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The tick response to Rickettsial dissemination during typical and atypical Rickettsial infection

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THE TICK RESPONSE TO RICKETTSIAL DISSEMINATION DURING TYPICAL AND ATYPICAL RICKETTSIAL INFECTION

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ABSTRACT

Ticks are the only disease vectors for spotted fever group (SFG) *Rickettsia* which are obligate intracellular bacteria belonging to the genus *Rickettsia*. In nature, ticks maintain the infection of SFG *Rickettsia* via vertical and horizontal transmission. However, the prevalence of rickettsial transmission is limited to certain species of ticks, and this limitation is known as a specific tick/*Rickettsia* relationship. Due to the continuous increase of tick-borne rickettsial disease cases in the United States, which contrasts with very low prevalence of *Rickettsia* in tick vectors, the study of vector competence of tick to *Rickettsia* is needed in order to understand the ecology and epidemiology of tick-borne rickettsioses. Here we characterized the role of *Dermacentor variabilis* α-catenin during rickettsial infection in tick ovaries suggesting a role in rickettsial infection in tick ovaries. We demonstrated that the typical nonpathogenic (*R. montanensis*) and typical pathogenic (*R. rickettsii*) *Rickettsia* persistently infect *Dermacentor variabilis* compared to atypical *Rickettsia* (*R. amblyommii*), and only *R. montanensis* is able to disseminate to tick ovaries. *D. variabilis* glutathione S-transferase1 (DvGST1) has been identified as a tick immune-like molecule that specifically responds to atypical rickettsial challenge in tick midguts suggesting a role in controlling atypical rickettsial infection in tick midguts. DvGST1 is highly upregulated in tick midguts during bloodmeal acquisition. The function of GST is known to be involved with detoxification and oxidative stress reduction, and acaricide resistance in ticks. Silencing of DvGST1 gene demonstrates significant reduction of mRNA and enzyme activity of DvGST1 in tick midguts; however, further characterization of DvGST1 is needed due to the off-target effect of negative control dsRNA. Continued study on the tick/*Rickettsia* interaction influencing tick vector competence for *Rickettsia* will lead to a better understanding of ecology and epidemiology of tick-borne rickettsioses.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Ticks

Ticks are ectoparasites of vertebrates belonging to the class Arachnida of the phylum Arthropoda. Arachnids include ticks, mites, spiders and scorpions. Ticks and mites are in the subclass Acari which is divided into the superorder Parasitiformes and Acariformes (Beaty B.J. and Marquardt W.C. 1996). The superorder Parasitiformes contains the order Ixodida, Holothyrida, and Mesostigmata. The order Ixodida contains three families: the Nuttalliellidae, Argasidae, and Ixodidae (Mullen G and Durden L. 2002). The family Nuttalliellidae contains only one species, *Nuttalliella namaqua.* The family Argasidae is subdivided into five genera containing approximately 170 species. The family Ixodidae is subdivided into five subfamilies consisting of 12 genera and 650 species. Approximately, 80% of all the tick species belong to the family Ixodidae (Table 1.1) (Mullen G and Durden L. 2002).

The body structure of the tick is divided into two major regions which are the capitulum and idiosoma. The capitulum is a mouthpart containing the basis capituli, segmented palps, the chelicerae, and the hypostome. The idiosama consists of the podosoma (legs) and the opisthosoma (body) (Beaty B.J. and Marquardt W.C. 1996). Ixodid ticks have an external morphology distinct from that of argasid ticks. Ixodid ticks, or hard ticks, have hard cuticle plate, scutum, covering the dorsum surface. The scutum is only present on the anterior half of the dorsum of females. During blood feeding, new cuticle is synthesized, and the posterior of female ticks expands tremendously. For male ixodid ticks, scutum completely covers the dorsum which limits the physical expansion of male ticks during blood feeding. The mouthparts of ixodid ticks are dorsally visible. Argasid ticks, or soft ticks, have a soft leathery cuticle and no scutum. In nymphs and adults of the family argasid, the mouthparts are not visible from above because the

Table 1.1: Taxonomy of the Order Ixodida. The order Ixodida is divided into three families containing *Ixodidae* (hard tick), *Argasidae* (soft tick), and *Nuttalliellidae*. The family *Ixodidae* is divided to two subgroups: Prostriata and Metastriata.

Family	Subfamily (subgroup)	Genus
Ixodidae	Ixodinae (Prostriata)	*Ixodes*
	Amblyomminae (Metastriata)	*Amblyomma, Aponomma*
	Haemaphysalinae (Metastriata)	*Haemaphysalis*
	Hyalomminae (Metastriata)	*Hyalomma*
	Rhipicephalinae (Metastriata)	*Dermacentor, Cosmiomma, Margaropus, Nosomma, Anomalohimilaya, Rhipicentor, Rhipicephalus*
Argasidae	Argasinae	*Argas*
	Ornithodorinae	*Ornithodoros*
	Otobinae	*Otobius*
	Antricolinae	*Antricola*
	Nothoaspinae	*Nothoaspis*
Nuttalliellidae		*Nuttalliella*

(Modified from Medical and veterinary entomology, 1st edition. (Mullen G and Durden L. 2002)

capitulum is protected by the anterior projection of the body (hood) (Mullen G and Durden L. 2002; Sonenshine 1993).

1.1.1 Life Cycle and Feeding Behavior

The life cycle of ticks consists of four stages; the embryonated egg, six-legged larva, eight-legged nymph, and eight-legged adult (Figure 1.1). The larval, nymphal, and adult stages are parasitic. In most species, a bloodmeal is needed for development to the next life stages (Sonenshine 1993).

For ixodid ticks, eggs hatch into larvae which then seek hosts, attach, feed, detach, and molt to nymphs. Nymphs seek hosts, attach, feed, detach, and molt to adults. For argasid ticks, which have two or three nymphal stages, the first stage nymphs molt to further nymphal stages before molting to adults. Adults then seek hosts, attach, and feed. After they are fully engorged, females drop off and deposit their eggs. Compared to other hematophagous arthropods, ticks are long-lived and can live more than one year without feeding (Goodman J.L. et al. 2005; Mullen G and Durden L. 2002).

In nature, ticks utilize one of two strategies for seeking hosts; ambush or hunter strategies (Goodman J.L. et al. 2005; Sonenshine 1993). Ticks that use the ambush strategy climb onto grass, bushes, or leaves and wait for passing hosts. In most species, larvae which feed on small mammals remain close to ground, and adults climb onto higher vegetation in order to encounter large animals, e.g. deer, dogs, and humans. Ticks stay clinging to vegetation and remain on the leaves until stimulated by passing hosts. The ticks then cling to the hair, fur, or cloths of the hosts. This behavior is called questing. Questing ticks respond to many factors, e.g. odors, tactile cues, sounds, vibration, radiant heat, and carbon dioxide.

The second strategy is the hunter strategy. Hunting ticks are buried in sand or soil to

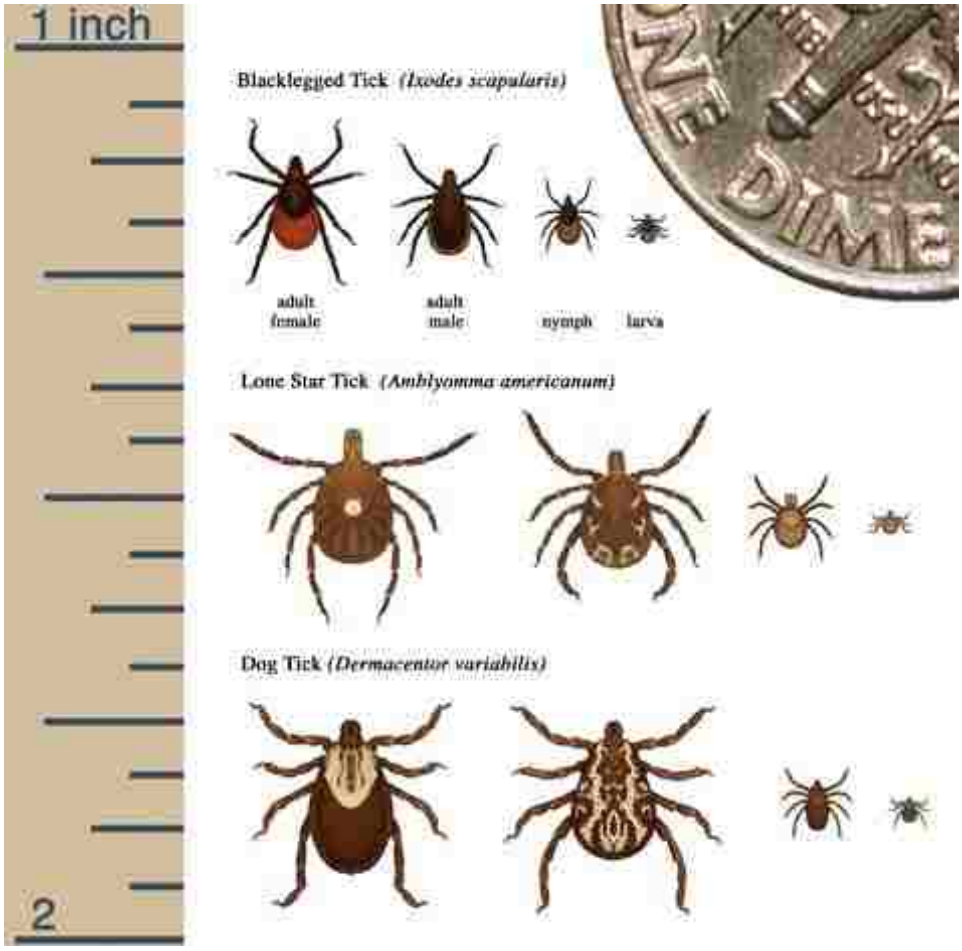

Figure 1.1: Tick life cycle. The relative size of ixodid ticks in three life stages: larva, nymph, adult male and adult female including Blacklegged Tick (*Ixodes scapularis*), Lone Star Tick (*Amblyomma americanum*), and American dog tick (*Dermacentor variabilis*). (Courtesy of Centers of Disease Control and Prevention)

shelter from heat and desiccation, and when they are excited by a host, ticks emerge, move to and attack the host.

On hosts, ticks then search for a suitable feeding site. They puncture the skin down to the dermis using chelicerae and use teeth-like hypostome to anchor themselves at the bite site. In most *Ixodid* species, after biting the tick rapidly secrete a cement substance surrounding the mouthparts which quickly hardens. After cement secretion, ticks start sucking blood, and the salivary glands produce an array of chemical compounds immunomodulatory and homeostatic modulators which facilitates successful feeding (Goodman J.L. et al. 2005; Sonenshine 1993).

1.1.1.a Ixodid Ticks

Life cycles of ixodid ticks include three types; one-host, two-host, and three-host life cycles (Figure 1.2). In the one-host tick life cycle, all life stages feed and molt on the same host. Examples of one-host ticks include *Dermacentor albipictus* (winter tick), and *Rhipicephalus microplus* (cattle tick). For two-host ticks, larvae feed and molt to nymphs on the same host. Fed nymphs then drop off and molt to adults. The adults attach and feed on a new host. An example of a two-host tick is *Hyalomma dromedarii* (camel tick). The three-host life cycle is characteristic of most ixodid ticks, as more than 90% of ixodid species are three-host ticks (Mullen G and Durden L. 2002). In this life cycle, larvae feed, drop off, and find a sheltered microenvironment in which to molt into nymphs. The emerging nymphs find new hosts, feed, and the engorged nymphs drop off and molt into adults. Sexual dimorphism is present only in the adult stage. Emerging adults seek hosts, feed, mate, and drop off. Replete females find a sheltered microenvironment and deposit several thousand eggs and then die. The time required to complete a life cycle is dependent on host availability and microenvironment: temperature and humidity. With limited environmental resources (e.g. food availability) three-host ticks may

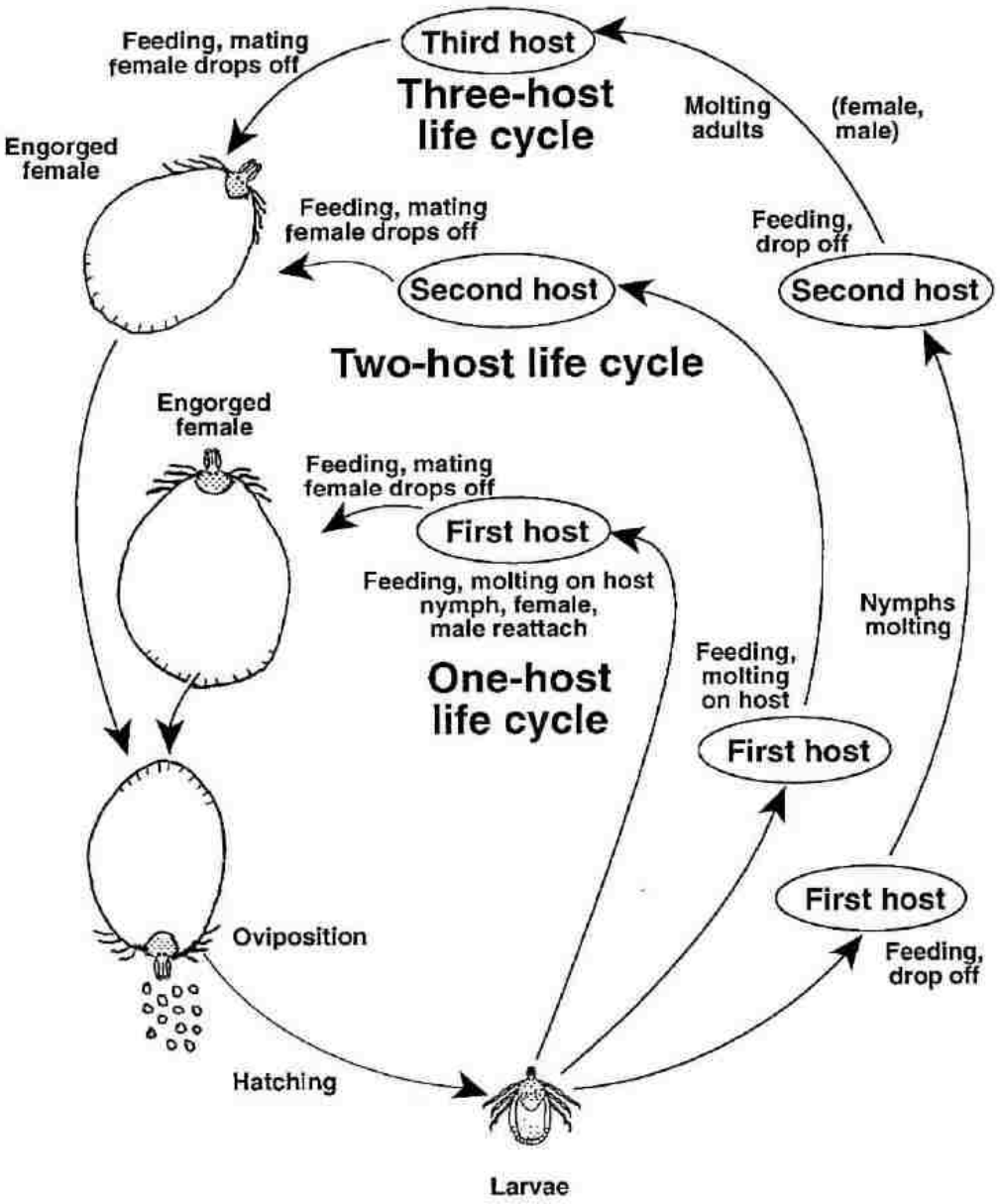

Figure 1.2: Three life cycles of ixodid ticks. (1) One-host life cycle (inner circle): All three stages (larva, nymph, and adult) feed and develop on the same hosts, for example, *Rhipicephalus annulatus*. (2) Two-host life cycle (middle cycle): Larvae and nymphs feed on the same hosts, and adults feed on the second hosts, for example, *Hyalomma dromedarii*. (3) Three-host life cycle (outer circle): Larvae, nymphs, and adults feed on different hosts, for example, *Dermacentor variabilis*. Figure from Medical and veterinary entomology, 1st edition (Mullen G and Durden L. 2002).

take three years to complete their life cycle (Sonenshine 1993).

Ixodid ticks and most argasid larvae are slow feeders, meaning they require several days to complete their feeding. For *D. variabilis*, larvae take three to four days, nymphs take four to five days, and females take seven to eight days to finish their blood feeding (Goodman J.L. et al. 2005). During blood feeding, ixodid ticks synthesize new cuticle to accommodate for the enormous volume of blood meals. The tick's body gradually grows during the long feeding period, and engorged larvae or nymphs weigh about 10 to 20 times their unfed weight. Female ixodid ticks feed only once and have only one gonotrophic cycle. The females feed slowly before mating, and feed rapidly after mating. The engorged females are found to weight 100 to 120 times more than unfed females (Goodman J.L. et al. 2005). Females die following the completion of oviposition, while males feed more than one time and remain on their hosts to mate with several females.

1.1.1.b Argasid Ticks

The Argasid tick life cycle is a multi-host life cycle and is distinct when compared to the ixodid tick life cycle. Larvae feed, drop from their hosts and molt into the first nymphal stage. Emerging nymphs feed, drop off, and molt into the next nymphal stage. There are often two or more nymphal stages in their life cycle; however, the number of nymphal stages varies, even within the same species (Sonenshine 1993). Unlike ixodid ticks, argasid females have multiple gonotrophic cycles; female ticks take many small bloodmeals and lay small egg batches (< 500 eggs/cycle) (Mullen G and Durden L. 2002; Sonenshine 1993).

Argasid ticks are rapid feeders (15-30 minutes) except for larvae of certain *Argas* and *Ornithodoros* species (Sonenshine 1993). In contrast to ixodid ticks, argasid adults do not synthesize new cuticle during feeding. Instead, the existing cuticles stretch during feeding,

thus limiting bloodmeal size and producing small clutches of eggs. The time between adult bloodmeals can be from one week to several months.

1.1.1.c Laboratory Ticks

In order to study tick biology, tick/pathogen interaction, and tick/vertebrate host relationships, tick colonization in the laboratory is required to produce large numbers of specific pathogen-free ticks. In nature, three-host ticks may take two or more years to complete their life cycle (Sonenshine 2005). However, in the laboratory they may only take four or five months depending on the tick species (Troughton and Levin 2007).

The Louisiana State University (LSU) *D. variabilis* colony was started from a laboratory tick colony maintained by Dr. Daniel E. Sonenshine at Old Dominion University (ODU). The ODU *D. variabilis* colony is reared on a combination of host species. Immature *D. variabilis* feed on rats, guineas pigs, or small rodents, while adult ticks feed on dogs, rabbits, or medium to large-sized hosts (Sonenshine 1993).

As for the LSU *D. variabilis* colony, it requires about three months to complete the life cycle in the laboratory. Larvae feed on BALB/c mice for three to five days. Fed larvae molt within two weeks. Nymphs feed on BALB/c mice or Sprague Dawley rats; they require four to seven days to feed until repletion. Engorged nymphs molt into adults within three weeks. Adults feed on Hartley guinea pigs or New Zealand White rabbits (Figure 1.3). Females complete their feeding within seven to ten days. Replete females start ovipositing their eggs within one week and oviposition lasts six days. Between feedings, all tick stages are kept in an environmental chamber at 27 ± 1°C and 87 ± 2% relative humidity with a photoperiod of 16:8 (L:D) h.

1.1.2 Distribution of Medically Important Ticks in the United States

Tick distribution is defined by suitable habitats including forests, meadows and other

Figure 1.3: LSU *Dermacentor variabilis* colony maintenance. *D. variabilis* larvae feed on BALB/c mice. Nymphal stage feed on BALB/C mice or Sprague Dawley rats using encapsulation technique. Adult ticks feed on Hartley guinea pigs or New Zealand white rabbits.

clearings, grasslands, savannahs, and semi-deserts. Some ticks have developed the ability to survive in many habitats; however, some ticks have limited adaptation. Besides habitat other factors including host availability, rainfall, and winter temperature also contribute to the geographic distribution of ticks. Below is the list of human diseases transmitted ticks and tick distribution in the United States.

