host for *A. maculatum* is an intriguing possibility for another vertebrate player in the *R. parkeri* cycle. These rodents are heavily infested with *A. maculatum*, and the cotton rat should be investigated for infection with *R. parkeri* as well.

The research described in this body of work has advanced the field of rickettsiology considerably by developing an animal model for *R. parkeri* rickettsiosis and clearly establishing a role for the tick vector in the proliferation of *Rickettsia* within the vertebrate host. This work has also added to the general knowledge of *R. parkeri* through identifying the domestic dog as a probable reservoir or amplification host. These additions to the field will provide a strong platform for continued research into the pathogenesis of SFG *Rickettsia* and outline a road map for early investigation into emerging rickettsioses.

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APPENDIX A COMMONLY USED ABBREVIATIONS

ADP – adenosine diphosphate

ANOVA – analysis of variance

Arp2/3 – Actin related protein 2/3

ATP – adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

CCL2 - Chemokine (C-C motif) ligand 2

CD4+ - cluster of differentiation 4

cDNA – complementary DNA

cfD – complement factor D

cpd – copies per 5 µl extracted DNA

CT – crossing threshold

dpi – days post-inoculation

gDNA – genomic DNA

gltA – citrate synthase gene

H&E – hematoxylin and eosin

IACUC – Institutional Animal Care and Use Committee

IFN- γ – interferon γ

IL-1 – interleukin 1

IL1b – interleukin 1 beta

IL-6 – interleukin 6

IL-10 – interleukin 10

IL-12p40 – interleukin 12p40

kDa - kiloDalton

Ku70 – subunit of DNA-dependent protein kinase

LPS – lipopolysaccharide

Itgax – Integrin, alpha X

MCP1 – monocyte chemotactic protein 1

MHC – major histocompatibility complex

NCBI - National Center for Biotechnology Information

PCR – polymerase chain reaction

qPCR – real-time quantitative polymerase chain reaction

RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted

RMSF – Rocky Mountain spotted fever

rOmpA – rickettsial outer membrane protein A

rOmpB – rickettsial outer membrane protein B

Sca – surface cell antigen

SEM – standard error of the mean

SFG – spotted fever group

SPG – sucrose-phosphate-glutamic acid

TBRD – tick-borne rickettsial disease

Th1 – type 1 helper T cell response

Th2 – type 2 helper T cell response

TLR4 – toll-like receptor 4

TNF- α – tumor necrosis factor α

APPENDIX B REAGENTS AND PROTOCOLS

1. *Rickettsia* partial purification

1.1 *Rickettsia*-infected cells are harvested using a cell scraper

1.2 Cells are lysed by aspirating and expelling 10 times through a 27 gauge needle attached to a 5 cc syringe.

1.3 Suspension is centrifuged at 275 x gravity at 4°C for 10 minutes

1.4 The supernatant is forced through a 2 micron filter attached to a 5 cc syringe

1.5 Suspension is transferred to 1.5 ml centrifuge tubes and spun at 13000 rpm at 4°C for 10 min

1.6 Pellet is *Rickettsia* and is resuspended in SPG buffer

2. Sucrose-phosphate-glutamic acid buffer

2.1 Added the following reagents to double distilled water

2.2 218 mM sucrose

2.3 3.8 mM KH_2PO_4

2.4 7.2 mM K_2HPO_4

2.5 4.9 mM monosodium L-glutamic acid

2.6 Adjusted to a pH of 7.0

3. *Rickettsia* counting

3.1 Take 100 µl of partially purified *Rickettsia*, and transfer to 1.5 ml tube and spin at 16,000 x gravity at 4°C for 10 min

3.2 Resuspend *Rickettsia* pellet with 500 µl 0.85% NaCl, and spin at 16,000 x gravity at 4°C for 10 min

3.3 Resuspend in 100 µl 0.85% NaCl and dilute 1:50 and mix 100 µl of cell suspension with 0.3 µl of dye mixture (LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen)

3.4 Incubate in dark for 15 minutes

3.5 Pipet 10 µl to count using bacteria counting chamber

Calculation

$Rickettsia/ml = (\text{rickettsial organisms in 5 squares}) \times 5 \times 0.05 \times 10^6 \times \text{dilution factor (50)}$

4. Hematology

4.1 Dilute whole, EDTA anti-coagulated blood 1:100 with 2% acetic acid

4.2 Incubate for 10 minutes at room temperature then pipette 10 µl of solution into hemacytomer

4.3 Incubate for 5 minutes at room temperature on microscope stage

4.4 Count nuclei present in four corner squares of each counting grid

Calculation

Absolute leukocyte concentration = ((average number of nuclei per counting grid) x dilution factor (100))/(number of squares counted (4) x volume of one square (0.1 µl))

APPENDIX C CONSENT FORMS

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Britton Grasperge was born in Asheville, North Carolina, and moved to New Orleans Louisiana at the age of 7. He is the son of Mr. Claude Grasperge and Mrs. Linda McKee, and he is the older brother of Ms. Rachel Kimberly Turley. Britton graduated with his bachelor of sciences from Louisiana State University in 2003 at which point he was a year into his veterinary training also at Louisiana State University. He finished his Doctor of Veterinary Medicine degree in 2006 and enrolled in the graduate program in the Department of Pathobiological Sciences as a resident in veterinary clinical pathology. Britton passed the certifying examination for veterinary clinical pathology in 2009 and transitioned into the Vector Biology Research Laboratory to begin his research into vector-borne disease under the guidance of Dr. Kevin Macaluso. Britton finished his dissertation and will graduate in May, 2012. After graduation, Britton will continue to pursue his research interests in the field of tick-borne diseases at the Tulane National Primate Research Center in Covington, Louisiana.