1.1.2.a *Amblyomma americanum* (Lone Star Tick)

Amblyomma americanum (Lone star tick) is distributed in the southeastern and eastern United States (Figure 1.4). It is a three-host species that feeds on a variety of hosts; however, the major host of lone star ticks is the white-tailed deer. The lone star tick is a vector of *Ehrlichia chaffeensis, E. ewingii, Coxiella burnetti,* and *Francisella tularensis* (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.b *Amblyomma maculatum* (Gulf Coast Tick)

Amblyomma maculatum (Gulf Coast tick) is distributed in coastal areas of the United States along the Atlantic coast and the Gulf of Mexico (Figure 1.5). It has a three-host life cycle. Immature stages feed on birds and small rodents. Adults feed on deer and other animals. Gulf Coast ticks can transmit *Rickettsia parkeri* to humans (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.c *Dermacentor andersoni* (Rocky Mountain Wood Tick)

Dermacentor andersoni (Rocky mountain wood tick) is distributed around the Rocky Mountain States, in the northwestern part of the United States (Figure 1.6). It is a three-host tick. Larvae and nymphs feed on small mammals, such as ground squirrels, chipmunks, woodrats, and mice. Adult ticks feed on larger mammals, including deer, livestock, and humans. The Rocky mountain wood tick is a vector of *Rickettsia ricketsii*, *F. tularensis,* Powassan viruses

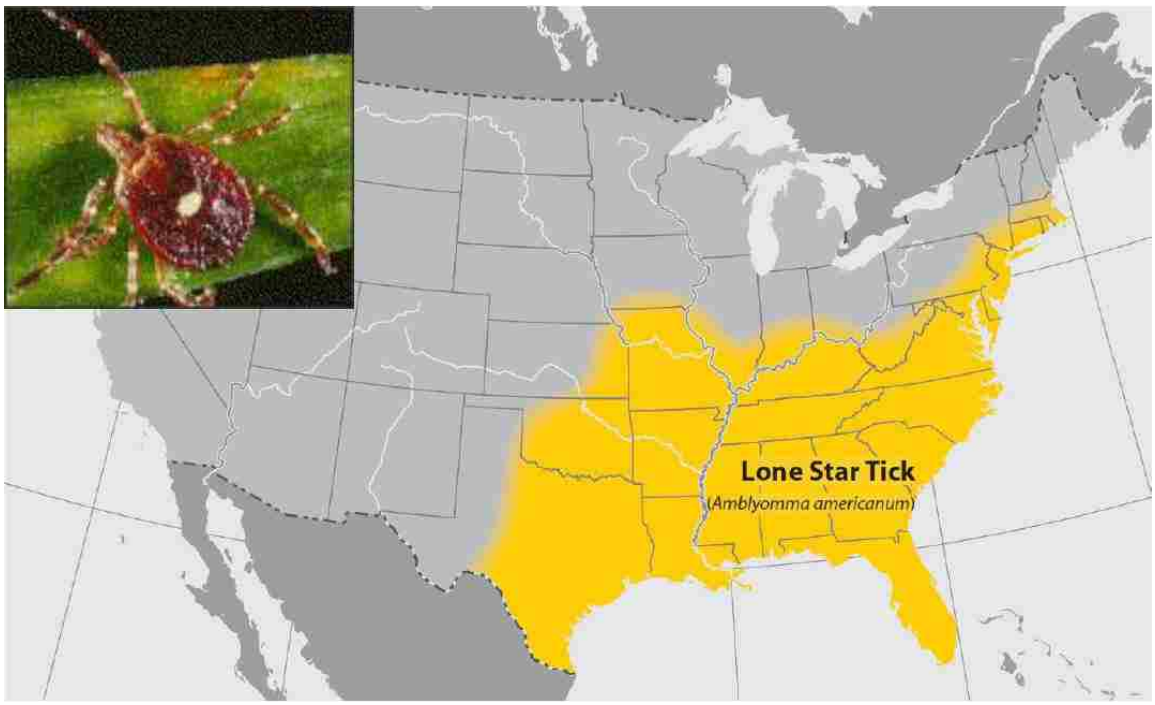

Figure 1.4: Distribution of *Amblyomma americanum* (Lone star tick) in the United States.
(Courtesy of Centers of Disease Control and Prevention)

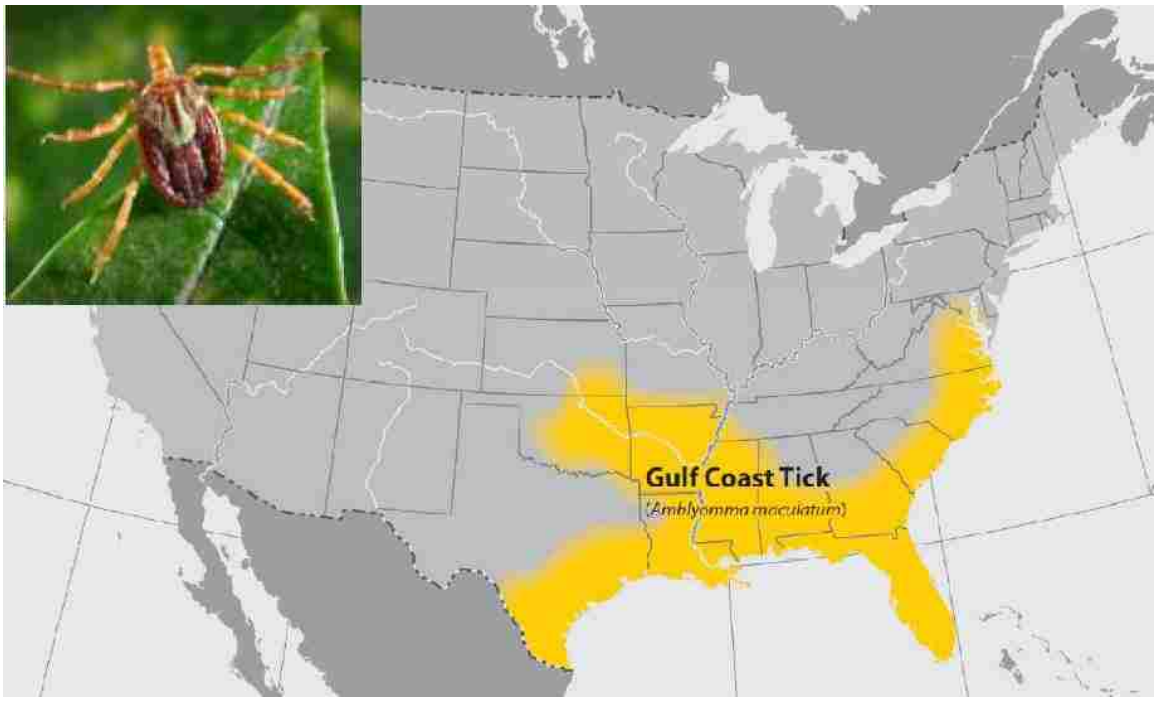

Figure 1.5: Distribution of *Amblyomma maculatum* (Gulf Coast tick) in the United States.
(Courtesy of Centers of Disease Control and Prevention)

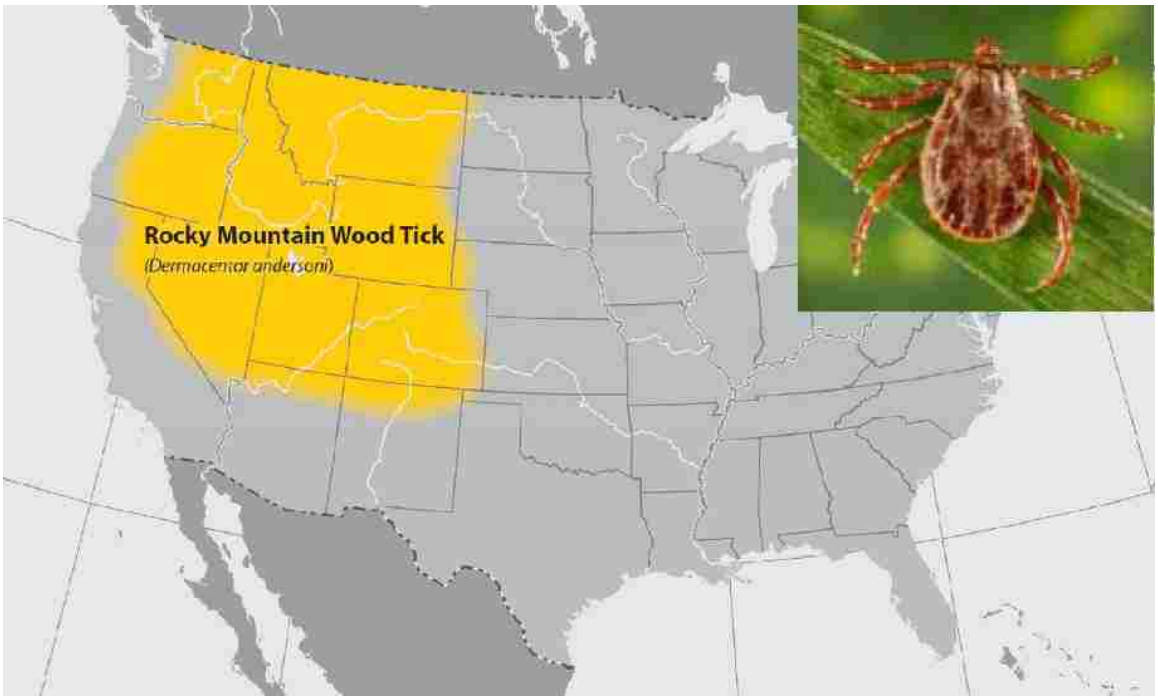

Figure 1.6: Distribution of *Dermacentor andersoni* (Rocky Mountain Wood tick) in the United States. (Courtesy of Centers of Disease Control and Prevention)

(PWV), and Colorado tick fever virus (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.d *Dermacentor variabilis* (American Dog Tick)

Dermacentor variabilis (American dog tick) is distributed east of the Rocky Mountains and some areas on the Pacific Coast of the United States (Figure 1.7). This three-host tick feeds on a variety of hosts. Immature stages feed on small rodents, including deer mice, rice rats, voles, chipmunks, and tree squirrels, while adults feed on larger animals and humans. American dog ticks can transmit *R. rickettsii* and *E. chaffeensis*, and *F. tularensis* to human (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.e *Ixodes pacificus* (Western Blacklegged Tick)

Ixodes pacificus (Western blacklegged tick) is distributed along the Pacific coast region of the United States (Figure 1.8). It is a three-host tick, and immature ticks feed on small mammals, birds, and lizards. Adults feed on larger mammals including Columbian black-tailed deer and humans. It is a vector of *Babesia*, *Anaplasma phagocytophilum* and *Borrelia* species (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.f *Ixodes scapularis* (Blacklegged Tick)

Ixodes scapularis (blacklegged tick or deer tick) is distributed in the northeastern and upper midwestern United States (Figure 1.9). It is a three-host tick. Larvae and nymphs of blacklegged ticks feed on small mammals and birds while adults feed on larger mammals including deer, livestock, and humans. It can transmit *Babesia*, *A. phagocytophilum*, *Borrelia* species, *F. tularensis*, and PWV to humans (Goodman J.L. et al. 2005) (www.cdc.gov).

1.1.2.g *Rhipicephalus sanguineus* (Brown Dog Tick)

Rhipicephalus sanguineus (Brown dog tick) is distributed throughout the United States

Figure 1.7: Distribution of *Dermacentor variabilis* (American dog tick) in the United States.
(Courtesy of Centers of Disease Control and Prevention)

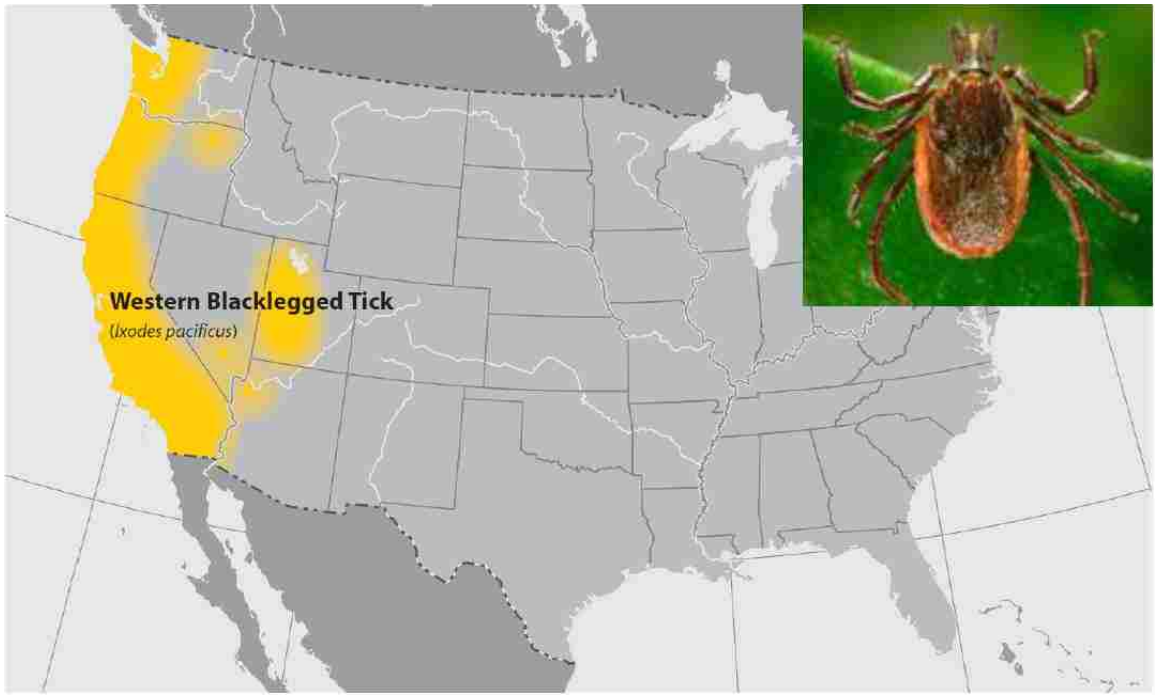

Figure 1.8: Distribution of *Ixodes pacificus* (Western Blacklegged tick) in the United States.
(Courtesy of Centers of Disease Control and Prevention)

Figure 1.9: Distribution of *Ixodes scapularis* (Blacklegged tick) in the United States.
(Courtesy of Centers of Disease Control and Prevention)

and the world (Figure 1.10). It is a three-host tick which all life stages primarily feed on dogs; however, they occasionally feed on other mammals. The brown dog tick is a vector of *F. tularensis* and many *Rickettsia* species in Europe and Asia. Recently, it has been reported as an important vector in the transmission of *R. rickettsii* in the United States (Demma et al. 2005; Wikswo et al. 2007) (www.cdc.gov).

1.1.3. The Importance of Ticks in Veterinary and Human Health

There are approximately 850 known species of ticks in every continent except Antarctica (Goodman J.L. et al. 2005). The direct effect of tick feeding is problematic, especially in livestock, causing economic losses (Jongejan and Uilenberg 2004). Large numbers of ticks infesting a host may cause severe blood loss leading to anemia, reducing growth rate and milk production. Some species of ticks produce a toxin that causes paralysis also known as tick paralysis. Also, host immune response to tick attachment can cause skin damage, for example, inflammation, itching, and pain as well as destruction of hide quality.

Moreover, ticks are the second only to mosquitoes as disease vectors and have been reported to transmit a variety of infectious microorganisms, e.g. bacteria, viruses, protozoa, fungi, and helminthes (Sonenshine and Hynes 2008). It was first acclaimed as a disease vector in 1891 by Smith and Kilbourne who demonstrated that *Rh.* (previously *Boophilus*) *annulatus* (cattle tick) served as a vector of *Babesia bigemina*, the disease agent of Texas cattle fever (Smithcors 1981). The importance of ticks in association with public health is summarized in Table 1.2.

1.2. Tick-borne Rickettsioses

Rickettsioses are worldwide zoonoses that are biologically transmitted by arthropod vectors including fleas (e.g. murine typhus), lice (e.g. epidermic typhus), mites (e.g. scrub

Figure 1.10: Distribution of *Rhipicephalus sanguineus* (Brown dog tick) in the United States. (Courtesy of Centers of Disease Control and Prevention)

Table 1.2: Tick-borne diseases of humans. Tick-borne diseases, causative agents, primary tick vectors, and associated hosts.

Disease	Causative agent	Primary tick vector species	Associated host
Human Babesiosis	*Babesia microti, B. divergens, B. major*	*Ixodes scapularis, I. ricinus,* other	Rodent, cattle, humans
Rocky Mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor variabilis, D. andersoni, Rhipicephalus sanguineus*	Small mammals, humans
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*A. americanum, D. variabils*	Deer, humans
Human anaplasmosis	*Anaplasma phagocytophilum*	*I. scapularis, I. pacificus, I. ricinus*	Rodents, dogs, humans
Q fever	*Coxiella burnetii*	Many tick species	Large domestic livestock, humans
Lyme disease	*Borrelia burgdorferi, B. afzelii, B. garinii, B. bissettii*	*I. scapularis, I. ricinus, I. pacificus, I. persulcatus,* others	Small mammals, some birds, humans
Tick-borne relapsing fever	*Borrelia* spp.	*Ornithodoros* spp.	Various mammals
Tularemia	*Francisella tularensis*	*D. variabilis, D. andersoni, D. reticulutus, A. americanum, I. apronophorus, I. ricinus* complex, *Haemaphysalis leporispalustris,* others	Lagomorphs, rodents, humans
Powassan encephalitis	Flavivirus; family Reoviridae	*Ixodes, Dermacentor*, and *Haemaphysalis* spp.	Rodents, hares
Colorado tick fever	Coltiivirus; family Reoviridae	*D. andersoni*	Rodents, domestic animals, humans
Crimean-Congo hemorrhagic fever	Nairovirus; family Bunyaviridae	*Hyalomma m. marginatum, H. m. rufipes,* others	Hares, hedgehogs, small mammals, humans

(Goodman J.L. et al. 2005)

typhus), and ticks (e.g. RMSF). Tick–borne rickettsioses is caused by members of the spotted fever group (SFG) *Rickettsia* in the genus *Rickettsia* (Rickettsiales: *Rickettsiaceae*) belonging to the class *Alphaproteobacteria*. *Rickettsia* is a gram negative, obligate intracellular bacterium. The typical *Rickettsia* morphology is small, coccobacilli (rod-shaped) with size ranging from 0.3 to 0.5 µm in width and 0.8 to 2.0 µm in length (Hackstadt 1996). Polymorphic rickettsiae (irregular bacillary, filamentous or long-form) have been reported both in tick vectors (Burgdorfer et al. 1981; Philip et al. 1981) and in cultivated isolates (Gulevskaia et al. 1975; Sunyakumthorn et al. 2008; Kekcheeva et al. 1992; Wisseman, Jr. and Waddell 1975; Labruna et al. 2004; Labruna et al. 2007; Philip et al. 1983). It is suggested to be the adaptive form of *Rickettsia* during nutrient exhaustion or unfavorable conditions (Labruna et al. 2007; Sunyakumthorn et al. 2008).

In general, the clinical manifestations of all tick-borne rickettsioses are similar. Symptoms include fever, headache, rash, myalgia, nausea and, sometimes eschars, which are local dermal and epidermal necroses at the bite sites (Walker and Ismail 2008; Parola et al. 2005). The classic symptom for diagnosis is a skin rash which is why it is called spotted fever. In RMSF patients, the rash initially appears on the wrists and ankles and spreads to the trunk; however, a rash does not develop in some cases (Goodman J.L. et al. 2005). In severe cases when vascular endothelial cells are infected, the infection causes hypovolaemia and hypotensive shock resulting in acute renal failure. Doxycycline is the typical drug of choice for rickettsioses treatment (Holman et al. 2001). The dosage for adults and children older than 8 years old is 100 mg every 12 hours for five to ten days. However, for pregnant women and children younger than 8 years doxycycline is not recommended, and chloramphenicol may be drug of choice (Goodman J.L. et al. 2005).

RMSF was the first rickettsiosis that was described. It was identified more than 100 years ago in Montana (Mullen G and Durden L. 2002). It is caused by *R. rickettsii* and is primarily transmitted by *D. andersoni*, and *D. variabilis*, and recently *Rh. sanguineus* was demonstrated to be another competent vector of RMSF (Demma et al. 2005; Wikswo et al. 2007; Piranda et al. 2011). Unlike other rickettsioses, tick-borne rickettsioses have a limited geographic distribution which depends on their tick vector's distribution (Azad and Beard 1998). In 1910, Mediterranean spotted fever (MSF) or boutonneuse fever was described in Tunis. It is the most common tick-borne rickettsioses in the Mediterranean area. It is caused by *R. conorii* subsp. *conorii*, and *Rh. sanguineus* is its primary tick vector. Many years after the discovery of MSF, many other spotted fever rickettsioses were described including Israeli spotted fever first reported in Israel, Siberian tick typhus or North Asian tick typhus first described in Primorye and Queensland tick typhus first recognized in eastern Australia. Many more tick-borne rickettsioses have been characterized with a peak in description occurring from 1984 through 2005, for example, Japanese or Oriental spotted fever, Astrakhan fever, African tick bite fever, Flinders Island spotted fever, *R. parkeri* rickettsiosis, and many unnamed tick-borne rickettsial diseases. Today, more than 20 species of bacteria in SFG *Rickettisia* have been described and characterized around the world; however, not all of them are pathogenic for humans (Goodman J.L. et al. 2005; Parola et al. 2005).

1.2.1. History

Rickettsia was first described in the 1890s when Howard Taylor Ricketts (1871-1910), an American microbiologist, discovered the causative organism (*R. rickettsii*) of RMSF in the blood of experimentally infected guinea pigs and monkeys (Ricketts H.T. 1906) and in the tissues and eggs of *D. andersoni* (Ricketts H.T. 1907a). Ricketts' first attempt is to identify a disease agent

in patient blood, and he was unable to identify the pathogen using microscopic examination and bacteriologic culture; however, inoculation of patient blood into uninfected animals caused disease in the animals with similar manifestations. Ricketts demonstrated that infection can pass from infected animals to uninfected animals and be maintained by serial animal passage (Ricketts H.T. 1906). The infectious agent was preliminarily thought to be a virus because it was noncultivatable. He also demonstrated the mode of transmission of RMSF by *D. andersoni*. The female ticks that previously fed on infected guinea pigs were able to transmit the infection to uninfected guinea pigs (horizontal transmission) (Ricketts H.T. 1907b). Ricketts also showed evidence of vertical transmission in *D. andersoni* (ticks to their eggs, eggs to larvae, and larvae to nymphs) (Philip 2000; Ricketts H.T. 1907b)

In 1909, during an outbreak of epidemic typhus in Mexico City, Ricketts went to investigate the cause of infection. While attempting to isolate the organism, Ricketts was infected with *R. prowazekii* and died in 1910. The scientific community named a family Rickettsiaceae and the order Rickettsiales in honor of Ricketts (Gross and Schafer 2011).

1.2.2. *Rickettsia* Taxonomy

The family *Rickettsiaceae* contains two genera; genus *Rickettsia* and genus *Orientia*. There is only one species, *O. tsutsugamushi*, in the genus *Orientia.* It is the etiological agent of scrub typhus, a mite-borne disease of the Asia-Pacific region. Scrub typhus is transmitted to humans by larval mites also called chiggers. The genus *Leptotrombidium* is the primary vector, of which only the larval stage is parasitic. Therefore, vertical transmission of *O. tsutsugamushi* is critical to maintain the infection in nature.

The genus *Rickettsia*, was recently reorganized into four defined groups which are the ancestral group (AG), the typhus group (TG), the transitional group (TRG), and the spotted fever

group (SFG) (Figure 1.11). The new classification was proposed using phylogeny analysis of chromosomal and plasmid genes based on genetic evolutionary analysis (Gillespie et al. 2007, 2008). The SFG contains the majority of rickettsial species. Until recently, many emerging rickettsial species have been described and designated as a new species and subspecies; however, the rickettsial taxonomy is still controversial because there are no universal criteria for classification (Parola et al. 2005; Walker and Ismail 2008).

1.2.3. Pathogenicity and Pathogenesis

Since its discovery, many different species or strains of SFG *Rickettsia* have been recognized. Nevertheless, many of them are considered nonpathogenic (e.g. *R. montanensis, R. peacockii*) for vertebrates based on human case reports and pathogenicity testing with laboratory animals such as guinea pigs and voles. However, using animal model testing for human diseases is still questionable due to the route of infection and host specificity.

Rickettsial pathogenicity involves two major components because *Rickettsia* is able to infect both invertebrate and vertebrate hosts. Due to the small size of the genome, rickettsial species lack many genes responsible for amino acid synthesis, nucleotide synthesis and lipid and sugar metabolism; therefore, *Rickettsia* requires host cells in order to survive (Walker 2007). The first component to consider is inside the tick vector. *Rickettsia* needs to be able to escape from the gut barrier and tick immunity and then disseminate to the salivary glands in order to be transmitted to the vertebrate host during feeding. Some rickettsial species (e.g. *R. peacockii*) heavily infect tick ovaries but do not disseminate to salivary glands resulting in the absence of horizontal transmission (Niebylski et al. 1999). The recent study of rickettsial actin-based motility of *R. parkeri* in *Drosophila* cells using RNAi demonstrated that *Rickettsia* employs host actin organization in order to be motile and invade arthropod cells (Serio et al. 2010).

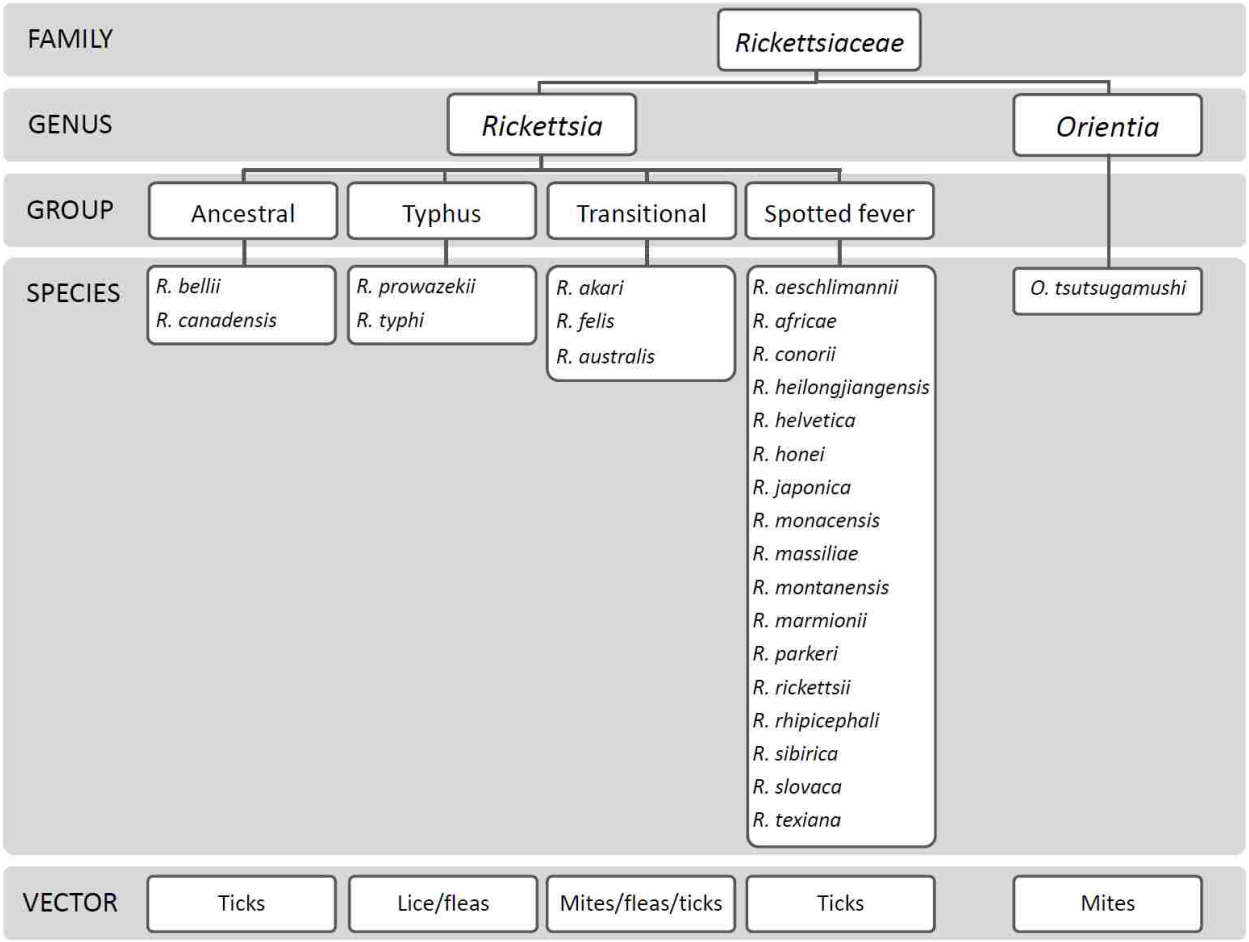

Figure 1.11: Taxonomy and arthropod vectors of the genus *Rickettsia.* The family *Rickettsiaceae* contains two genera: *Rickettsia* and *Orientia*. Within the genus *Rickettsia*, there are four groups which are ancestral group, typhus group, transitional group, and spotted fever groups. In nature, bacteria in family *Rickettsiaceae* are maintained and transmitted by arthropod vectors (Gillespie et al. 2008; Parola et al. 2005).

The second component of rickettsial pathogenesis, is when *Rickettsia* internalizes into vertebrate host endothelial cell where they multiplies and disseminate to other target cells. *Rickettsia* is initially transmitted to the host with tick saliva during bloodmeal acquisition. *Rickettsia* binds to a host cell receptor and forms a ligand/receptor complex which induces phagocytosis. In cell culture, *R. conorii* binds to host receptors (Ku70) on the surface of non-phagocytic cells via rickettsial outer membrane protein B (OmpB) also known as surface cell antigen 5, and recruits actin to the attachment site leading to rickettsial internalization (Martinez et al. 2005). Recently, surface cell antigen 2 and surface cell antigen 1 autotransporter proteins of *R. conorii* have been demonstrated as rickettsial adhesion proteins involved with invasion of host cells (Cardwell and Martinez 2009; Riley et al. 2010). The role of rickettsial outer membrane protein in the adhesion and invasion of host cell has been demonstrated in many pathogenic SFG *Rickettsia* species, for example, *R. rickettsii* outer membrane protein A (OmpA) (Li and Walker 1998) and *R. japonica* OmpB (Chan et al. 2009; Uchiyama 2003). In contrast to *R. rickettsii*, *R. peacockii* which is closely related to *R. rickettsii* and considered a nonpathogenic *Rickettsia* is not able to express OmpA and surface cell antigen 1 (Felsheim et al. 2009) suggesting that rickettsial outer membrane proteins likely contribute to the differential pathogenicity of *Rickettsia.*

Inside the phagosome, *Rickettsia* produces membranolytic phospholipase D and haemolysin to lyse the phagosome membrane, and escapes from the vacuole, and then resides freely in the cytosol. At this step, many candidate genes (e.g. Phospholipase D, Haemolysin A and D, and Actin-tail polymerization genes) are suspected to be rickettsial virulence genes playing a role in rickettsial survival in host cells (Parola et al. 2009; Walker and Ismail 2008). In the cytosol, SFG *Rickettsia* replicates by binary fission and invades the nearby cells by

inducing filopodia formation using an actin polymerization mechanism (Gouin et al. 2004; Walker 2007; Walker and Ismail 2008).

In the host cells, SFG *Rickettsia* utilizes host actin cytoskeleton proteins that control actin polymerization and depolymerization to facilitate rickettsial motility and cell invasion. RickA protein of *R. conorii* was identified as actin related protein 2/3 (Arp2/3) complex activators which induced actin nucleation and actin filament (Gouin et al. 2004). Genome comparison of *R. rickettsii* and *R. peacockii* suggests that RickA protein may be a virulent factor (Felsheim et al. 2009); however functional characterization is lacking.

1.2.4. Laboratory Tools to Study Tick-borne Rickettsioses

The PCR-based assay is the most efficient method to detect rickettsial infection during the acute phase when antibody titers are undetectable. It has high sensitivity, and all types of clinical specimens (e.g. whole blood, serum, and skin biopsy specimens) and tick specimens can be used; however, the clinical specimens need to be collected before antibiotic treatment. The PCR technique is based on the detection of gene portions in rickettsial genomes using a pair of gene specific primers (e.g. 17 kDa surface antigen, 16S rRNA, citrate synthase, OmpA, and OmpB genes) (Higgins et al. 1998). Moreover, the PCR products can be further identifying using restriction fragment length polymorphism or DNA sequencing; therefore, it is also commonly used in research laboratories in order to molecularly characterize new emerging rickettsial species.

Rickettsial isolation has been performed using several methods including embryonated chicken egg yolk, animal inoculation, and cell cultures. Embryonated chicken egg yolk and animal inoculation are used with many rickettsial species such as *R. rickettsii* and *R. felis*; however, cell cultures are currently widely used for primary isolation. Rickettsial inoculation

has been shown possible in both mammalian (e.g. L929 and Vero cells) and tick cells (e.g. ISE6 cells) (La and Raoult 1997).

Being obligate intracellular bacteria, *Rickettsia* cannot typically be cultured in any cell-free medium. The culture of SFG *Rickettsia* isolates in cell culture is widely used to propagate *Rickettsia* for characterization and experimental studies. The study of the interactions between *Rickettsia* and host cells has been performed using both mammalian and tick cell cultures. For example, recent molecular studies have investigated cell invasion of *R. conorii* in Vero and HeLa cells (Cardwell and Martinez 2009; Chan et al. 2009; Martinez and Cossart 2004; Martinez et al. 2005; Riley et al. 2010) and the immune response of ISE6 cells during SFG rickettsial infection (Mattila et al. 2007).

1.2.5. Transmission Cycle

All vector-borne diseases are involved in a classic triangle of pathogen-vector-host interaction which is divided to three components: (1) pathogens which cause diseases in humans or animals, (2) competent vectors which are able to acquire the pathogens from infected vertebrate hosts and transmit to the next susceptible hosts, (3) the susceptible vertebrate hosts which can be infected by arthropod vectors (Figure 1.12).

For SFG rickettsioses, ixodid ticks are the main vectors transmitting *Rickettsia*. They are not only vectors (horizontal transmission), but also reservoir hosts (vertical transmission); therefore, the interaction between SFG *Rickettsia* and the tick is important to study. Ixodid ticks feed only once in each life stage; therefore, after acquisition of rickettsial infection they cannot transmit the infection until the next blood feeding of the next life stage. In order for ticks to transmit diseases to vertebrate hosts, they must exhibit either transstadial transmission, or transovarial transmission. Horizontal transmission to mammalian reservoirs helps maintain and

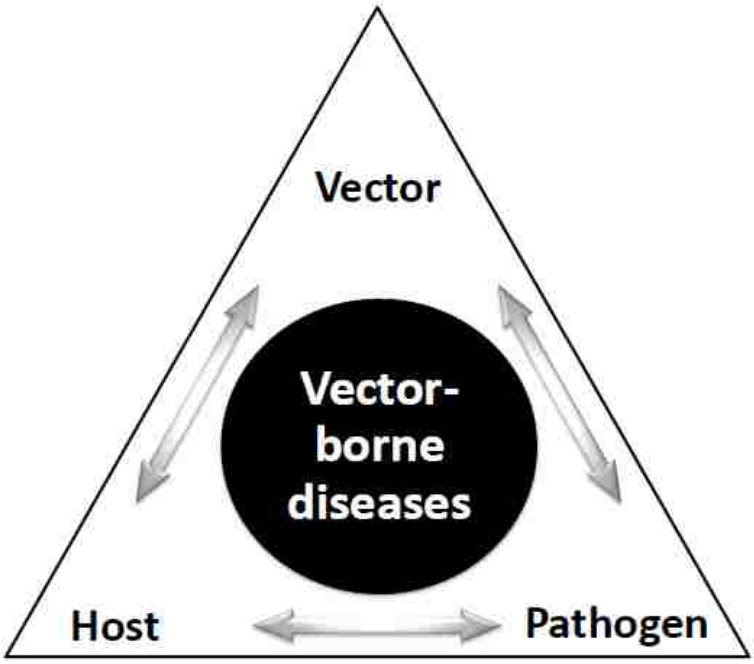

Figure 1.12 pathogen-vector-host interaction diagram of vector-borne diseases.

introduce infection into new populations of ticks.

In 1907, transstadial and transovarial transmission of SFG *Rickettsia* in ticks were first documented by Ricketts who described a complete transmission mechanism of *R. rickettsii* in each *D. andersoni* life cycle stage. He demonstrated the successful transstadial transmission of *R. rickettsii* from laboratory-infected larvae to nymphs and laboratory-infected nymphs to adults and transovarial transmission from infected females to their eggs. His experiment also demonstrated multiplication of rickettsiae in ticks during bloodfeeding suggesting biological transmission (Philip 2000; Ricketts H.T. 1907a; Ricketts H.T. 1907b).

Due to the low level of transovarial transmission in ticks, Ricketts suggested that reservoir hosts of rickettsial infection are required in order to maintain the infection in nature. He examined the susceptibility of several animal species including ground squirrel, woodchuck, rock squirrel, wood rat, pine squirrel, rock rabbit, and three other lagomorphs to rickettsial infection (Ricketts H.T. 1907b). Rock squirrels, chipmunks, and woodchucks were susceptible to rickettsial infection and considered possible reservoir hosts of RMSF in western Montana (Philip 2000). Therefore, Ricketts clearly demonstrated that *D. andersoni* is a vector of *R. rickettsii* which is maintained in nature by small mammals, and humans do not necessarily contribute to the transmission cycle (Figure 1.13).

1.2.6. Vector Competence

Vector competence is the ability of arthropod vectors to acquire infection and transmit pathogens to new susceptible hosts (Mullen G and Durden L. 2002). Several yet undetermined factors likely contribute to tick vector competence for rickettsial species.

For SFG *Rickettsia*, ticks acquire novel rickettsial infection while feeding on infected animals. *Rickettsia* is ingested with the bloodmeal into the midguts which is the first site of

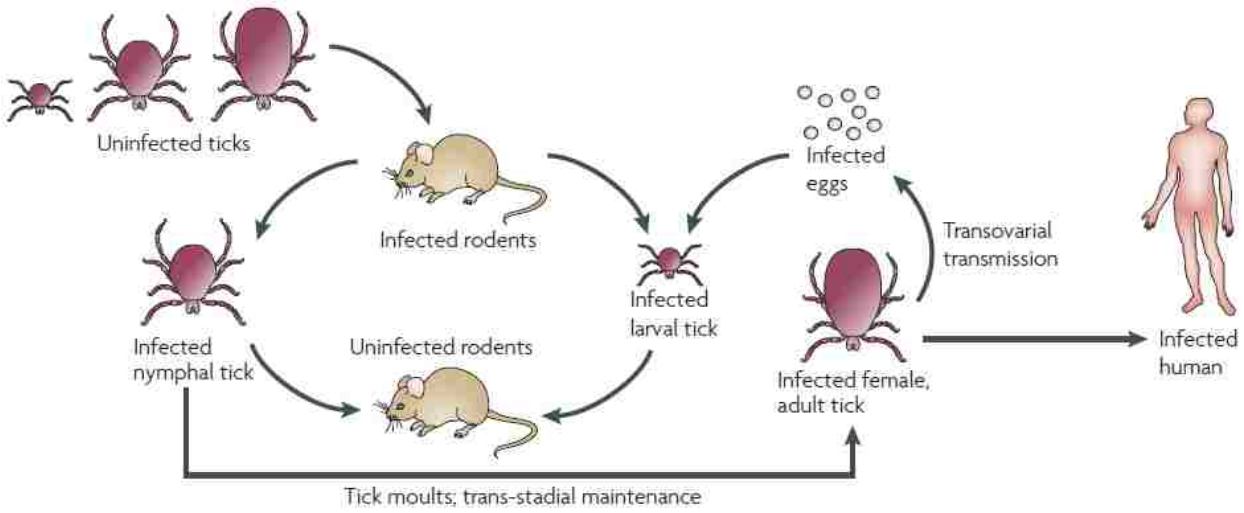

Figure 1.13 Transmission cycle of spotted fever group rickettsiosis. In nature, spotted fever group *Rickettsia* is maintained in infected ticks via transstadial and transovarial transmissions and reservoir hosts (e.g. rodents) via horizontal transmission. Humans are incidental hosts. (Figure from Walker and Ismali , 2008)

contact. *Rickettsia* then escapes from gut barriers and infects midgut epithelial cells. The *Rickettsia* replicates in the midgut and disseminate to hemolymph which causes systemic infection when all tick tissues including hemolymph, salivary gland, midgut, and ovary are potentially infected with *Rickettsia* (Munderloh and Kurtti 1995; Socolovschi et al. 2009). The infected tick horizontally transmits *Rickettsia* to the next susceptible host while taking the next bloodmeal. The infected tick can also vertically transmit *Rickettsia* via transstadial and transovarial transmission. The list of SFG *Rickettsia* in the United States and their competent vectors are shown in Table 1.3.

1.2.7. Tick-borne Rickettsiosis in the United States

In the United States, many species of SFG *Rickettsia* are identified including pathogenic and nonpathogenic rickettsiae, and their geographic distribution is limited to their tick vectors (Azad and Beard 1998). RMSF is considered to be the most common tick-borne rickettsiosis causing human disease in the United States; however, many emerging tick-borne rickettsioses have been reported and characterized. Below is a brief description of the tick-borne SFG *Rickettsia* associated with human disease and other common SFG *Rickettsia* not currently associated with human diseases (Table 1.3).

Rickettsia rickettsii is the causative agent of RMSF which has been reported throughout the United States. Most cases occur in southeastern and eastern United States (e.g. Delaware, Maryland, Washington D.C., Virginia, West Virginia, North Carolina, South Carolina, Georgia, and Florida); the highest numbers of reported cases are in North Carolina and Oklahoma (www.cdc.gov). The primary vectors of *R. rickettsii* are *Dermacentor* ticks (i.e. *D. andersoni* and *D. variabilis*), but recently *Rh. sanguineus* has also been reported as a competent vector (Demma et al. 2005).

Table 1.3: Spotted fever group *Rickettsia*, primary tick vector, and distribution in the United States.

***Rickettsia* species**	**Disease**	**Recognized tick vector**	**Distribution in the US**
Pathogenic *Rickettsia*			
R. rickettsii	Rocky Mountain spotted fever	*D. andersoni*, *D. variabilis*, *Rh. sanguineus*	Widespread Arizona
R. parkeri	*Rickettsia parkeri* rickettsiosis	*A. maculatum*	Alabama, Texas, Georgia, Mississippi, Kentucky
Rickettsia 364D (*R. phillipi*)	364D rickettsiosis	*D. occidentalis*	California
R. massiliae	unnamed	*Rh. sanguineus*	Arizona
Nonpathogenic *Rickettsia*			
R. montanensis	None recognized	*D. andersoni*, *D. variabilis*	Widespread
R. peacockii	None recognized	*D. andersoni*	Montana, Colorado
R. amblyommii	None recognized	*A. americanum*, *A. maculatum*	Widespread
R. rhipicephali	None recognized	*Rh. sanguineus*, *D. andersoni*, *D. variabilis*, *D. occidentalis*	Mississippi, Texas, North Carolina, South Carolina, Montana, California

(Demma et al. 2005; Goodman J.L. et al. 2005; Parola et al. 2005; Parola et al. 2009; Eremeeva et al. 2006)

Rickettsia parkeri, a *Rickettsia parkeri* rickettsiosis agent, was identified in 1939 by Parker from *A. maculatum* in the Texas Gulf Coast region. It was considered as a nonpathogenic species until 2004; Paddock et al. (2004) clearly demonstrated the first human case of *R. parkeri* rickettsiosis. The patient presented with mild febrile illness, headache, fever, arthralgia, and multiple eschars on his legs. The infection was identified by serological assay, immunohistochemical staining, and molecular assay. The organism was isolated in cell culture from an eschar specimen, and a *Rickettsia* culture isolate was confirmed by PCR assays (Paddock et al. 2004). Human infection has been reported in Florida, Mississippi, and South Carolina, while *A. maculatum* infection is found in many southeastern states (Sumner et al. 2007; Edwards et al. 2010).

Rickettsia 364D was first isolated in 1981 from *D. occidentalis* in California (Philip et al. 1981). The 364D isolate had similar serologic characteristics to *R. rickettsii*. *D. occidentalis* is likely a vector, and in eight California counties, 11% of *D. occidentalis* are infected with 364D. The first human case was reported in 2010 in northern California consisting of swelling and erythema and an eschar developing seven days later after tick bite. The PCR assay result from the eschar biopsy was identical to those of 364D (Shapiro et al. 2010).

Rickettsia massiliae was first isolated in 1992 from *Rh. sanguineus* in Marseilla, France and designated as a new rickettsial species (Parola et al. 2009). In 2006, the first human infection of *R. massiliae* was identified from frozen blood specimen of a 45-year-old man who was hospitalized in Italy. The patient presented with fever, an eschar, rash on palms and soles, and mild hepatomegaly. He was first presumed to have *R. conorii*, and after 20 years, the isolate was molecularly identified as *R. massiliae* (Vitale et al. 2006). Recently, *R. massiliae* was detected in *Rh. sanguineus* in California (Beeler et al. 2011) and isolated from *Rh. sanguineus* in

eastern Arizona (Eremeeva et al. 2006) United States; however, human cases have not been recognized in the United States.

Rickettsia montanensis was first isolated in 1963 from *D. variabilis* and *D. andersoni* in Montana (Bell et al. 1963). It is considered to be a nonpathogenic *Rickettsia* because it is not pathogenic for guinea pigs; however, it has been isolated from rodents (genera *Microtus* and *Peromiscus*) (Raoult and Roux 1997). *R. montanensis* is widespread, but commonly found in the south central region of the United States. Many researchers have used *R. montanensis* as a nonpathogenic model to examine tick response to rickettsial infection (Ceraul et al. 2007, 2008, 2011; Macaluso et al. 2003; Mulenga et al. 2003).

Rickettsia peacockii was first described in 1925 from *D. andersoni* collected from the east side of the Bitterroot Valley. Burgdorfer et al (1981) demonstrated that ticks on the east side of the Bitterroot Valley were infected with this nonpathogenic *Rickettsia*, and approximately 70% of ticks vertically transmit *Rickettsia* in order to maintain the infection. It was originally designated the east side agent, and it was suggested that the agent interfered with rickettsial maintenance of *R. rickettsii* in the east side of the Bitterroot Valley where the incidence of RMSF was low compared to the west side of the valley (Burgdorfer et al. 1981).

Candidatus Rickettsia amblyommii was first isolated in 1981 from *A. americanum* collected in Tennessee (Burgdorfer et al. 1981). The organism was originally designated the WB-8-2 agent and considered to be a nonpathogenic *Rickettsia* (Burgdorfer et al. 1981); however, it has not been formally classified. Recently, *R. amblyommii* has been implicated as a causative agent of tick-borne rickettsiosis in North Carolina due to the abundance of *A. americanum* in the endemic areas and low population of *D. variabilis* (Apperson et al. 2008); however it has not yet been clearly implicated as a cause of disease in humans.

Rickettsia rhipicephali was first isolated in 1975 from *Rh. sanguineus* removed from dogs in Mississippi (Burgdorfer et al. 1975). The organism was pathogenic for voles, but nonpathogenic for guinea pigs. It has been detected in *D. variabilis, D. occidentalis,* and *D. andersoni* (Wikswo et al. 2008).

1.2.8. Tick Distribution and Rickettsiosis Epidemiology

According to CDC reports, the number of human cases of tick-borne rickettsial diseases has continuously increased since 1998. In 2008, there were 4,727 confirmed and unconfirmed cases of tick-borne rickettsial diseases and up to 2,500 cases of RMSF (Figure 1.14) (Dumler 2010). In contrast, very low prevalence of *R. rickettsii* infection in *Dermacentor* ticks has been demonstrated in many areas including endemic areas despite an increase in RMSF cases (Ammerman et al. 2004; Stromdahl et al. 2010).

The distribution of SFG rickettsiosis depends on the distribution of tick vectors. Based on overlapping tick distribution and seasonality (section 1.1.3), there is the potential for ticks to share the same reservoir hosts such as small mammals. However, in large part the prevalence of rickettsial infection is specific to tick genera. For example, in North America *Dermacentor* ticks primarily carry *R. rickettsii* and *R. montanensis*, and *Amblyomma* ticks primarily carry *R. parkeri* and *R. amblyommii*. Little is known about the factors that play a role in tick/*Rickettsia* specificity in nature.

1.3. Tick/*Rickettsia* Relationship

Ticks are the only competent vectors for all pathogenic SFG *Rickettsia* species, and they are known to have a specific relationship. Ticks serve as long-term reservoir hosts which can maintain rickettsial infection up to a year depending on the tick life cycle stage and environmental conditions. However, rickettsial infection in tick vectors is not always favorable.

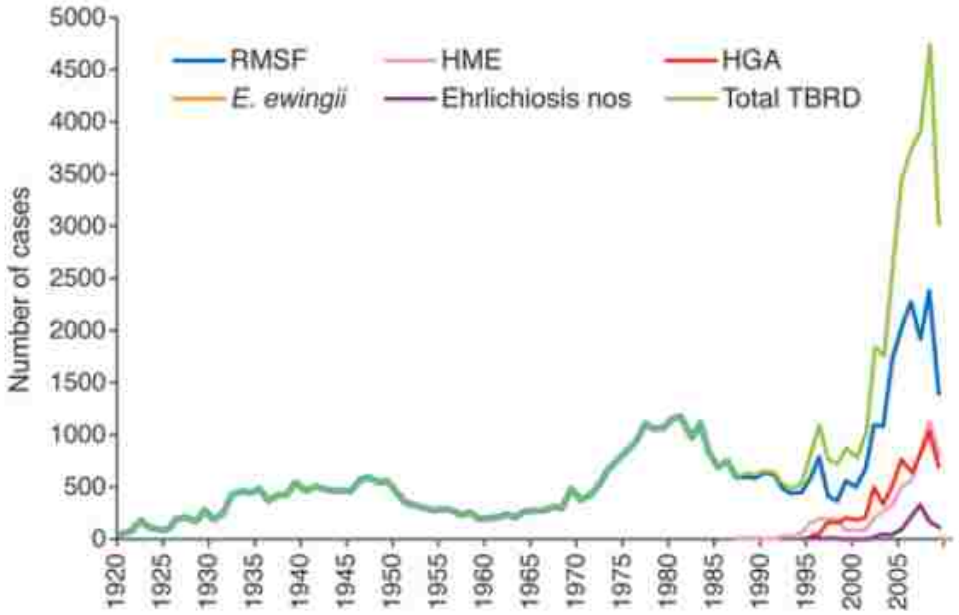

Figure 1.14 Confirmed and unconfirmed cases of tick-borne rickettsial diseases (TBRD) in the United States form 1920 to 2009. RMSF, Rocky Mountain spotted fever; HME, human monocytic ehrlichiosis; HGA, human granulocytic anaplasmosis; nos, not otherwise specified. Figure from (Dumler 2010).

For example, experimental infection of *R. rickettsii* in *D. andersoni* significantly decreased tick viability during molting (Niebylski et al. 1999) and female fecundity (Mcdade and Newhouse 1986). Recently, the study of tick and *Rickettsia* relationship has focused on rickettsial adhesion/internalization (Macaluso et al. 2003) and tick immune/stress response against rickettsial infection (Ceraul et al. 2007, 2008, 2011; Mulenga et al. 2003) which may play a role in controlling rickettsial infection in ticks.

1.3.1. Interspecific Relationship of *Rickettsia* in Ticks

In 1981, Burgdorfer discovered *R. peacockii* which was found in *D. andersoni* predominantly on the east side of the Bitterroot Valley. *R. peacockii* infected most tick tissues and was able to be transmitted to tick offspring. It was described as a nonpathogenic or symbiotic *Rickettsia* because they were not able to establish infection in embryonated eggs, animals, and cell cultures (Burgdorfer et al. 1981; Mcdade and Newhouse 1986). Burgdorfer found that 80% of *D. andersoni* were infected with *R. peacockii* on the east side, but only 8-16% of *D. andersoni* were infected with *R. peacockii* on the west side where there was a high number of RMSF cases (Burgdorfer et al. 1981). His experimental infection bioassays demonstrated that infection of *R. peacockii* in *D. andersoni* interfered with transovarial transmission of *R. rickettsii*. It was suggested that infection by nonpathogenic *Rickettsia* inhibits the transovarial transmission of pathogenic *Rickettsia* (Burgdorfer et al. 1981). This was called the interference phenomenon.

Recently, Macaluso et al. (2002) have shown that the interference of transovarial transmission by a second *Rickettsia* is not specifically associated with rickettsial pathogenicity. *D. variabilis* was capillary fed with two species of nonpathogenic *Rickettsia*; *R. montanensis*, (*Dermacentor*-associated *Rickettsia*) and *R. rhipicephali*, (*Rhipicephalus*-associated *Rickettsia*)

in two reciprocal challenges. Eggs from individual females were collected and examined for rickettsial infection by PCR. The infection prevalence of *R. montanensis* was higher compared to *R. rhipicephali*, and only *R. montanensis*-infected ticks were resistant to interspecific challenge. Additionally, only *R. montanensis* was maintained transovarially to F1 and F2 suggesting that the specificity of tick/*Rickettsia* pairing is important for successful transmission (Macaluso et al. 2002).

In the context of pathogen transmission, the relationship between ticks and *Rickettsia* is critical; however, interspecific competition between rickettsial species also plays a role in the ecology and epidemiology of tick-borne rickettsioses in nature.

1.4. Tick Immunity

Ticks are bloodsucking arthropods which are often infected with pathogens that are found in host's blood. Like other animals, ticks have a defense mechanism against pathogens which may enter into their bodies by injury or ingestion (Sonenshine and Hynes 2008). Similar to insects, ticks have only an innate immune system containing two components: cellular and humoral responses (Taylor 2006). Tick immunity, however, is not well-studied when compared to insect immunity. The most well known innate immune system of ectoparasitic insects is in *Anopheles* mosquitoes, the malaria vector (Sonenshine and Hynes 2008). They recognize non-self molecules using pattern recognition receptors (PPRs). For example, the peptidoglycan recognition proteins, (PGRPs) when exposed to bacteria, activate the Toll signal transduction pathways thereby inducing antimicrobial peptides (AMPs) secretion, phagocytosis, and other immune responses (Sonenshine and Hynes 2008).

1.4.1. Tick Cellular Immune Response

The coagulation of hemolymph is an important part of the tick immune system which

serves to prevent microbial infection and heal wounds (Muta and Iwanaga 1996). Tick hemolymph contains two important immune components: protein-rich plasma and hemocytes. There are four major types of hemocytes, namely prohemocytes, non-granular plasmatocytes, granulocytes, and spherulocytes (Sonenshine 1993; Sonenshine and Hynes 2008). Prohemocytes are small hemocytes (6 – 7 µm long) having little cytoplasm with no granules. They are the stem cells of other hemocytes and represent a small part of the hemocyte population. Plasmatocytes are larger elongated hemocytes that are 8 - 12 µm long. Granulocytes are the largest hemocytes (15 – 20 µm long) and they consist of type I and type II granulocytes; both contain numerous intracellular granules. The typical characteristics of granylocytes are numerous intracellular granules and filopodia. Sperulocytes (8 – 14 µm long) are oval or suboval hemocytes containing large fibril-filled granules.

An increase of hemocytes in response to bacterial challenge with organisms not naturally associated with the tick species has been demonstrated. In *D. variabilis,* when injected with the spores of *Bacillus subtilis,* the hemocyte population increased 6.4-fold in comparison to unchallenged ticks (Johns et al. 1998). When injected with *B. burgdorferi*, the Lyme disease spirochete, the introduced bacteria are lysed by AMP and ingested by phagocytosis (Johns et al. 2001).

1.4.1.a. Phagocytosis

Phagocytosis is a complex process involving many signal transduction pathways. In insects, focal adhesion kinase (FAK)/Src and mitogen activated protein kinase (MAPK) pathways play an important role in this process (Lamprou et al. 2007). Phagocytosis begins with recognition of microbes or foreign objects by plasmatocyte or granulocyte cell receptors, followed by induction of endocytosis into a vesicle which then fuses with lysosomes to form a

phagolysosomes. Cell receptors recognize gram positive bacteria, spirochetes, and also fluorescent-coated beads which are ingested by tick phagocytes (Inoue et al. 2001). Inside the phagolysosomes, digestive enzymes such as acid phosphatases and lysozyme are activated by signaling molecules (e.g. calreticulin) (Asgari and Schmidt 2003) leading to digestion of trapped microbes and objects. However, little is known about the surface receptors on tick hemocytes and the important signal factors that induce tick phagocytosis.

1.4.1.b. Nodulation

Nodulation is the aggregation of hemocytes that occurs when the tick hemocytes recognize components on the bacterial surface, such as lipopolysaccharide (LPS), and form a massive aggregation to surround the bacteria which is then digested. Tick nodulation is similar to melanotic encapsulation in insects except no melanin is involved in the process (Sonenshine and Hynes 2008). Opsonizing molecules such as lectins have been found in both soft ticks and hard ticks. Dorin-M was isolated from hemocytes of the soft ticks *O. moubata* (Kovar et al. 2000), and Ixoderin A was isolated from the midgut and hemocytes of *I. ricinus* (Rego et al. 2005).

1.4.1.c. Encapsulation

Encapsulation occurs when pathogens (e.g. parasites and nematodes) are too large for phagocytosis and nodulation. In insects, it is similar to nodulation but with melanin, in which melanization and toxic free radicals destroy the parasites. A similar process was observed in *D. variabilis* injected with plastic beads (Eggenberger et al. 1990). Degranulation of granulocytes results in deposits of matrix-like materials which aggregate around the bacteria or objects. The plasmatocytes attack the matrix and undergo apoptosis becoming a thick layer encapsulating the invading object (Sonenshine and Hynes 2008).

1.4.2. Humoral Response

In addition to the hemocyte response, insects and ticks also have a humoral response with functional secreted molecules (e.g. AMPs, lectins, lysozymes, coagulation factors, proteases and protease inhibitors) and play an important role in insect and tick innate immunity.

1.4.2.a. Antimicrobial Peptides

Antimicrobial peptides are small molecules (4 – 20 kDa) (e.g. defensin, cercopins, lysozyme) mainly found in the midgut and hemocytes. They are effective and fast acting molecules that bind to invading microbes and disrupt microbe membrane integrity. Many AMPs have been identified and characterized in insects including defensins, cercropins, attacins, and sarcotoxins. For ticks, there are five known AMPs including defensins, lysozymes, lectins, proteases and protease inhibitors.

Defensins are small (3 – 6 kDa) cysteine-rich cationic peptides initially described in the hard tick, *D. variabilis* (Johns et al. 2001) and the soft tick, *O. moubata* (Nakajima et al. 2001). The immature peptide ranges from 67 – 92 amino acids in length, and the mature peptide ranges from 37 – 61 amino acids in length. To date, more than 20 different tick defensins have been identified from 11 different tick species. In *D. variabilis*, there are two isoforms of defensin: defensin1 (varisin) and defensin2. Defensin1 is the major isoform and is primarily produced in the hemocytes, but is also found in the midgut and fat body. It is found in all life cycle stages (embryonic egg, larva, nymph, and adult) and is upregulated during bloodfeeding (Ceraul et al. 2007). When challenged with *B. burgdorferi* and *B. subtilus* defensin is upregulated and lyses the invading bacteria (Johns et al. 2001). Interestingly, 65% of *B. burgdorferi* are dead within 1 hour when chicken lysozyme was added indicating synergism of defensin and lysozyme. Defensin2 was later identified from tick midgut (Ceraul et al. 2007). *I. scapularis* defensin,

scapularisin, was identified, and only one isoform was found in hemolymph, midgut, and fat body (Hynes et al. 2005). However, scapularisin protein was not detectable in ticks that were injected with *B. burgdorferi* (Hynes et al. 2005). It is not clear whether scapularisin protein was not expressed or did not function. *B. burgdorferi* remained viable for 24 hours after injection, and when *B. burgdorferi* was incubated with *I. scapularis* hemolymph it was still alive and active (Johns et al. 2001). Most defensins from ticks (e.g. *D. variabilis, I. scapularis, A. americanum, H. longicornis*) are similar; they are small cationic peptides (Todd et al. 2007), except for *A. hebraeum* defensin, hebrasein, which has little amino acid similarity to other defensins (Sonenshine and Hynes 2008). In the soft tick *O. moubata*, all four defensin isoforms have four exons and three introns similar to defensins identified in aquatic mussels. Most of defensins from hard ticks have no introns (Hynes et al. 2005; Todd et al. 2007); however, the defensin from *I. ricinus,* a European tick, has two introns.

Lysozymes are small digestive enzymes (14 kDa) which also have an antimicrobial function. Lysozymes destroy bacterial cell walls by hydrolyzing the sugar bonds in the peptidoglycan backbone. In *D. variabilis*, lysozyme is highly expressed in hemolymph, and expressed at low levels in other organs (Sonenshine et al. 2005). In hemolymph, mRNA expression of *D. variabilis* lysozyme was upregulated when injected with *E. coli* (Simser et al. 2004; Sonenshine et al. 2005), and it may also act synergistically with defensin increasing the antimicrobial effect in tick hemolymph (Johns et al. 2001). The antimicrobial effect has been reported in many tick species for both ticks and tick (*I. ricinus, D. andersoni, I. persulcatus,* and *I. scapuralis*) cell lines (Kuhn KH and Haug T 1994; Mattila et al. 2007; Podboronov 1990). In *I. scapuralis* and *D. andersoni* cell lines, when challenged with *R. peacockii,* lysozyme expression was not upregulated; however, defensin expression was upregulated when injected

with *E. coli* and *Micrococcus luteus* (Mattila et al. 2007). It is suggested that *R. peacockii*, an endosymbiont *Rickettsia*, is able to avoid the recognition of the tick innate immune response.

1.4.2.b. Proteases

Proteases (e.g. serine proteases) are reported to be important factors involved in the refractoriness of mosquitoes to malaria parasites (Xu et al. 2006). In ticks, immune-responsive factor D-like serine protease was identified during *E. coli* challenge; however, its antimicrobial function and specificity or reactivity to *Rickettsia* has not been characterized (Simser et al. 2004).

1.4.2.c. Protease Inhibitors

Protease inhibitors are important innate immune molecules because most infectious pathogens secrete proteases during host tissue invasion (Sonenshine and Hynes 2008). In ticks, many protease inhibitors have been described including a serine proteinase inhibitor (serpin), α–macroglobulins, and Kunitz-type serine protease inhibitor. In *D. variabilis*, serpin is predominant in hemocytes and contains a clip domain that is found in vertebrates (Simser et al. 2004). Serpin has been reported in other hard tick species, *A. americanum* (Chalaire et al. 2011), and the soft tick *O. moubata* (Kadota et al. 2002). Alpha–2 macroglobulin was found in *O. moubata* and *I. scapularis* (Saravanan et al. 2003; Valenzuela et al. 2002). It forms a molecular cage, traps proteases in the bait region and destroys the proteases. Additionally, upregulation of α–2 macroglobulin was demonstrated in *R. montanensis*-infected *D. variabilis* (Mulenga et al. 2003). Kunitz protease inhibitor (KPI) was recently identified and characterized from *D. variabilis* as a novel anti-rickettsial peptide (Ceraul et al. 2008, 2011). Ceraul et al. (2011) suggested that DvKPI associates with rickettsiae and limits the invasion of *R. montanensis* in *D. variabilis* midgut.

1.4.2.d. Lectins

Lectins are proteins or glycoproteins containing specific oligosaccharide binding sites for binding pathogen surfaces. They also function in an opsonization process engulfing invading microbes. Moreover, lectins can bind one another and form an immobilized complex with invading microbes causing aggregation (Sonenshine and Hynes 2008). Many lectins have been identified from tick hemolymph and some from tick saliva, for example, Dorin M from *O. munbata* and Ixoderin A from *I. ricinus*. Lectins are thought to be involved in many processes in tick innate immunity such as pathogen recognition, opsonization, phagocytosis, and encapsulation (Sonenshine and Hynes 2008).

1.4.3. Tick Response to Spotted Fever Group *Rickettsia*

The molecular interaction of tick and *Rickettsia* has been studied in both ticks and tick cell lines. Most studies compared tick gene expression of uninfected and *Rickettsia*-infected tick cells in order to identify tick factors during rickettsial infection. The response of *D. variabilis* during *R. montanensis* infection has been studied using molecular techniques (Macaluso et al. 2006) including subtractive hybridization (Mulenga et al. 2003b) and differential display PCR (Macaluso et al. 2003a). The first attempt was to identify tick-derived molecules that inhibit transovarial transmission of a second *Rickettsia* (Macaluso et al. 2003a, 2003b). Tick ovaries from uninfected and *R. montanensis*-infected ticks were used to identify the ovarian specific tick response. Eleven cDNA fragments were differentially expressed by subtractive hybridization and nine cDNA fragments were identified from differential display PCR. Identified tick molecules were classified to categories based on their predicted function as shown in Table 1.4. Most molecules are suspected to be related to rickettsial invasion (e.g. ATPase of clathrin-coated vesicles, α-catenin) and tick immunity (e.g. ferritin, glutathione S-transferase). However,

Table 1.4 *D. variabilis* molecules in response to *R. montanensis* infection, their predicted functions and primary tick tissues.

Predicted function	Putative protein identification	Primary tick tissue
Receptor/adhesion	ATPase of clathrin-coated vesicles	Ovary
	α-Catenin	Ovary
	Clathrin adaptor protein	NC
	Mucin-like protein	NC
	Protein inhibitor of signal	NC
	Tetraspanin	NC
	Transducer and activator of transcription 1/3	NC
Tick immune and stress response	Ferritin	NC
	α-Dehypdrgenase reductase	NC
	Glutathione S-transferase	Gut
	Nucleosome assembly protein	NC
	Cyclin A2 protein	NC
	Cu^{2+} –transporting ATPase	NC
	Tubulin α-chain	Ovary
	Serine protease	NC
	Prophenoloxidae-activating factor	NC
	Defensin1	Hemolymph
	Lysozyme	Hemolymph, midgut
	Kunitz protease inhibitor	Midgut
Tick-host interaction	α-2 macroglobulin	NC
	Salivary glue precursor	Salivary gland
	IgE-dependent histamine release factor	NC
	ENA vasodilator	NC
	Calreticulin	NC
	Histamine release factor	NC
Unknown	Probable elongation factor	NC
	Glycine-rich protein	Ovary

NC = none characterized

functional characterization of these tick molecules during rickettsial infection has not been done (Ceraul et al. 2007, 2008; Macaluso et al. 2003; Mulenga et al. 2003). Unlike other hematophagous arthropods, ticks digest blood intracellularly. During the beginning of bloodfeeding, a peritrophic membrane (PM) is formed (Munderloh and Kurtti 1995) and remains intact for many days after repletion (Sonenshine and Hynes 2008). The PM acts as a barrier against many microbes; however, some parasites are still able to penetrate the PM (Rudzinska et al. 1983). *Anaplasma marginale* binds to an unknown receptor on the midgut surface of *D. variabilis* and *D. andersoni* via an outer membrane protein: MSP1a (de la Fuente et al. 2004). For SFG *Rickettsia*, many outer membrane proteins were demonstrated to interact with vertebrate host cell receptors, for example, OmpB and Ku70 (Martinez et al. 2005). Although rickettsial ligand/receptor binding has not been yet identified in tick vector, receptor/adhesion tick molecules have been identified from *Rickettsia*-infected ticks, for example, vATPase and α-catenin (Macaluso et al. 2003a).

In the tick midguts, many immune genes (e.g. defensin) respond to bloodfeeding due to the presence of blood pathogens. In *D. variabilis*, immune molecules including defensin1, defensin2 (Ceraul et al. 2007), Kunitz protease inhibitor (DvKPI) (Ceraul et al. 2008) and glutathione s-transferase (DvGST) (Dreher-Lesnick et al. 2006) are upregulated in the tick midguts following bloodfeeding. Defensin1 expression was increased 35-fold, while defensin2 expression was increased 5-fold after 4 days of feeding. When challenged with *R. montanensis*, at 24 and 48 hours post-challenge (hpc) defensin1 expression was upregulated 2.6- and 1.7-fold, respectively, while defensin2 expression was increased 1.9-fold only at 24 hpc (Ceraul et al. 2007). DvKPI was highly expressed in the midgut compared to other tick tissues such as hemocytes, fat body, ovary and salivary gland. Bloodfeeding and rickettsial infection

upregulated DvKPI gene expression (Ceraul et al. 2007). Functional assays of DvKPI demonstrated that DvKPI limits the dissemination of *R. montanensis*. DvGST was first identified from *R. montanensis*-infected *D. variabilis* containing two isoforms: DvGST1 and DvGST2. DvGST1 is constitutively expressed in the midguts, while DvGST2 is highly expressed in tick ovaries (Dreher-Lesnick et al. 2006). Gene expression of both GSTs was downregulated when challenged with *E. coil*; however, it is not known how GST functions during rickettsial infection (Dreher-Lesnick et al. 2006).

1.5 Summary

Ticks maintain infection of SFG *Rickettsia* in nature via horizontal and vertical transmission. In the United States, despite the sympatric population of tick species, the prevalence of rickettsial transmission is limited to certain species of ticks. Even if an individual tick can be infected with two rickettsial species in the laboratory, only certain rickettsial species (typical *Rickettsia*) can be maintained via vertical (transstadial and transovarial) transmission, and the successful transmission is dependent on tick/*Rickettsia* pairing in nature. This is believed to be related to the specific relationship between tick and *Rickettsia*; however, the specific tick/*Rickettsia* interaction has not yet been identified.

In vertebrate hosts, the pathogenesis of *Rickettsia* has been described and involves many mechanisms including signal transduction and cytoskeleton rearrangement. Also, the immune mechanisms of mammalian hosts to *Rickettsia* have been examined. For ticks, which serve as a disease vectors and reservoir hosts, many tick-derived molecules related to rickettsial invasion and tick immune response have been identified and characterized over the past few years. During rickettsial invasion, SFG *Rickettsia* utilizes host cytoskeleton proteins to induce actin-based motility and spread to adjacent cells. Alpha–catenin, a cytoskeleton protein which

regulates actin dynamic in the cells, was upregulated in *R. montanensis*-infected *D. variabilis* ovaries. Little is known about the specific mechanism of α-catenin in response to rickettsial infection. Many studies have focused on tick humoral immune response during rickettsial infection (i.e. AMPs). Many tick AMPs demonstrated antimicrobial effect upon rickettsial challenge; however, most studies determined the immune response upon typical or endosymbiont rickettsial infection. Specific tick responses that regulate atypical rickettsial invasion and rickettsial survival in ticks may contribute to vector competence of ticks for *Rickettsia* and the interspecific relationship of *Rickettsia* in ticks. Studying *Rickettsia*/tick relationships will lead to a better understanding of ecology and epidemiology of tick-borne rickettsioses. The hypothesis of this dissertation is that the tick response is specific to individual rickettsial species and specific tick-derived molecules control rickettsial infection and rickettsial survival in ticks. The specific aims of this dissertation were to: (1) molecularly characterize and examine gene expression of *D. variabilis* α–catenin during *R. montanensis* (typical *Rickettsia*) and *R. amblyommii* (atypical *Rickettsia*) infection; (2) determine rickettsial dissemination and specific tick immune response during *R. montanensis* and *R. amblyommii* infection; (3) functionally characterize identified tick immune molecules and examine their effects on rickettsial infection.

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CHAPTER 2
MOLECULAR CHARACTERIZATION AND TISSUE-SPECIFIC GENE EXPRESSION OF *DERMACENTOR VARIABILIS* α-CATENIN IN RESPONSE TO RICKETTSIAL INFECTION

2.1 Introduction

Ticks are known as arthropod vectors for many pathogenic and nonpathogenic organisms of the genera *Anaplasma*, *Babesia*, *Borrelia*, *Ehrlichia* and *Rickettsia* (Sonenshine 1993). In the United States, ticks are responsible for the transmission of more vector-borne diseases than any other group of arthropods (Dumler 2010; Spach et al. 1993), and recently human case reports of tick-borne rickettsioses, such as Rocky Mountain spotted fever (RMSF), human monocytic ehrlichiosis, and human granulocytic anaplasmosis (HGA) have increased extensively (Dumler 2010). Despite sympatric distribution of multiple tick and *Rickettsia* species, there appear to be established relationships between tick and *Rickettsia* that influence the paring of species. The American dog tick, *Dermacentor variabilis,* is a common vector for spotted fever group (SFG) *Rickettsia*, including *Rickettsia rickettsii*, the causative agent of RMSF and *Rickettsia montanensis*, a *Rickettsia* considered nonpathogenic to humans (Mcdade and Newhouse 1986). Despite the assessment infection of these ticks with other species of *Rickettsia* (Williamson et al. 2010), the most commonly encountered is *R. montanensis*. This is likely due to tick-borne SFG *Rickettsia* and their tick hosts which often exist in a benign relationship, in which the tick serves as both the vector and the reservoir (Mcdade and Newhouse 1986). The SFG *Rickettsia* are maintained vertically in ticks via transstadial and transovarial transmission; however, the molecular interactions between tick and SFG *Rickettsia* are not well-defined.

A previous study utilizing differential-display PCR to determine SFG *Rickettsia* infection-induced regulation of tick molecules in tick ovaries identified nine tick-derived molecules which were differentially expressed when the ticks were infected with SFG *Rickettsia*

(Macaluso et al. 2003). In addition to immune and stress-response molecules, a putative cytoskeleton associated molecule, α-catenin, was identified. In humans, α-catenin is able to bind various cytoskeleton proteins and regulates actin dynamics in the cells (Gates and Peifer 2005; Drees et al. 2005). Alpha-catenin has two forms: monomeric α-catenin which binds β-catenin thereby forming an E-cadherin-dependent cell-cell adhesion complex and links the complex to actin filaments, and homodimeric α-catenin which binds actin filaments and inhibits the formation of Arp2/3 and actin filament complex (Hartsock and Nelson 2008). During bacterial infection, many species of bacteria utilize host α-catenin to mediate actin rearrangement in infected cells. For example, enterohemorrhagic and enteropathogenic *Escherichia coli* secrete a bacterial effector protein (EspB) into host cells that binds to many host-derived proteins including α-catenin. EspB promotes α-catenin dimerization by competing with Arp2/3 complex (Hamaguchi et al. 2008; Kodama et al. 2002). Likewise, during internalization of *Listeria monocytogenes* into epithelial cells, *Listeria* Internalin A binds to E-cadherin-β-catenin complex on the host cell membrane, which is linked via α-catenin to actin filaments and recruits the cytoskeleton protein to the entry site (Sousa et al. 2005). It has been demonstrated that in some species of SFG *Rickettsia*, actin polymerization is also required for bacterial invasion and motility during infection (Martinez and Cossart 2004; Serio et al. 2010). *R. conorii* binds to host Ku70 and mediates actin polymerization via the Arp2/3 complex during internalization (Martinez and Cossart 2004; Martinez et al. 2005). Additionally, recent studies have identified a core set of actin cytoskeletal proteins associated with motility of *R. parkeri* in *Drosophila* cells (Serio et al. 2010). In *Ixodes scapularis* cells (ISE6), *R. felis*, the flea-borne rickettsiosis agent, associates with tick cell surface via the binding of rickettsial outer-membrane protein B and tick histone H2B. Depletion of histone H2B by RNAi and enzymatic treatment decreased rickettsial

infection in the tick cells, suggesting a role of histone H2B in *R. felis* internalization into tick cells (Thepparit et al. 2010). Nevertheless, the invasion mechanism of SFG *Rickettsia* in ticks has not been identified.

The objectives of this study were to identify and characterize the α-catenin (Dvα-catenin) gene from *D. variabilis* and examine its association with rickettsial infection. We hypothesize that differential regulation of tick α-catenin during rickettsial infection is tissue-specific. Functional bioassays were used to test the hypothesis. According to the specific *Rickettsia*/tick relationship, tick tissues (backless tick) were used for typical (*Dermacentor*-associated *Rickettsia; R. montanensis*) or atypical (*Dermacentor* non-associated *Rickettsia; R. amblyommii*) rickettsial infection in order to determine the specific regulation of Dvα-catenin in response to two different rickettsial species. Understanding the molecular mechanism of rickettsial infection in tick ovaries will provide insight into the successful transovarial transmission of *Rickettsia* in tick vectors.

2.2 Materials and Methods

2.2.1. Tick Dissection

D. variabilis colonies were routinely maintained on rats and rabbits at Old Dominion University, as described previously (Macaluso et al. 2001). Unmated female ticks partially fed for 3-5 days were forcibly detached from host animals, washed twice in 70% ethanol, and rinsed with distilled water. Selected tick tissues (salivary glands, midguts, and ovaries) were dissected out of the ticks, washed in sterile diethyl pyrocarbonate (DEPC) treated water or fresh phosphate buffer saline (PBS, pH 7.4) and placed in either RNA*later* (Ambion) for RNA extraction or in protease inhibitor cocktail (PIC), (Roche) for protein preparation. Tissues were immediately processed for nucleic acid or protein extraction or stored at -80 °C until used for extraction.

2.2.2. Nucleic Acid Extraction from Tick Tissues and Cloning of Dvα-catenin cDNA

As previously described (Mulenga et al. 2004), ovaries from at least five *D. variabilis* were pooled, total RNA and subsequently mRNA were extracted using the NucleoSpin RNAII and NucleoTrap mRNA Mini kit (Clontech) according to the manufacturer's protocol. All RNA was stored at -80 °C until used.

Cloning of full-length cDNA for Dvα-catenin was carried out using rapid amplification of cDNA ends (RACE) as described by Mulenga et al. (2004). Briefly, 1 µg of mRNA extracted from ovaries was used to generate templates for 3' and 5' RACE using the SMART RACE cDNA synthesis kit (Clontech) according to the manufacturer's protocol. Following DNA sequencing of the Dvα-catenin gene fragment obtained by differential display PCR (Macaluso et al., 2003), gene specific (GSP) sense and antisense primers were designed to amplify the 3' and 5' ends, respectively. PCR products were routinely cloned into TOPO TA cloning vectors (Invitrogen). The clones were sequenced by the dye terminator method on a 373 automated fluorescence sequencing system (Applied Biosystems) in the biopolymer laboratory at the University of Maryland, Baltimore. MacVector software program (Accelrys) was used for DNA sequence analysis. Similarity, comparisons to known proteins in the database were made by scanning DNA sequences against the GenBank database using tblastx.

2.2.3. Construction of Dvα-catenin Expression Plasmid.

In order to produce Dvα-catenin using the Baculovirus Expression System (Invitrogen), cDNA encoding Dvα-catenin was cloned into the pENTR/D-TOPO entry vector (Invitrogen) and then transferred to the pDEST10 vector (N-terminal His fusion vector, Invitrogen). The clone containing full-length Dvα-catenin was transformed into DH10Bac *E.coli* (Invitrogen), which contains the baculovirus shuttle vector (bacmid), to produce recombinant bacmid harboring

Dvα-catenin. The positive colonies (white colony) that contain recombinant bacmid DNA were selected and cultured in selective (50 µg/ml kanamycin, 7 µg/ml Gentamycin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml isopropyl-beta-D-thiogalactopyranosid) medium. The Dvα-catenin bacmid DNA was isolated and used to infect the *Spodoptera frugiperda* (Sf9) cell line (Invitrogen).

2.2.4. Expression and Purification of Recombinant Dvα-catenin (rDvα-catenin)

Sf9 cells were cultured in SF900 II serum-free medium (Invitrogen) supplemented with penicillin/streptomycin (50 U/ml and 50µg/ml, respectively, Invitrogen). One microgram of Dvα-catenin bacmid DNA was used to transfect 9 x 10^5 Sf9 cells (Invitrogen) using Cellfectin reagent (Invitrogen) according to the manufacturer's protocol. The transfected Sf9 cells were incubated at 27°C for 7 days and the culture medium containing the recombinant baculoviruses was collected as a primary viral stock (Passage 1). The viral titer was determined using end-point dilution as described by O'Reilly et al. (1994); the amplified virus was diluted 10-fold from 10^{-3} to 10^{-8}. Optimal multiplicity of infection (MOI) was determined by infecting at an MOI of 0.1, 0.5, 1, 2, 5, and 10 for 4 days, and in order to optimize the harvest time point, Sf9 cells were infected at MOI of 1 for 2, 3, 4, 5, and 6 days. The product was analyzed by SDS-PAGE and Western blotting with anti-His antibody to confirm expression of His-tagged protein.

A large scale production of rDvα-catenin for purification utilized Sf9 cells infected with rDvα-catenin baculovirus at a MOI of 1 for 4 days. The infected cells were then collected and washed with PBS buffer and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) supplemented with 1% (v/v) Nonidet P40 (NP-40). The mixture was sonicated on ice for 5 min twice and centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant containing the soluble 6xHis-α-catenin was incubated with 50% (v/v) Ni-NTA

agarose, which was previously equilibrated in lysis buffer, at 4°C with gentle rocking for 2 h. The mixture was then loaded on a 10 ml column under gravity flow. The column was washed twice with PBS buffer. 6xHis-α-catenin was eluted from the column 8 times with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8, 1X PIC). Eluted fractions were pooled and dialyzed against PBS overnight at 4°C.

2.2.5. Protein Identification

In order to confirm a peptide sequence, the purified rDvα-catenin protein (96 kDa) was identified as previously described by Sunyakumthorn et al. (2008). The protein band on the Coomassie-stained protein gel was excised using the Proteome Works Spot Cutter (Bio-Rad) and digested using a MassPrep Station (Waters/Micromass). The peptides were then extracted, and a Q-Tof (quadrupole time-of-flight) Micro (Waters/Micromass Corp) hybrid mass spectromer (MS) was used for analysis. ProteinLynx Global Server, version 2.0 (Waters/Micromass) was used for data acquisition and analysis. Database comparative analysis was performed using an online Mascot (Matrix Science) tandem MS (MS/MS) ion search against the NCBInr/Proteobacteria.

2.2.6. Production of Polyclonal Antibody

Polyclonal antibodies to rDvα-catenin were generated in BALB/c mice. Three mice were subcutaneously injected with 30 µg of purified rDvα-catenin protein mixed with an equal volume of TITERMAX GOLD adjuvant (Sigma). First (100 µg) and second booster injection (200 µg) of purified rDvα-catenin protein in TITERMAX GOLD adjuvant was given at two week intervals. An equal volume of PBS buffer was mixed with TITERMAX GOLD adjuvant and used to inject another mouse as a negative control. Sera were collected a week after the final booster.

2.2.7. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Salivary gland, midgut, and ovary were dissected from 40 unfed female *D. variabilis*. The tissues were homogenized using a sterile plastic pestle in 100 µl of PBS buffer containing 1% NP-40 and 2X PIC. The tissue extracts were sonicated for 10 min in a bath sonicator (Crest Ultrasonic) and centrifuged at 16,000 × *g* at 4°C for 10 min. Protein concentration was measured using the Bradford assay (Bio-Rad) according to manufacturer's protocol. One hundred micrograms of each tick tissue extract (salivary gland, midgut, and ovary) were subjected to SDS-PAGE using 4-12% Bis-Tris gradient gels (Invitrogen). Separated proteins then were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad), and membranes were blocked with 5 % (w/v) skim milk in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1 % (v/v) Tween 20, pH 7.5) for 1 h at room temperature. The membranes were then incubated with the mouse anti-α-catenin polyclonal antibody in a dilution of 1:400 for 2 h, followed by a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (KPL) at a dilution of 1:20,000 for 1 h. The protein was detected using a SuperSignal West Pico chemiluminescent substrate kit (Pierce).

2.2.8. Tissue-specific Expression and Blood Feeding

To determine the specific expression of α-catenin in tick tissues and response of α-catenin gene expression during blood feeding, unfed (3 ticks) and 5 day fed female ticks (3 ticks) were dissected. Tick tissues (salivary gland, midgut, and ovary) were collected and stored in RTL buffer (QIAGEN) for RNA extraction.

2.2.9. Tick Cells and Rickettsial Culture.

Dermacenter variabilis-derived (DVE1) and *Amblyomma americanum*-derived

(AAE12) cell line (Kurtti, 2005), provided by T. Kurtti (University of Minnesota), were maintained in L15B growth medium (Munderloh UG 1989) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 10% tryptose phosphate broth (Becton, Dickinson and Company) at pH 6.8 to 7.0 in a humidified 5% CO_2 incubator at 34°C.

Two rickettsial species, *R. montanensis* strain M5/6 and *R. amblyommii* strain Darkwater (provided by Dr. Christopher Paddock) were routinely maintained and propagated in an African green monkey kidney cell line (Vero E6) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS (Hyclone) in a humidified 5% CO_2 incubator at 34°C. For rickettsial infection, rickettsiae were semi-purified from *Rickettsia*-infected Vero E6 cells when more than 80% of cells are infected with rickettsiae. Briefly, *Rickettsia*-infected Vero E6 cells were detached from the tissue culture flask, transferred to an Erlenmeyer flask containing sterile 3-mm borosilicate glass beads (Sigma), and vortexed at high speed for 5 min. The cell lysate was filtered through a 2 micron pore size syringe filter (Millipore). The rickettsiae in the filtrate were collected by centrifugation at 16,000 × *g* at 4°C for 10 min. Rickettsial viability and enumeration were assessed as previously described by Sunyakumthorn et al. (2008).

2.2.10. RNA Isolation and Relative Quantitative RT-PCR (qRT-PCR) Assay.

Total RNA was isolated from tick tissues using the RNasey Mini kit (QIAGEN) according to the manufacturer's protocol. RNA was then treated with Dnase (Ambion) and purified using an RNA cleanup kit (Zymo Research). The synthesis of cDNA was carried out using 200 ng total RNA in 25 µl reaction volumes of an iScript reverse transcription kit (Bio-Rad).

The PCR reaction reagents were mixed in 96-well plates containing 5 µl of cDNA, 2X iTaq SYBR Green Supermix with ROX (Bio-Rad), 100 mM each forward and reverse primers in

a total volume of 35 µl per reaction. The following qRT-PCR primers were used: DvCat2555F (5'-CACCGATTGTTGTGTGGAAG-3'), DvCat2661R (5'-CTTTTTCTGTGAGCCCTTGC-3'), DvAct1424F (5'-CTTTGTTTTCCCGAGCAGAG-3'), and DvAct1572R (5'-CCAGGGCAGTAGAAGACGAG-3'). No RT reaction (water was added instead of Reverse transcriptase) was performed to confirm an absence of genomic DNA. Ten microlitres of each reaction mixture were transferred into 3 wells of a 384-well plate and reacted in an ABI 7900HT unit (Applied Biosystems) at Louisiana State University, School of Veterinary Medicine using condition system (SDS v2.3) software. Data for each sample was initially calculated as the percent difference in threshold cycle (C_T) value ($\Delta C_T = C_T$ *Actin* - C_T *α-catenin*)

2.2.11. Rickettsial Infection Bioassay in Backless *D. variabilis*

In order to determine tissue-specific responses of ticks during rickettsial infection, backless ticks were generated according to a modified protocol of Bell (1980) and used for rickettsial infection. In a laminar flow hood, thirty-six unfed female *D. variabilis* ticks were cleaned with 70% ethanol for 2 min, 10% benzalkonium chloride solution for 5 min, and rinsed with sterile water 3 times. The ticks were then air-dried on sterile filter paper. Mouthparts and legs were excised to minimize contamination, cleaned ticks were transversely cut along the perimeter of alloscutum with a scalpel, and the dorsal cuticle was taken off as shown in Figure 2.1. The backless ticks were placed individually in wells on a 96-well plate containing 200 µl of complete L15B medium and incubated at 34ºC. After 24 h, any contaminated ticks were removed from the experiment. The backless ticks were divided to three groups (12 ticks per group); the first group, unexposed, was incubated in L15B medium, the second and third group were exposed to *R. montanensis* or *Rickettsia amblyommii* (2.4×10^8 rickettsiae per tick per well), respectively. After 1 and 12 hours-post inoculation (hpi), the tick tissues were dissected

Figure 2.1. Tick tissue culture (Backless tick). Unfed female *D. variabilis* were cleaned, and the dorsal cuticle was removed. The backless ticks were placed in 96-well plates containing 200 µl complete L15B medium for 24 h prior to rickettsial exposure.

out and kept in 100 µl RNA*Later* at -20ºC. Total RNA was extracted using the RNasey Mini kit (QIAGEN) and digested with DNase (Ambion) according to the manufacturer's protocol. Total RNA (40 ng) was used for cDNA synthesis with an iScript reverse transcription kit (Bio-Rad). Two microlitres of cDNA template were subjected to qPCR assay as described above.

2.2.12. Statistical Analysis.

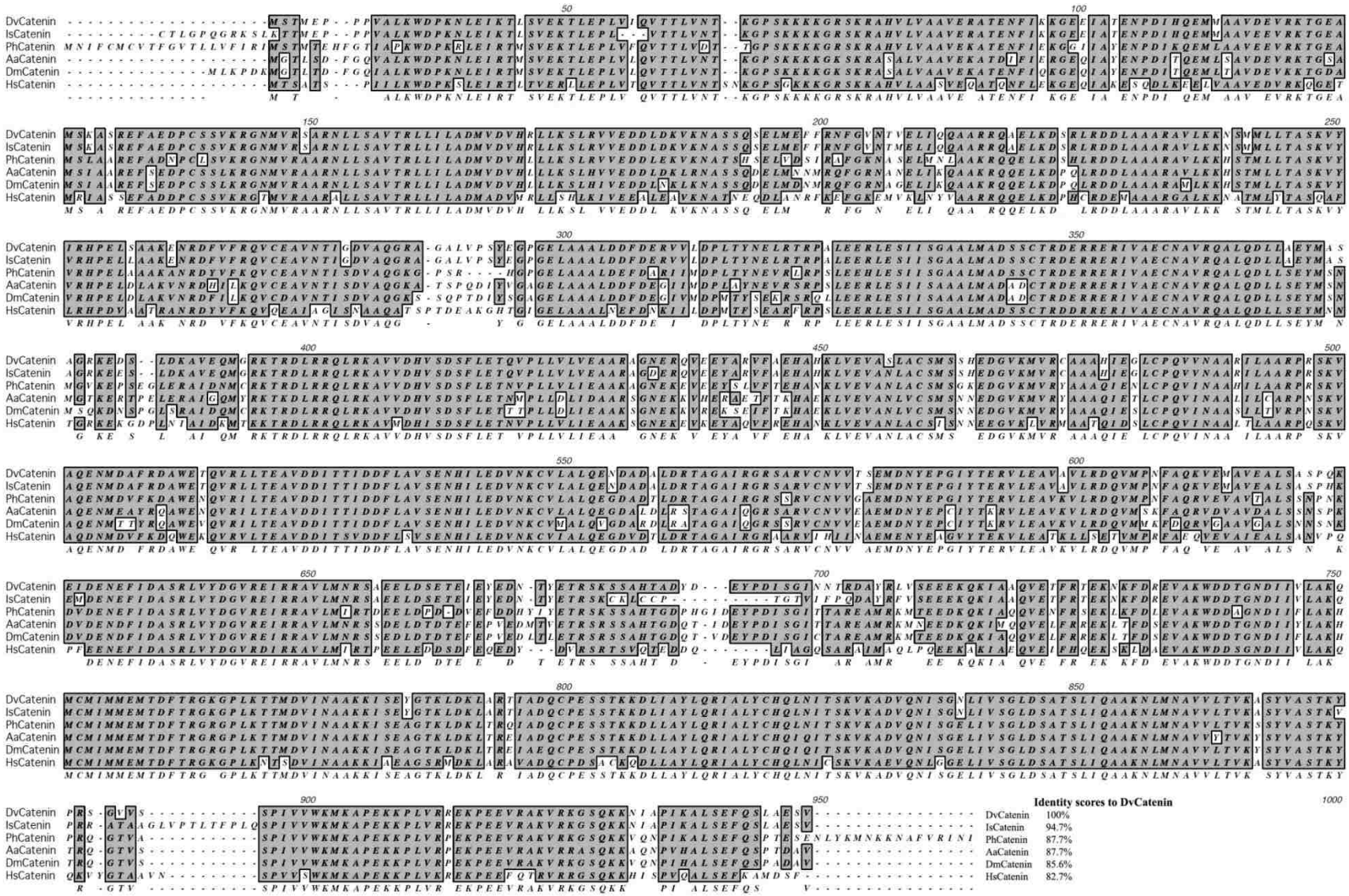
The Analysis of variance was analyzed using SAS statistical package GLM procedure ANOVA Version 9.1.3. The relative gene expression of α–catenin of unfed and 5 day fed tick tissues was examined for potential difference. For the backless tick bioassay, the relative gene expression was analyzed after rank transformation and performed two-way interaction (rickettsial infection and tick tissues) analysis. When overall significance was found, Tukey's honestly significant difference (HSD) post hoc test was performed to determine the pairwise difference of means of main effects. Pairwise *t*-tests of least squares means were performed to determine any interaction effects of relative expression of α-catenin between unfed and 5 day fed ticks, and unexposed and *Rickettsia*-exposed backless ticks. *P*-values of < 0.05 were considered significantly different.

2.3 Results

2.3.1. Full-length Dvα-catenin cDNA and Sequence Analysis

Gene specific primers designed from a Dvα-catenin gene fragment obtained by differential display PCR (Macaluso et al. 2003) were used to clone the full-length α-catenin cDNA. After sequence analysis (BlastX), the 3069 bp full-length cDNA was designated Dvα-catenin (Genbank accession number HM755938). A putative 2718 bp ORF, encodes an expected 905 amino acid protein with a calculated molecular weight of 96 kDa. The deduced amino acid sequence is shown in Figure 2.2. A multiple sequence alignment of Dvα-catenin

Figure 2.2. (Following page). Multiple sequence comparison of α-catenin amino acid sequences. The Dvα-catenin deduced amino acid sequence was aligned with *Ixodes scapularis* α-catenin (IsCatenin, accession No. XP002413819), *Pediculus humanus corporis* α-catenin (PcCatenin, accession No. XP002429770) *Aedes aegypti* α-catenin (AaCatenin, accession No. XP001657216), *Drosophila melanogaster* α-catenin (DmCatenin, accession No. NP524219), and *Homo sapiens* α-catenin (HsCatenin, accession No. NP004380). Alignment was performed using MacVector software. Shaded gray indicates conserved amino acid residues. The identity scores to Dvα-catenin were derived from pairwise alignment using ClustalW 1.83 software.


DvCatenin
IsCatenin
PhCatenin
AaCatenin
DmCatenin
HsCatenin
Identity scores to DvCatenin
DvCatenin 100%
IsCatenin 94.7%
PhCatenin 87.7%
AaCatenin 87.7%
DmCatenin 85.6%
HsCatenin 82.7%

amino acids showed the highest similarity to tick and insect α-catenin with 94.7% identity to *Ixodes* tick α-catenin (Genbank accession number XP002413819), 87.7% to body louse α-catenin (Genbank accession number XP002429770) and *Aedes* mosquito α-catenin (Genbank accession number XP001657216), and 85.6% to fruit fly α-catenin (Genbank accession number NP524219), compared to human α-catenin (82.7% similarity, Genbank accession number NP004380) (Figure 2.2).

Conserved domains were identified using NCBI Conserved Domain Search Service (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd). The Dvα-catenin amino acid sequence is homologous to a vinculin conserved domain at amino acid positions 19-865, and contains a putative F-actin binding region (697-905) at the C-terminus as well as a β-catenin binding (57-146) and α-catenin dimerization regions (82-264) at the N-terminus (Figure 2.3) (Pokutta et al. 2008).

2.3.2. Expression of rDvα-catenin in a Baculovirus System.

The ORF of Dvα-catenin was subcloned into pET (Novagen) and transferred to Bacmid. The rDvα-catenin was expressed as a soluble form and purified using affinity chromatography (Ni-NTA column). The analysis of purified rDvα-catenin using SDS-PAGE and Coomassie staining showed a protein band on the gel with a mass of approximately 96 kDa. The band was excised and quadruple time-of-flight micro MS was performed.

The data was matched to α-catenin protein of *Ixodes* ticks. The yield of purified rDvα-catenin after dialysis was 1.25 mg/L. The purified rDvα-catenin was used for polyclonal antibody production in mice.

2.3.3. mRNA Expression of Dvα-catenin in Tick Tissues and Response to Feeding.

To determine the mRNA expression profile of Dvα-catenin in different tick tissues and its

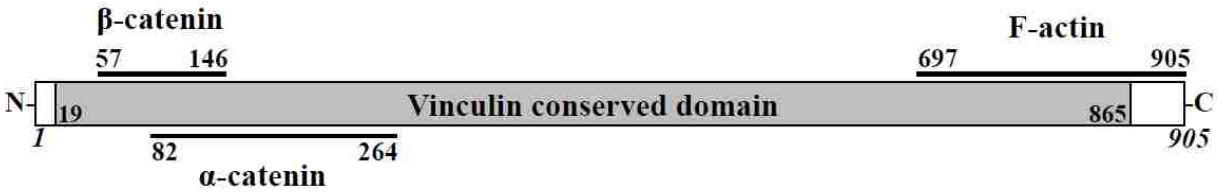


Figure 2.3. Putative protein binding sites of Dvα-catenin. Numbers correspond to amino acids of the protein sequence. Shaded gray region is vinculin conserved domain. Amino acid position 57-146 is β-catenin binding site. Amino acid position 82-264 is α-catenin binding site. Amino acid position 697-905 is F-actin binding site.

responses to blood feeding, total RNA samples from different tick tissues (salivary glands, midguts, and ovaries) of unfed and 5 day fed ticks were subjected to qRT-PCR assay. Results showed that Dvα-catenin transcripts were significantly predominant in tick ovaries compared to salivary glands and midguts (Figure 2.4). After 5 days of feeding, expression of Dvα-catenin was still highest in ovaries. Although, overall Dvα-catenin expression was downregulated compared to unfed ticks, downregulation of Dvα-catenin expression in salivary glands and midguts individually was not significant. Results suggested that Dvα-catenin may not be responsive to tick feeding. The decrease of gene expression may be due to the fact that during tick feeding, ticks need to produce more enzymes and proteins related to blood digestion.

2.3.4. Detection of Dvα-catenin in Tick Tissues and Tick Cell Lines Using Mouse Anti-α-catenin Polyclonal Antibody

In order to examine Dvα-catenin protein expression, polyclonal antibodies generated against rDvα-catenin were used to detect α-catenin protein in different tick tissues and tick cell lines. Protein (100 µg) from salivary glands, midguts, and ovaries extracted from 5 day fed ticks were separated and transferred to PVDF membranes. The western blotting results corresponded with the mRNA expression analysis; α-catenin was predominantly expressed in tick ovaries. The Dvα-catenin polyclonal antibody reacted strongly with a ~96 kDa protein band from tick ovaries (Figure 2.5A). There was no band from either salivary gland or midgut samples. As shown in Figure 2.5B, α-catenin protein was detected in unfed and 5 day fed *D. variabilis* ovaries and DVE1 cell line but not in AAE12 cell line. However, there is another band at 112 kDa. It is possible that there are two isoforms of Dvα-catenin in embryonic cells.

2.3.5. Tissue-specific Gene Expression of Dvα-catenin in Response to Rickettsial Infection.

Previous examination suggested that Dvα-catenin expression is tissue-specific in

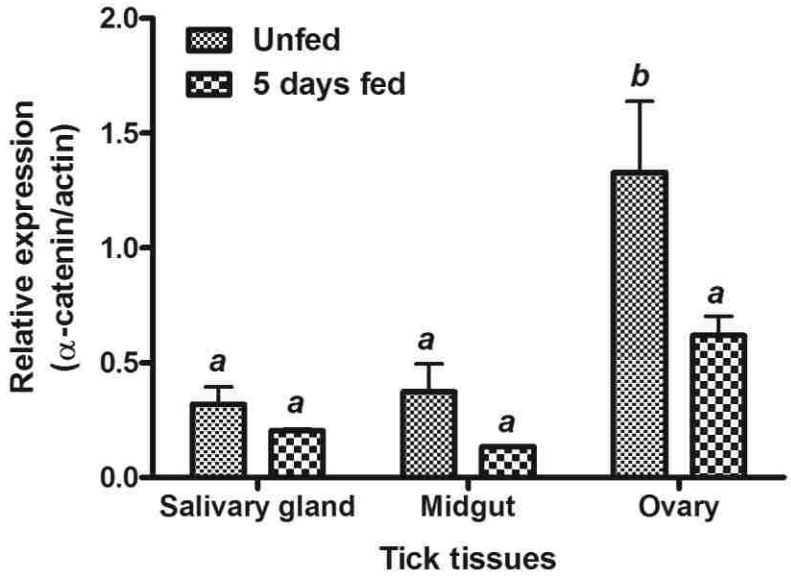


Figure 2.4. Tissue-specific expression of Dvα-catenin mRNA expression in unfed and 5 days fed *D. variabilis*. Total RNA was extracted from tick tissues (salivary glands, midguts, and ovaries) and performed qRT-PCR assay. Transcription level of Dvα-catenin was normalized to tick *actin*. Data shown are mean relative expression. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$).

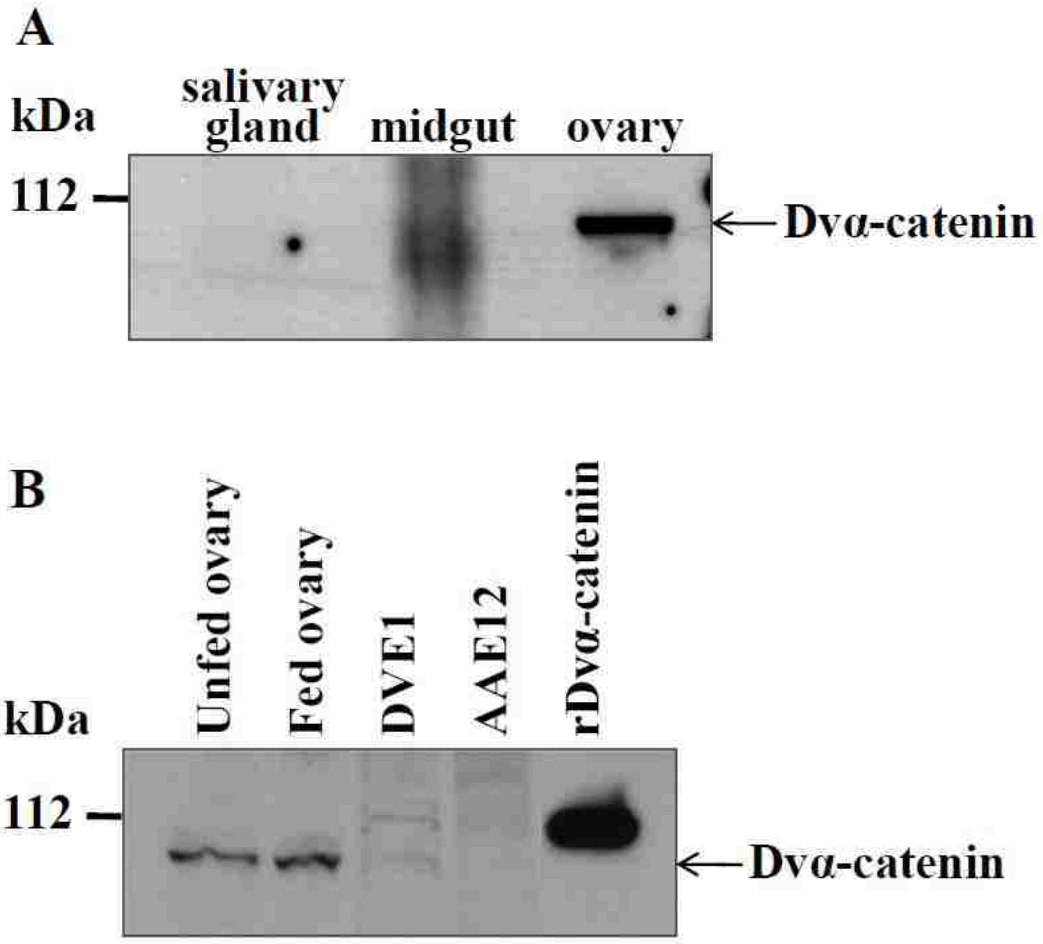


Figure 2.5. Dvα-catenin protein in tick tissues and tick cell lines. One hundred microgram protein of tick tissues (salivary glands, midguts, and ovaries), DVE1 (*D. variabilis* cell line), AAE12 (*A. americanum* cell line), and 0.2 µg protein of rDvα-catenin was performed western blot analysis using mouse anti-α-catenin polyclonal antibody. (A) Dvα-catenin protein expression in 5 day fed tick tissues. Dvα-catenin protein was highly expressed in tick ovaries compared to salivary glands and midguts. (B) Dvα-catenin protein was detected in unfed and 5 day fed tick ovaries, DVE1 and AAE12 cells, and rDvα-catenin.

Rickettsia-infected ticks (Macaluso et al. 2003). In order to determine whether the different gene expression of Dvα-catenin in response to rickettsial infection is specific to tick tissues and rickettsial species, an *ex vivo* study of tick tissues (backless tick) was performed. In order to expose tick tissues to rickettsiae the tick dorsal integument was cut and removed, and backless ticks were exposed to either *R. montanensis* or *R. amblyommii*. After 1 and 12 hpi, total RNA of salivary glands, midguts, and ovaries from unexposed and *Rickettsia*-exposed ticks were subjected to qRT-PCR assay. Results showed a significant decrease of Dvα-catenin gene expression in *R. montanensis*-exposed tick ovaries after 12 h and no significant differences in *R. amblyommii*-exposed tick ovaries in comparison to uninfected ticks (Figure 2.6). Although we were able to detect the Dvα-catenin mRNA in salivary glands and midguts, a significant difference post infection and between species was not observed.

2.4 Discussion

Previous studies utilized differential-display PCR to identify a partial cDNA with similarity to α-catenin in partially fed *Rickettsia*-infected ticks. The present study describes α-catenin from the American dog tick, *D. variabilis*. Multiple alignments demonstrate that α-catenin is conserved among species of ticks and other arthropods, as well as in humans; however, its full characterization in hematophagous arthropods had not been examined prior to this study. Consistent with other organisms, the characteristics of the deduced amino acid sequence shows homology with vinculin protein, containing putative α-catenin dimerization, β-catenin, and actin-binding domains.

Female ixodid adult ticks are known to feed for extended periods of time (1-2 weeks), and dynamic changes in tick gene activity is associated with tick feeding (Aljamali et al. 2009; Chalaire et al. 2011; Mulenga and Khumthong 2010a, 2010b). Most tick genes that are

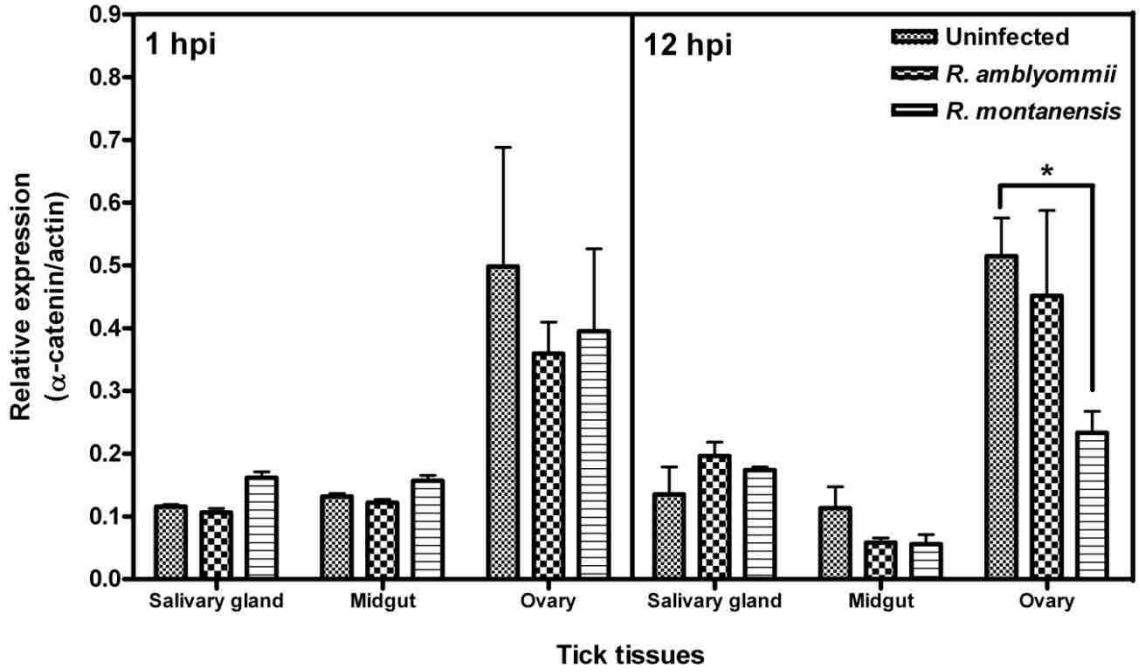


Figure 2.6. Dvα-catenin mRNA expression in backless ticks during *R. montanensis* and *R. amblyommii* infection. Unfed *D. variabilis* female ticks were transversely cut along the perimeter of alloscutum, and the dorsal cuticle was taken off. Then, backless ticks were exposed to *R. montanensis* and *R. amblyommii* and incubated at 34°C. After 1 and 12 hpi, tick tissues were dissected and preformed RNA extraction. Total RNA were subjected to qRT-PCR assay. Transcription level of Dvα-catenin was normalized to tick *actin*. Data shown are mean relative expression from two experiments. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$).

responsive to blood feeding are related to manipulation of blood flow and host immune responses, and are upregulated in salivary glands during tick feeding (Aljamali et al. 2009). Dvα-catenin is constitutively expressed in tick ovaries, and its expression is downregulated during the slow phase of feeding before mating (5 dpf) suggesting that Dvα-catenin is not responsive to tick feeding. The expression of unnecessary genes is decreased in order to conserve resources for other responsive genes during tick feeding. Dvα-catenin is one of nine tick-derived molecules which were differentially expressed in *R. montanensis*-infected tick ovaries. Its expression was higher in *R. montanensis*-infected tick ovaries compared to uninfected ticks. The function of Dvα-catenin in *Rickettsia*-infected ovaries is unknown. It was suggested that SFG *Rickettsia* uses α-catenin to modulate actin rearrangement in order to invade neighboring host cells, and the upregulation of invasion genes in *Rickettisia*-infected tick tissues during feeding may be responsive to the reactivation of rickettsiae (Hayes and Burgdorfer 1982) in ovaries (Macaluso et al. 2003). During tick feeding after attachment and ingestion of host blood, oocytes begin to further develop (Sonenshine 1993) in tick ovaries. It is possible that *Rickettsia* invades oocytes or other cells during this period which results in an increase in Dvα-catenin gene expression.

The use of host molecules by SFG *Rickettsia* is not unprecedented. In vertebrate host cells, many species of bacteria are able to modulate rearrangement of actin cytoskeleton in order to invade host cells *e.g. Listeria*, *Shigella, Rickettsia*, and recently *Burkholderia* and *Mycobacterium* (Dramsi and Cossart 1998; Gouin et al. 2004; Hamaguchi et al. 2008; Sousa et al. 2005). However, different bacteria use different strategies. For *Rickettsia,* multiple species utilize actin-based motility in order to invade neighboring cells (Gouin et al. 2004; Heinzen et al. 1999; Heinzen 2003). Spotted fever group rickettsiae including *R. conorii* and *R. rickettsii* have

demonstrated the formation of actin structures, also called actin tails, in host cell cytosol. These actin tails facilitate bacterial movement inside the cell and invasion of other nearby cells. This study demonstrated that infection of *R. montanensis* which is a typical *Rickettsia* for *D. variabilis* downregulated Dvα-catenin gene expression in ovaries at 12 hpi but not 1 hpi suggesting a role of Dvα-catenin in typical *Rickettsia* infection in tick ovaries. Interestingly, the decrease of Dvα-catenin gene expression only occurred when ticks were exposed to *R. montanensis.* Presumably, ticks control the level of rickettsial infection in ovaries by downregulation of the Dvα-catenin gene preventing cell invasion. Moreover, controlling rickettsial infection in tick ovaries may be responsible for the infection blocking mechanism or an interference phenomenon of *R. montanensis* in ovaries. Macaluso et al. (2003) has demonstrated that *R. montanensis*-infected *D. variabilis* were resistant to transovarial transmission of *R. rhipicephali* infection, and only *R. montanensis* demonstrated successful transovarial transmission to offspring. However, the mechanism of Dvα-catenin during rickettsial infection in tick ovaries was not identified in that study.

Transmission of SFG *Rickettsia* among ticks is complex as the tick serves as the vector and reservoir. However, not all rickettsial species are horizontally transmitted by ticks and vertical transmission occurs with specificity as demonstrated transovarial transmission is limited to few parings. Combined, field and laboratory studies suggest that the biological association between ticks and rickettsial species is specific. To further examine these relationships, unfed ticks were used for backless tick experiments. *Rickettsia*-uninfected ticks were exposed to *R. montanensis* and *R. amblyommii*. *R. amblyommii* is predominantly found in *Amblyomma* ticks. It was used as a non *Dermacentor*-associated *Rickettsia* (atypical *Rickettsia*) in order to examine the specific response to different rickettsial species. The alteration of Dvα-catenin gene

expression only during *R. montanensis* infection suggested that the tick response was specific to certain rickettsial species.

Due to different tissues being involved in vertical and horizontal transmission, it was known that the close relationship between tick and *Rickettsia* is tissue-specific. In order to study the tick tissue-specific response during rickettsial infection, we used a modified tick tissue culture of backless ticks (Bell 1980) and a primary tick tissue culture (Mosqueda et al. 2008) for rickettsial infection. Both techniques supported the idea of tissue-specific analysis during rickettsial infection; however, during preliminary experiments the backless tick technique provided better results. Therefore, the backless tick technique was modified and used for rickettsial infection. The technique was developed by Bell (1980) to study the development of *Theileria parva* in *Rhipicepphalus appendiculatus* salivary glands. Our backless tick experiment demonstrated that it can be used as a model for tick tissue culture.

Arthropods and microbes are known to have intimate relationships. Mutual relationships are found between arthropods and their endosymbionts, for example, insects and *Wolbachia* (Werren et al. 2008). Recently, Neelakanta et al. (2010) demonstrated the beneficial effect of the agent of HGA, *Anaplasma phagocytophilum*, in *Ixodes scapularis*. Infection with *A. phagocytophilum* upregulated *I. scapularis* antifreeze glycoprotein (IAFGP) gene expression which is important for tick survival in the cold environment. In the Northeast and Upper Midwest of United States, *I. scapularis* overwinters as adults; therefore, more *A. phagocytophilum*-infected *I. scapularis* survive overwintering compared to uninfected *I. scapularis*. This may enhance coexistence of both *A. phagocytophilum* and *I. scapularis* in nature which directly affects the epidemiology of HGA and other *I. scapularis*-borne diseases. Studying specific interaction between tick vectors and their microbes will lead to a

better understanding of ecology and epidemiology of tick-borne diseases in nature.

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CHAPTER 3
RICKETTSIAL DISSEMINATION AND SPECIFIC TICK IMMUNE RESPONSE DURING TYPICAL AND ATYPICAL RICKETTSIAL INFECTION

3.1 Introduction

Tick-borne rickettsioses are caused by members of spotted fever group (SFG) *Rickettsia,* which are obligate intracellular bacteria belonging to the genus *Rickettsia* (Rickettsiales: *Rickettsiaceae*). SFG *Rickettsia* can infect both vertebrate and invertebrate hosts, and ticks serve as both the disease vector and a reservoir host. Ticks are able to transmit and maintain the bacteria by horizontal and vertical transmission. In the United States, despite the sympatric population of tick species, the prevalence of *Rickettsia* is limited to certain species of ticks.

Rickettsia rickettsii is the etiologic agent of Rocky Mountain spotted fever (RMSF) which is the most common tick-borne rickettsiosis in the United States. *R. rickettsii* is predominantly found in *Dermacentor variabilis* (the American dog tick) in the midwestern and eastern United States and *D. andersoni* (the Rocky Mountain wood tick) in the western United States (Mcdade and Newhouse 1986). However, *Rhipicephalus sanguineous* has recently been described as a competent vector of *R. rickettsii* in Arizona (Demma et al. 2005). Recently, Center for the Disease Control and Prevention reported a continuous increase in human cases of tick-borne rickettsial diseases since 1998, and in 2008 there were 2,500 cases of RMSF (Dumler 2010). However, there is no evidence of an increase in *R. rickettsii* prevalence in the tick vector even in endemic areas (Ammerman et al. 2004; Stromdahl et al. 2010). Therefore, further study of the ecology and epidemiology of tick-borne rickettsioses is needed.

Burgdorfer et al. (1981) demonstrated that *D. andersoni* population on the east side of the Bitterroot valley harbored of *Rickettsia peacockii*, a nonpathogenic spotted fever group *Rickettsia*, and it presence affected the prevalence of vertically maintained *R. rickettsii*. This

suggested that transovarial interference by nonpathogenic *Rickettsia* (Burgdorfer et al. 1981) may play a crucial role in RMSF epidemiology in Montana. However, a study by Macaluso et al. (2002) demonstrated that the inhibition of transovarial transmission by a second rickettsial species is not specifically associated with rickettsial pathogenicity and suggested that tick/*Rickettsia* pairing is important for successful transmission. The presence of *R. montanensis,* a nonpathogenic typical *Rickettsia* in *D. variabilis,* also affects the infectivity of other nonpathogenic *Rickettsia* within individual ticks. Likewise, an atypical *Rickettsia* (*R. rhipicephali*) was not able to be maintained in *D. variabilis* through multiple generations (Macaluso et al. 2002). This is believed to be related to the close association between tick and rickettsial species.

Recently, the molecular interaction between ticks and SFG *Rickettsia* has focused on the rickettsial adhesion/internalization (Macaluso et al. 2003) and the tick immune/stress response (Ceraul et al. 2007, 2008; Mulenga et al. 2003) during rickettsial infection. Many tick immune-like molecules which are related to rickettsial survival have been identified (Ceraul et al. 2007, 2008, 2011). In *D. variabilis,* it has been shown that the expression of multiple tick antimicrobial genes was upregulated during rickettsial challenge, for example, defensin1 (varisin) (Ceraul et al. 2007), lysozyme (Ceraul et al. 2007), glutathione S-transferase1 (GST1) (Dreher-Lesnick et al. 2006; Mulenga et al. 2003), and Kunitz protease inhibitor (KPI) genes (Ceraul et al. 2008). Likewise, in response to rickettsial infection, tick-derived molecules were differentially expressed in a tissue-specific manner (Ceraul et al. 2007, 2008; Macaluso et al. 2003; Mulenga et al. 2003). Most studies have focused on the tick response to infection with typical *Rickettsia;* (Ceraul et al. 2007, 2008, 2011; Macaluso et al. 2003; Mulenga et al. 2003) therefore, little is known about how ticks respond to rickettsial infection with an atypical

Rickettsia. The nature of tick-specific reaction to infecting rickettsial species may have broad implication relating to the ecology of tick-borne rickettsial diseases.

To better understand the mechanistic nature of tick/*Rickettsia* relationships, the objectives of this study were to 1) monitor the dissemination and survival of *R. amblyommii*, an atypical *Rickettsia*, *R. montanensis*, a nonpathogenic typical *Rickettsia*, and *R. rickettsii*, a pathogenic typical *Rickettsia* in *D. variabilis*; and 2) assess specific tick immune response during typical and atypical rickettsial infection. The hypothesis being tested is that *R. amblyommii*, which is a nonpathogenic *Amblyomma*-related *Rickettsia*, is not able to survive and disseminate from the midgut to infect other tissues, and specific tick immune molecules control rickettsial dissemination and survival of the atypical *Rickettsia*. Studying the specific tick/*Rickettsia* relationship of typical and atypical *Rickettsia* will lead to better understanding of the ecology and epidemiology of tick-borne diseases in the United States.

3.2 Materials and Methods

3.2.1. LSU *Dermacentor variabilis* Colony

Uninfected *D. variabilis* (Say) were provided by Dr. Daniel Sonenshine, Old Dominion University. Ticks were maintained in an environmental chamber at 27±1°C, 87±2% relative humidity (RH), and a 16: 8 (light: dark) cycle. The tick life cycle is routinely maintained using mice for larval feeding, rats for nymphal feeding, and guinea pigs for adult feeding at the School of Veterinary Medicine, Louisiana State University (SVM-LSU). All animals were handled according to Louisiana State University's Institutional Animal Care and Use Committee regulations.

For bioassays, female ticks were allowed to feed on Hartley guinea pigs (*Cavia porcellus*), (Charles River) for 5 days. Partially fed female ticks were forcibly detached from

guinea pigs, washed with 1% bleach for 5 min and 70% ethanol for 5 min and rinsed with distilled H_2O and phosphate-buffer saline (PBS).

3.2.2. Rickettsial Culture and Purification

R. montanensis (M5/6; *Microtus* isolate), *R. amblyommii* (Darkwater), *R. rickettsii* (VR149), and *R. parkeri* (portsmouth) were maintained and propagated in Vero E6 cells. *R. amblyommii* and *R. parkeri* were kindly provided by Dr. Christopher Paddock. The *Rickettsia*-infected Vero E6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone) and maintained in a humidified 5% CO_2 incubator at 34°C. *R. rickettsii* was maintained and manipulated in the BSL-3 laboratory in the Department of Pathobiological Sciences, SVM-LSU. *R. felis* (LSU) was maintained and propagated in *Ixodes scapularis*-derived ISE6 cells. *R. felis*-infected cells were cultured in L15B growth medium supplemented with 10% heat-inactivated FBS (Hyclone) and maintained in a humidified 5% CO_2 incubator at 32°C as described by Pornwiroon et al (2006).

Rickettsiae were semi-purified from infected Vero E6 cells when more than 80% of cells were infected. The infected Vero E6 cells were detached and transferred to an Erlenmeyer flask containing sterile 3-mm borosilicate glass beads (Sigma), and vortexed at high speed for 3 min. The cell lysate was filtered through a 2 micron pore size syringe filter (Millipore). Viability and enumeration of *R. montanensis* and *R. amblyommii* were assessed by staining with a *Bac*Light viability stain kit (Invitrogen), and rickettsiae were counted in a Petroff-Hausser bacteria counting chamber (Sunyakumthorn et al. 2008) using a Leica microscope. For *R. rickettsii* quantification, a milliliter of rickettsial solution was aliquoted for DNA extraction and qPCR assay using *R. rickettsii*-specific probe and primers (Smith et al. 2010). Rickettsiae were collected by centrifugation at 16,000 × *g* at 4 °C for 10 min. The rickettsial pellets were

resuspended in 1:125 diluted bovine blood which was heat-inactivated at 56 °C for 30 min.

3.2.3. Tick Immune Gene Expression in Response to Blood Feeding

To determine the response of *D. variabilis* immune genes during blood feeding, groups of 3 unfed and partially fed (5 day) female *Rickettsia*-free ticks were dissected. Tick tissues (hemolymph, salivary gland, midgut, and ovary) were collected and stored in RLT buffer (QIAGEN) for RNA extraction. Total RNA was isolated using the RNeasy kit (QIAGEN) according to the manufacturer's protocol. Then, qRT-PCR assay (see below) was performed on the extracted RNA.

3.2.4. Tick Feeding Using Capillary Feeding Techniques (CFT)

In order to assess the uptake of solution after capillary feeding, Rhodamine B was used as a feeding biomarker. Ten female ticks were restrained ventrally on double-sided adhesive foam in a large glass Petri dish. Ticks were capillary fed with 0.1% (W/V) Rhodamine B in 0.85% NaCl using 50-µl glass microcapillary tubes (KIMBLE). The feeding tubes were fit over the mouthparts and immobilized ticks restrained on modeling clay as previously described (Macaluso et al. 2001, 2002). Capillary feeding plates were incubated in an environmental chamber at 27±1°C with 87±2% RH. After 16 h, the capillary fed ticks were gently removed from the adhesive foam and rinsed with water three times and 70% ethanol three times to remove Rhodamine B on the tick surface. The cleaned ticks were visualized under a fluorescent microscope (MVX10 Research macro zoom system microscopy, OLYMPUS) in order to evaluate the uptake of feeding media.

3.2.5. Rickettsial Challenge-feeding

Five groups of partially fed female ticks (n = 3/time point) were allowed to imbibe 1:125 diluted bovine blood (Group 1), *R. amblyommii* (Group 2; 10^9 rickettsiae/ml), *R. montanensis*

(Group 3; 10^9 rickettsiae/ml), *R. rickettsii* (Group 4; 10^9 rickettsiae/ml) and combined *R. amblyommii* and *R. montanensis* (Group 5; 10^9 rickettsiae/ml) through a 50-µl microcapillary tube (KIMBLE) as described above. After 16 h of capillary feeding, three ticks from each group were sterilized by 1% bleach and 70% ethanol and rinsed with distilled H_2O and PBS. Tick legs were cut to collect the hemolymph, and ticks were then dissected to remove the salivary glands, midguts, and ovaries using standard microdissection techniques (Macaluso et al. 2003). Tick salivary glands, midguts, and ovaries were rinsed three times in PBS to remove hemocytes. Tissues from three ticks were pooled into the same tube and homogenized in 600 µl of RTL plus buffer (QIAGEN) by passage through a 27½ gauge needle ten times. The lysate was stored at -80°C until used for nucleic acid isolation. The remaining ticks were incubated at 27±1°C with 87±2% RH and their tissues were collected at 40, 88 and 184 hours post-challenge (hpc). Two independent experiments were performed.

3.2.6. Nucleic Acid Isolation

Total RNA and gDNA were isolated from tick tissues lysate using ALLPrep DNA/RNA Mini kit (QIAGEN) according to the manufacturer's protocol. Briefly, the homogenized lysate was passed through a DNA spin column, and the columns were stored at room temperature for later DNA purification. The flow-through which contained total RNA was mixed with 70% ethanol and transferred to RNA spin columns. After wash steps total RNA was eluted in 50 µl Nuclease-free water and stored at -80°C. The DNA spin columns were then washed with Buffer AW1 and AW2 and eluted with 50 µl Nuclease-free water.

3.2.7. Specificity Determination of Rickettsial Species-specific qPCR Assay

To evaluate the specificity of the *R. amblyommii, R. montanensis*, and *R. rickettsii* qPCR assays, gDNA from five rickettsial species including *R. amblyommii, R. montanensis*, *R.*

rickettsii, *R. parkeri* and *R. felis* were used as templates. gDNA was isolated from *R. amblyommii*, *R. montanensis*, *R. rickettsii*, *R. parkeri* and *R. felis*-infected cells using DNeasy kit (QIAGEN) according to the manufacturer's protocol. gDNA from the five rickettsial species were subjected to a qPCR assay using rickettsial species-specific molecular beacon probes and primers designed from rickettsial outer membrane protein (ompB): *R. montanensis*-HEX labeled probe (Smith et al. 2010) and *R. amblyommii*-FAM labeled probe (Jiang et al. 2009) and *R. rickettsii*-TYE665 labeled probe (Smith et al. 2010). The conventional PCR amplification of 17-kDa genus-specific antigen using *Rr*17.62p and *Rr*17.492n primers (Williams et al. 1992) was performed to confirm the presence of rickettsial gDNA. The 434 bp PCR products were analyzed on a 2% TAE agarose gel containing 1X SYBR safe DNA gel stain (Invitrogen). The primers and probes are shown in Table 3.1.

3.2.8. Construction of Standard Plasmids for Quantitative PCR (qPCR) Assay

To quantify the number of *R. amblyommii* and *R. montanensis* in infected *D. variabilis* samples, a standard plasmid was constructed and used in the qPCR assay to generate standard curves. OmpB*Ra*477F – OmpB*Ra*618R and OmpB*Rm*2832F – OmpB*Rm*2937R primers were used to amplify fragments of *R. amblyommii* (142 bp) and *R. montanensis* (106 bp) ompB genes (OmpB*Ra* and OmpB*Rm*), respectively, prior to cloning into the pCR4-TOPO vector (Invitrogen). The identity of each gene was confirmed by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Likewise, a 132 bp fragment of the *D. variabilis* calreticulin gene (CRT*Dv*) was amplified using CRT*Dv*321F – CRT*Dv*452R primers, cloned and sequenced. A gene-specific primer and either T3 or T7 were then used to amplify OmpB*Ra* and OmpB*Rm* genes from the recombinant plasmids. The amplicons were then digested with *Eco*RI (New England BioLabs) for 1 h at 37°C and ligated together. The OmpB*Ra* ligated OmpB*Rm*

Table 3.1. Primers for standard plasmid construction and qPCR assays.

Primer	Sequence (5'-3')	Experiment
OmpB*Rm*2937R$_{xbaI}$	AAAAAATCTAGACCTAAGTTGTTATAGTCTGTA GTG	Standard plasmid construction
CRT*Dv*321F$_{xbaI}$	AAAAAATCTAGAAGGAGAAAAGCAAGGGACTG	
OmpB*Ra*477F	GGTGCTGCGGCTTCTACATTAG	qPCR
OmpB*Ra*618R	CTGAAACTTGAATAAATCCATTAGTAACAT	
*Ra*OmpB_FAM	FAM/CGCGATCTCCTCTTACACTTGGACAGAATGCTT ATCGCG/BHQ_1	
OmpB*Rm*2832F	GCGGTGGTGTTCCTAATAC	qPCR
OmpB*Rm*2937R	CCTAAGTTGTTATAGTCTGTAGTG	
*Rm*OmpB_HEX	HEX/CGGGGCAAAGATGCTAGCGCTTCACAGTTACCC CG/IABkFQ	
OmpB*Rr*1370F	ATAACCCAAGACTCAAACTTTGGTA	qPCR
OmpB*Rr*1494R	GCAGTGTTACCGGGATTGCT	
*Rr*OmpB_TYE665	TYE665/CGCGATCTTAAAGTTCCTAATGCTATAACCCTT ACCGATCGCG/3BHQ_1	
CRT*Dv*321F	AGGAGAAAAGCAAGGGACTG	qPCR
CRT*Dv* 452R	CAATGTTCTGCTCGTGCTTG	
*Dv*CRT_TYE665	TYE665/TGGAGAAGGGCTCGAACTTGGC/IAbRQSp	
*Rr*17.61p	GCTCTTGCAACTTCTATGTT	Conventional PCR
*Rr*17.492n	CATTGTTCGTCAGGTTGGCG	

fragment (OmpB*RaRm*) was amplified using OmpB*Ra*477F – OmpB*Rm*2937R primers, cloned and sequenced. OmpB*Rm*2937R and CRT*Dv*321F primers were added with *Xba*I restriction site and were designated as OmpB*Rm*2937R$_{xbaI}$ and CRT*Dv*321F$_{xbaI}$, respectively. OmpB*RaRm* was then amplified using OmpB*Ra*477F and OmpB*Rm*2937R$_{xbaI}$ primers. Additionally, the primer pair CRT*Dv*321F$_{xbaI}$ and CRT*Dv*452R were used to amplify the CRT*Dv* gene. The amplicons of OmB*RaRm* and CRT*Dv* were then digested with *Xba*I (New England BioLabs) and ligated together. The ligation product was amplified using OmpB*Ra*477F and CRT*Dv*452R followed by cloning and sequencing (Figure 3.1). The resulting standard plasmid was linearized with *Xba*I (New England BioLabs) before being used as a standard template in the qPCR assay.

For the *R. rickettsii* ompB gene standard plasmid, the primers OmpB*Rr*1370F – OmpB*Rr*1494R were used to amplify a 124 bp fragment of the *R. rickettsii* ompB gene which was then subcloned into a pCR4-TOPO vector. The OmpB*Rr* standard plasmid was linerized with *Pst*I (New England BioLabs) restriction enzyme prior to the qPCR assay. All primers are shown in Table 3.1.

3.2.9. qPCR Amplification of the Rickettsial Outer Membrane Protein Gene

gDNA from tick tissues was subjected to qPCR assay using rickettsial species-specific molecular beacon probes and primers as described above. Tick calreticulin (CRT) gene primers and probe were designed by Primer3 software (http://frodo.wi.mit.edu/primer3/). All probes and primers were synthesized by Integrated DNA Technologies, Inc (Table 3.1). Serial 10-fold dilutions (1×10^8 to 10 copies) of standard plasmids were used as DNA templates to generate standard curves. Multi-plex qPCR assay of rickettsial (*R. amblyommii* or *R. montanensis*) and tick genes and single-plex qPCR assay of *R. rickettsii* gene were performed using a LightCycler 480® system II (Roche). The PCR reaction reagents were mixed in a 96-well plates with each

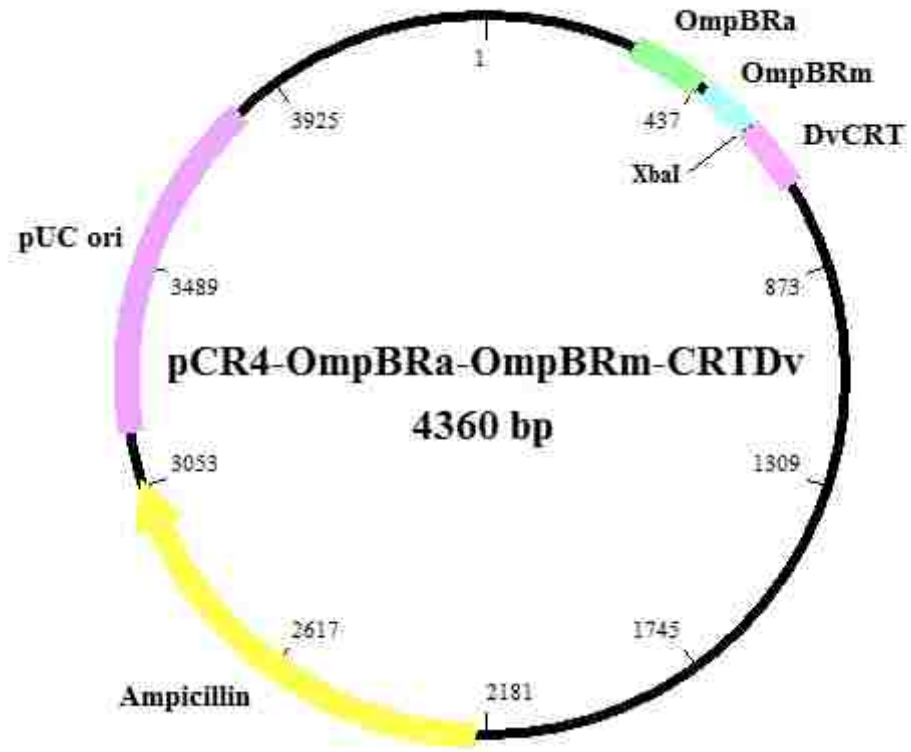

Figure 3.1. Schematic map of standard plasmid (pCR4-OmpB*Ra*-OmpB*Rm*-CRT*Dv*) for quantitative PCR amplification of rickettsial outer membrane protein (OmpB) and tick calreticulin (CRT) genes. A portion of *R. amblyommii* (142 bp) and *R. montanensis* (106 bp) OmpB genes and *D. variabilis* CRT (132 bp) gene were amplified and cloned into pCR4-TOPO vector and sequencing. The fragments of OmpB*Ra* and OmpB*Rm* genes were digested with *Eco*RI and ligated together. OmpB*Ra* ligated OmpB*Rm* fragment (OmpB*RaRm*) was amplified using OmpB*Ra*477F-Omp*Rm*2937R primers and cloned into pCR4-TOPO vector. OmpB*RaRm* and CRT*Dv* were amplified using OmpBRa477F-OmpB*Rm*2937R$_{xbaI}$ and CRT*Dv*321F$_{xbaI}$-CRTDv452R primers, respectively. The OmpB*RaRm* and CRT*Dv* amplicons were digested with *Xba*I, ligated, and amplified using OmpB*Ra*477F and CRT*Dv*452R followed by cloning. The pCR4-OmpB*Ra*-OmpB*Rm*-CRT*Dv* vector was linearized with *Xba*I prior to being used as a standard template in the qPCR assay.

well containing 2X LightCycler® 480 Probe Master (Roche), 0.2 µM each forward and reverse primers, 0.3 µM probes, and 5 µl of cDNA template in a total reaction volume of 35 µl. Ten microlitres of each reaction mixture were transferred into 3 wells of a 384-well plate. All reactions were subjected to the following conditions: a pre-incubation step of 95 °C for 10 min, 45 amplification cycles of 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 1 sec.

3.2.10. Quantitative Reverse Transcription-PCR (qRT-PCR) of Tick Immune Genes

Total RNA from tick tissues was treated with 4 units of Dnase Turbo (Ambion) for 1 h and purified using a RNA cleanup kit (Zymo Research). Synthesis of cDNA was carried out using 40 ng total RNA from hemolymph and 200 ng total RNA from salivary glands, midguts, and ovaries in 25 µl reaction volumes of iScript reverse transcription kit (Bio-Rad). No-RT reaction (distilled dH_2O was added instead of Reverse transcriptase) was performed to confirm the absence of gDNA. PCR reaction reagents were mixed in 96-well plates containing 2 µl of cDNA template, 2X LightCycler® 480 SYBR Green I master (Roche) and 0.1 µM each forward and reverse primers in a total volume of 35 µl. Ten microlitres of each reaction mixture were transferred into 3 wells of a 384-well plate. PCR assay conditions consisted of a 95 °C pre-incubation for 10 min, 45 amplification cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 5 sec followed by a melting curve step of 95 °C for 5 sec and 65 °C for 1 min. Primers used for amplification are listed in Table 3.2. Analysis of the crossing point (Cp) ratio of target (defensin1, lysozyme, KPI, and GST1) genes and reference (actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) genes values was conducted with LightCycler® 480 (1.5.0) software (Roche) using advanced relative quantification analysis (Efficiency method, a modified $\Delta\Delta C_T$ method, Roche). The relative gene expression of control unchallenged ticks was used to normalize those of *Rickettsia*-challenged ticks to generate the fold difference.

Table 3.2. Primers used in qRT-PCR assays.

Primer	Sequence (5'-3')	Reference
DvDefensin1For (Varisin)	CTTTGCATCTGCCTTGTCTTTCTC	Ceraul et al., 2007
DvDefensin1Rev (Varisin)	AATTCCTGTAGCAGGTGCAGG	
DvLyzFor	GATTGGATCTGCTTGGCAACAGC	Ceraul et al., 2007
DvLyzRev	TCAATATCGGCACCCCTTGACG	
DvKPIFor	CGAAGAATCAGAGTGCTGGAGAAC	Ceraul et al., 2007
DvKPIRev	CCGAGGTGGTTTTTAGGTCCTG	
DvGST1-416For	TATTTCCGGCCAAAGTGGTT	This study
DvGST1-590Rev	CCCAATCGCTACTCCCAGAG	
DvGAPDH-926For	ACTCCCACAGCAGCATCTTT	This study
DvGAPDH-1024Rev	TGCTGTAGCCGTACTCGTTG	
DvActin-1424For	CTTTGTTTTCCCGAGCAGAG	This study
DvActin-1572Rev	CCAGGGCAGTAGAAGACGAG	

3.2.11. Experimental Design

Dermacentor variabilis, a vector of RMSF, was used as a tick model for this study. In order to determine the specific tick immune response to rickettsial dissemination during atypical and typical rickettsial infection, three groups of partially fed female *D. variabilis* were capillary fed with *R. amblyommii* (nonpathogenic atypical *Rickettsia*), *R. montanensis* (nonpathogenic typical *Rickettsia*), or *R. rickettsii* (pathogenic typical *Rickettsia*). Dual challenge with *R. amblyommii* and *R. montanensis* was designed to minimize the variable volume of rickettsial feeding medium taken up by ticks. Equal amounts of *R. amblyommii* and *R. montanensis* organisms were mixed and capillary fed to partially fed female ticks. Genomic DNA and total RNA were extracted from the same tick tissues in order to determine the simultaneousness of tick immune response and presence of rickettsiae. Rickettsial infection was quantified using qPCR assay, and the gene expression of four tick immune genes was examined using qRT-PCR assay. The diagram of experimental design is shown in Figure 3.2. Two separate experiments were performed.

3.2.12. Statistical Analysis

The SAS statistical package (version 9.1.3) general linear model procedure in an analysis of variance was used to examine potential differences in populations of unchallenged and *Rickettsia*-challenged ticks. Data presented are from two separate bioassays. When overall significance was identified, Tukey's honestly significant difference (HSD) post-hoc test was used to examine pairwise differences of means of the main effects. Pairwise *t*-tests of least square means were performed for interaction effects to identify significant differences of tick immune gene expression between unfed and 5 day fed ticks and among *Rickettsia*-challenged and unchallenged tick tissues. For all comparisons, a P value of ≤ 0.05 was considered

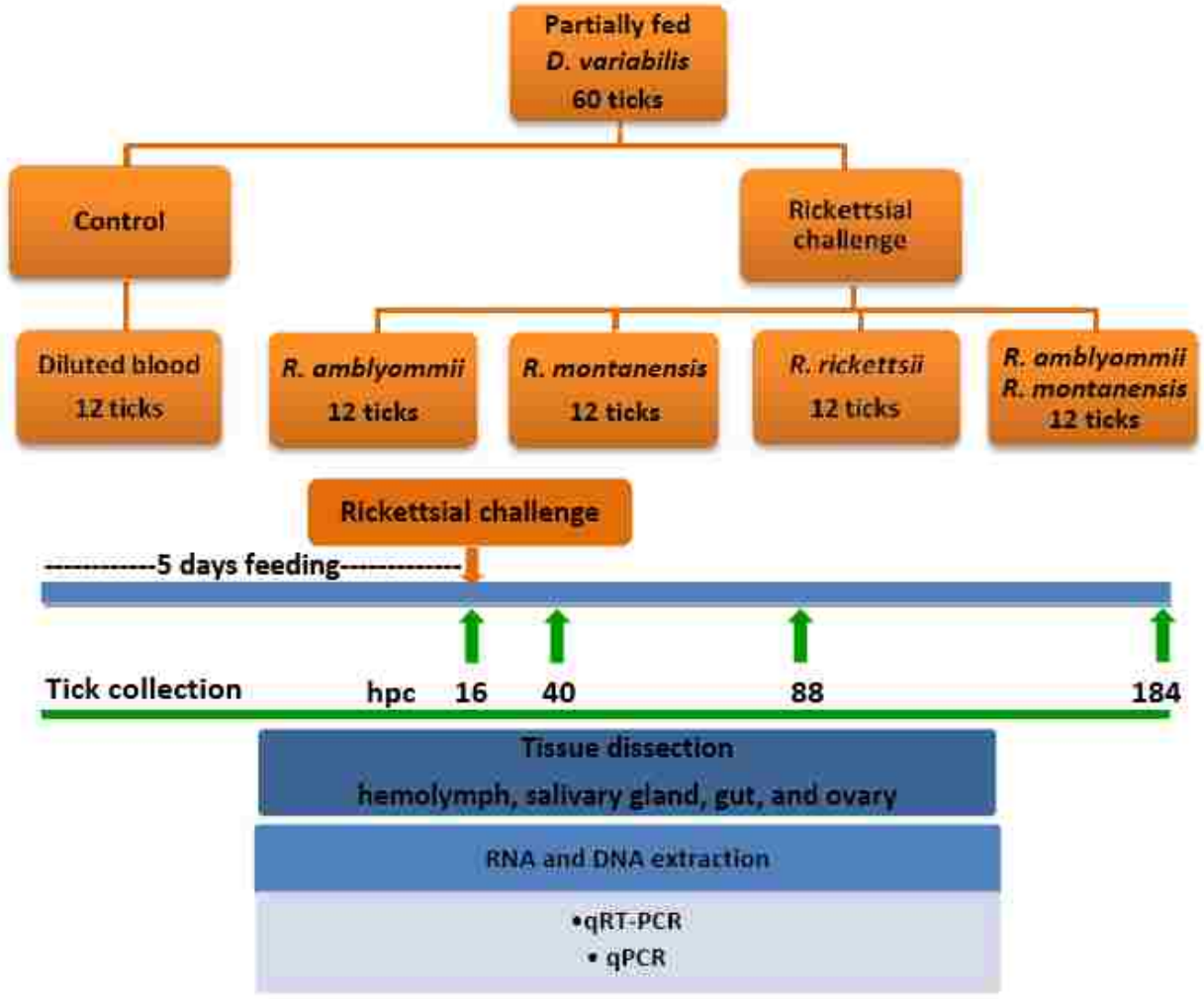

Figure 3.2. Diagram of experimental design of rickettsial challenge. Monitoring rickettsial dissemination and tick immune gene expression during typical (*R. montanensis* and *R. rickettsii*) and atypical (*R. amblyommii*) rickettsial challenge.

significantly different.

3.3. Results

3.3.1. Tick Immune Gene Expression in Response to Blood Feeding

During blood feeding a tick is confronted with microbes in the host blood. In order to determine the gene expression of *D. variabilis* immune genes (defensin1, lysozyme, GST1, and KPI) in response to blood feeding, total RNA samples from tick hemolymph, salivary glands, midguts, and ovaries of unfed and 5 day fed ticks was subjected to qRT-PCR assay. The gene expression of defensin1 was significantly upregulated in the tick hemolymph while KPI and GST1 was significantly upregulated in the tick midgut after 5 days of blood feeding (Figure 3.3A, 3.3C, 3.3D) suggesting that these tick genes may play an important role in controlling bacterial insult during tick feeding. In contrast, lysozyme gene expression was significantly downregulated in the midgut during blood feeding (Figure 3.3B).

3.3.2. Tick Capillary Feeding Technique

Rhodamine B is a chemical fluorescent dye that has been used as a biomarker for the fluorescent tracer technique to evaluate bloodfeeding of sandflies (Mascari and Foil 2009, 2010, ; Mascari et al. 2011). In order to evaluate the efficacy of tick feeding using CFT, ten female ticks were capillary fed with 0.1% Rhodamine B in 0.85% NaCl. After 16 h, all ticks were observed under fluorescent microscopy. Seven out of ten capillary fed ticks were fluorescent throughout the entire body (Figure 3.4), and three ticks were fluorescent only at the mouthparts suggesting that 70% of capillary fed ticks ingested the feeding media.

3.3.3 Specificity of Rickettsial Species-specific qPCR Assays

Specificity of the rickettsial species-specific qPCR assay was evaluated using five rickettsial species (*R. amblyommii, R. montanensis, R. rickettsii*, *R. parkeri*, and *R. felis*) gDNA

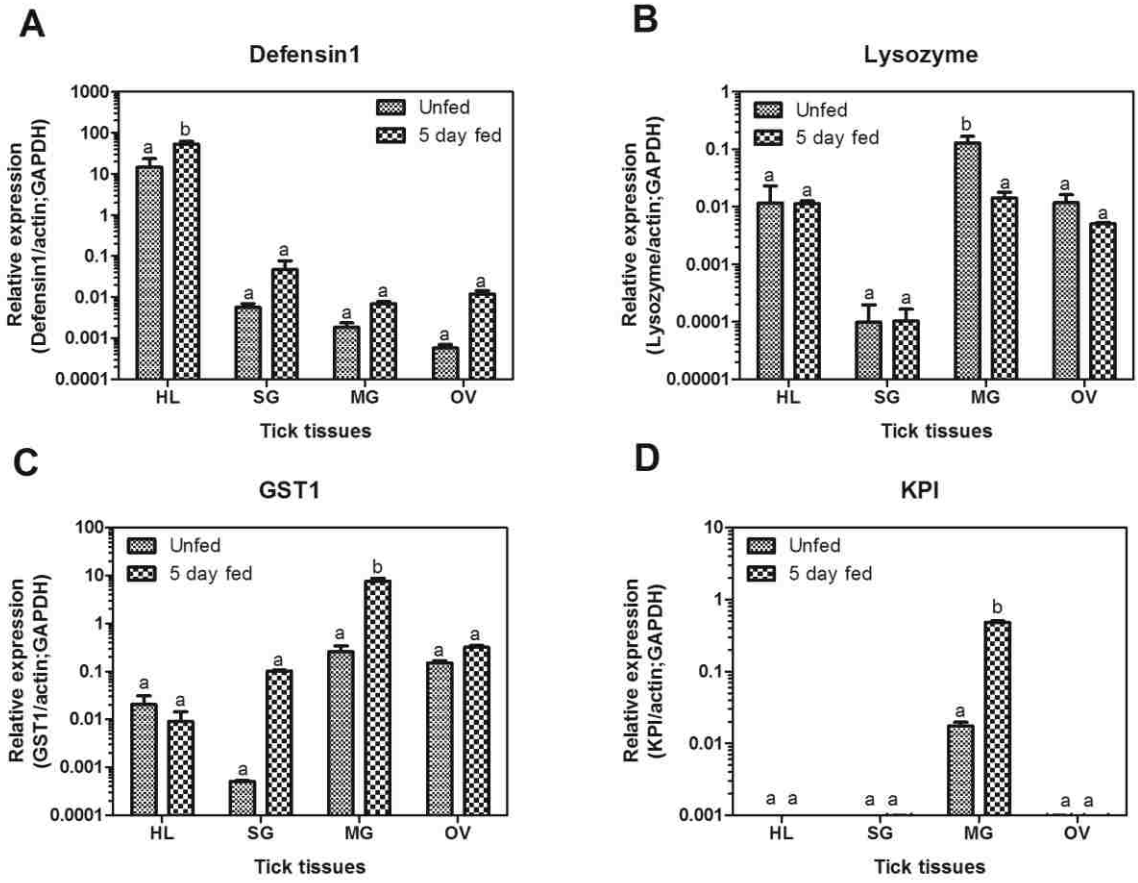

Figure 3.3. Tick immune gene expression in response to bloodfeeding (A; defensin1, B; lysozyme, C; Glutathione S-transferase, D; Kunitz protease inhibitor) in unfed and 5 day fed *D. variabilis*. Total RNA was extracted from tick tissues (hemolymph, salivary glands, midguts, and ovaries) and performed qRT-PCR assay. Transcription level of tick immune genes was normalized to actin and glyceraldehyde 3-phosphate dehydrogenase transcripts. Data shown are mean relative expression. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$). HL: hemolymph, SG: salivary gland, MG: midgut, OV: ovary.

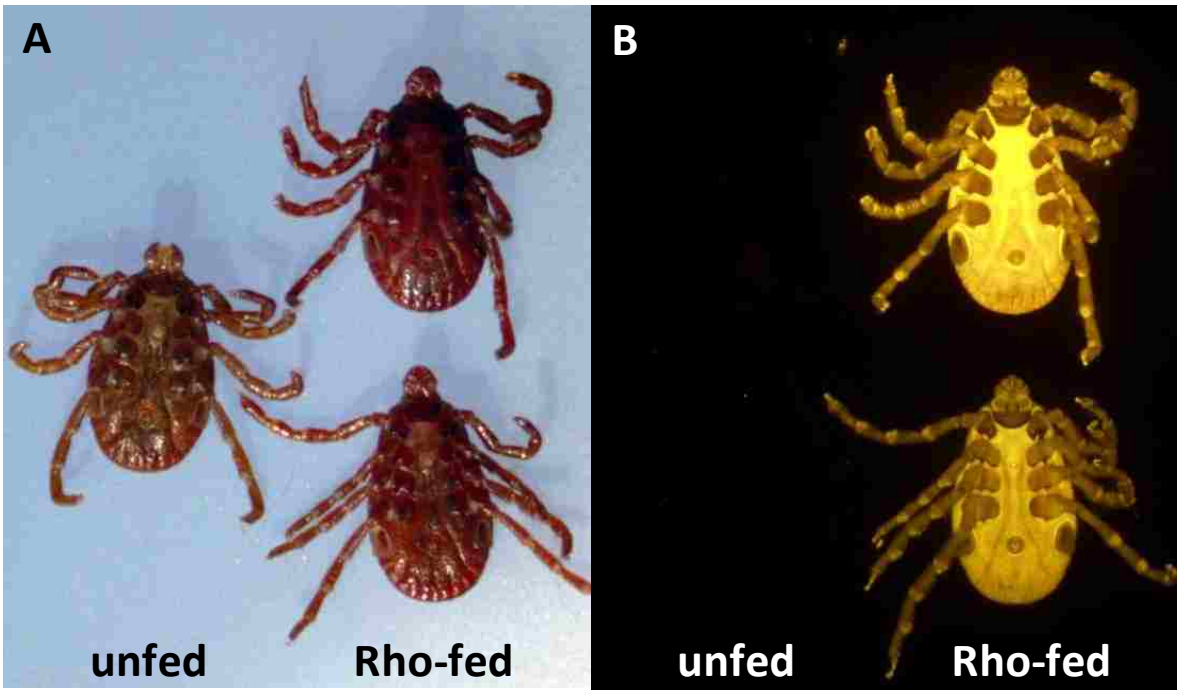

Figure 3.4. Rhodamine B feeding using capillary feeding technique (A:Bright-field, B:Red fluorescence)**.** Ten unfed female *D. variabilis* were capillary fed with 0.1% (W/V) Rhodamine B in 0.85% NaCl for 16 h. Unfed: unfed ticks, Rho-fed: Rhodamine B fed ticks.

as a template. The results demonstrated that rickettsial species-specific qPCR assays of *R. amblyommii, R. montanensis,* and *R. rickettsii* were species-specific, and PCR fragments of the ompB gene were confirmed by DNA sequencing (Table 3.3). The PCR amplification of the 17-kDa genus-specific antigen using *Rr*17.62p and *Rr*17.492n primers confirmed the presence of five rickettsial gDNA (Figure 3.5). The copy number of rickettsial ompB and tick calreticulin genes in tick tissues was determined using a standard curve of standard plasmids (pCR4-TOPO-OmpB*Ra*-OmpB*Rm*-CRT*Dv* and pCR4-TOPO-OmpB*Rr*).

3.3.4. Rickettsial Dissemination of Individual Rickettsial Challenge

To determine rickettsial dissemination of *R. amblyommii, R. montanensis,* and *R. rickettsii* in the tissues of *Rickettsia*-challenged ticks, gDNA from pooled three-tick tissues was subjected to a rickettsial species-specific qPCR assay. To determine the rickettsial infection level relative to tick tissue cells, serial dilutions of standard plasmids were used to generate standard curves of *R. amblyommii, R. montanensis,* and *R. rickettsii* ompB genes and the tick calreticulin gene. The minimum detection of all qPCR assays using standard plasmids was 10 copies. Only samples detected from three replicate wells were considered true signal and calculated. The results in Table 3.4 and 3.5 represent the copy number of rickettsial ompB genes in 10^8 copies of tick calreticulin gene from pooled three tick tissues.

For individual species challenges, after 16 h of capillary feeding all three rickettsial species were detected in tick hemolymph except for *R. amblyommii*-challenged ticks in experiment one, and only *R. montanensis* was detected in the tick midguts (Table 3.4). In experiment two, *R. montanensis* was also detected in tick ovaries. While the experiments had similar findings, there was variability in rickettsial dissemination with predominance of *R. montanensis* versus *R. amblyommii* in tick tissues over time. Similar to *R. montanensis,*

Table 3.3. The specificity of rickettsial species-specific qPCR assay.

Rickettsial gDNA	17-kDa conventional PCR	*R. amblyommii* ompB qPCR	*R. montanensis* ompB qPCR	*R. rickettsii* ompB qPCR	DNA sequencing results
R. amblyommii	+	+	-	-	*R. amblyommii*
R. montanensis	+	-	+	-	*R. montanensis*
R. rickettsii	+	-	-	+	*R. rickettsii*
R. parkeri	+	-	-	-	*R. parkeri*
R. felis	+	-	-	-	*R. felis*

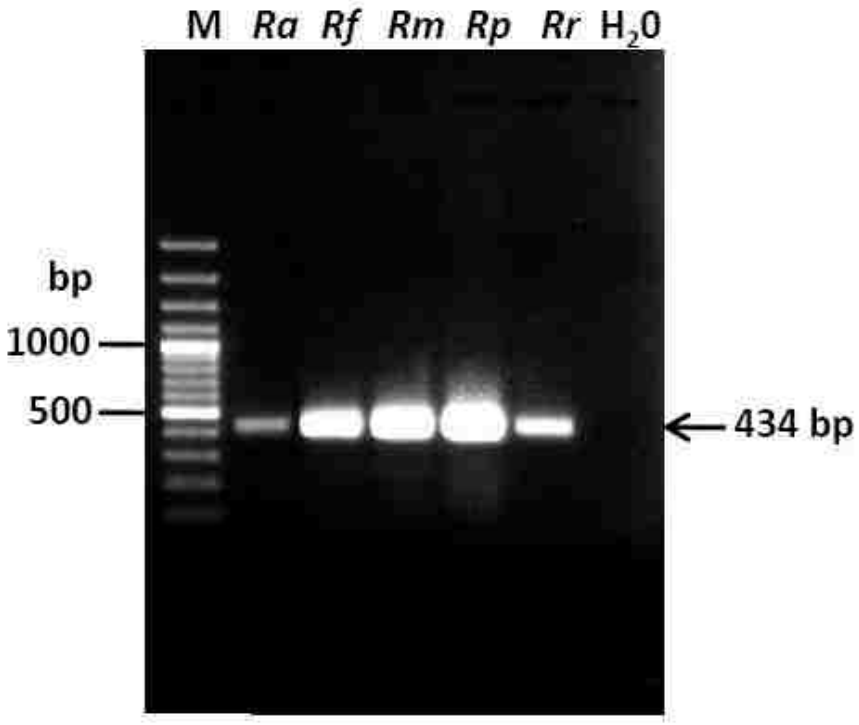

Figure 3.5. Confirmation of rickettsial gDNA using *Rickettsia* 17-kDa genus-specific antigen primers. gDNA was extracted from five *Rickettsia*-infected cells and used to performed PCR using *Rr*17.61p and *Rr*17.492n primers. The 434 bp PCR products were resolved on a 2% agarose gels containing 1X SYBR safe DNA gel stain (Invitrogen). M: GeneRuler 100 bp Plus DNA ladder (Fermentas), *Ra: R. amblyommii, Rf: R. felis, Rm: R. montanensis, Rp: R. parkeri, Rr: R. rickettsii*, H_2O: no template control.

Table 3.4. Copy number of rickettsial outer membrane protein B gene relative to 10^8 copies of tick calreticulin gene in individual rickettsial species-challenged ticks. Partially fed ticks were challenged with *R. amblyommii*, *R. montanensis*, or *R. rickettsii* for 16 h. Tick tissues were collected at 16, 40, 88, 184 hours post-challenge and performed qRT-PCR assays.

Individual rickettsial challenge		16 hpc				40 hpc				88 hpc				184 hpc			
		HL	SG	MG	OV	HL	SG	MG	OV	HL	SG	MG	OV	HL	SG	MG	OV
Experiment one	*R. amblyommii*	8.9E+00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R. montanensis	4.2E+04	-	7.5E+06	-	-	-	-	-	-	-	-	-	5.2E+03	-	-	-
	R. rickettsii	2.0E+06	-	-	-	ND	ND	ND	ND	-	-	2.2E+03	-	4.0E+05	-	-	-
Experiment two	*R. amblyommii*	-	-	-	-	5.8E+05	-	-	-	3.3E+05	-	3.0E+05	-	-	-	3.4E+04	-
	R. montanensis	1.0E+05	-	4.5E+07	8.3E+04	4.9E+05	-	3.7E+03	-	1.7E+06	-	-	-	6.3E+05	-	1.5E+04	-
	R. rickettsii	7.6E+05	-	-	-	ND	ND	ND	ND	-	-	-	-	2.5E+05	-	7.1E+03	-

HL = hemolymph
SG = salivary gland
MG = midgut
OV = ovary
ND = not determined

Table 3.5. Copy number of rickettsial outer membrane protein B gene relative to 10^8 copies of tick calreticulin gene in dual rickettsial species-challenged ticks. Partially fed ticks were challenged with combined *R. amblyommii* and *R. montanensis* for 16 h. Tick tissues were collected at 16, 40, 88, 184 hours post-challenge and performed qRT-PCR assay.

Dual rickettsial challenge (*R. amblyommii*/ *R. montanensis*)		**16 hpc**				**40 hpc**				**88 hpc**				**184 hpc**			
		HL	**SG**	**MG**	**OV**	**HL**	**SG**	**MG**	**OV**	**HL**	**SG**	**MG**	**OV**	**HL**	**SG**	**MG**	**OV**
Experiment one	***R. amblyommii***	-	-	-	-	-	-	-	-	-	1.4E+03	-	-	-	-	-	-
	R. montanensis	-	-	-	-	-	-	-	-	1.8E+04	-	-	-	3.3E+03	-	8.4E+02	-
Experiment two	***R. amblyommii***	-	-	5.7E+04	-	-	-	-	-	-	-	-	-	-	-	-	-
	R. montanensis	-	-	1.8E+04	4.8E+04	5.2E+05	-	-	-	3.9E+05	-	-	-	8.1E+05	-	-	-

HL = hemolymph
SG = salivary gland
MG = midgut
OV = ovary
ND = not determined.

the *D. variabilis*-associated *R. rickettsii* was found to consistently disseminate after introduction. Combined results from two separate experiments demonstrated that *D. variabilis* was persistently infected with *R. montanensis* and *R. rickettsii* compared to *R. amblyommii*.

3.3.5. Rickettsial Dissemination of Dual Rickettsial Challenge

To assess the specific ability of *Rickettsia* to disseminate, a dual rickettsial challenge bioassay was employed in which the same amount of *R. amblyommii* and *R. montanensis* was combined and fed to ticks (Table 3.5). Similar to the individual species challenges bioassay, only *R. montanensis* was able to disseminate to tick ovaries, and compared to *R. amblyommii*, *R. montanensis* was consistently detected in ticks at all time points. The combined results of dual challenge also suggested that compared to *R. amblyommii, R. montanensis,* which is a typical *Rickettsia,* persistently infected tick tissues with a higher infection level.

3.3.6. Tick Immune Response During Rickettsial Infection

In order to compare the specific tick immune in response to rickettsial infection, ticks were challenged with *R. amblyommii, R. montanensis*, *R. rickettsii*, and combined *R. amblyommii* and *R. montanensis*. In tick salivary glands and ovaries, no difference in defensin1, lysozyme, GST1, and KPI gene expression was observed between *Rickettsia*-challenged and unchallenged ticks (data not shown). Likewise, although rickettsial infection was observed in tick hemolymph, there was no significant difference in tick immune gene expression among *Rickettsia*-challenged and unchallenged ticks (Figure 3.6A, 3.6B). In tick midgut, which is the first site of contact, gene expression of lysozyme in *Rickettsia*-challenged ticks was not significantly different from unchallenged ticks (Figure 3.7A); however, GST1 gene expression was significantly upregulated at 184 hpc when challenged with *R. amblyommii* (Figure 3.7B). The slight increase of KPI gene expression during *R. amblyommii* challenge was also observed;

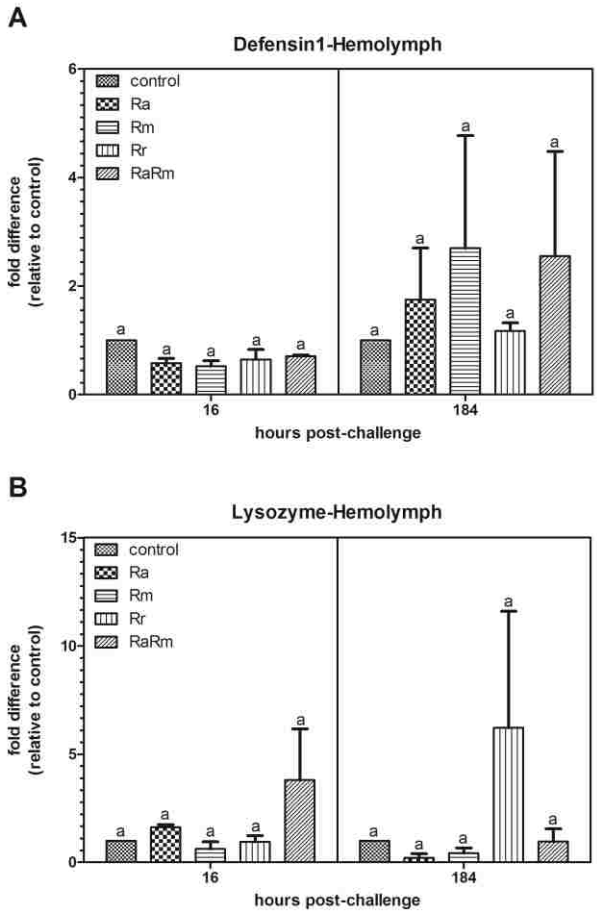

Figure 3.6. Gene expression of tick immune genes in tick hemolymph in response to rickettsial challenge (A: defensin1, B: lysozyme). Total RNA was extracted from tick hemolymph and performed qRT-PCR assay. Transcription level of tick immune genes was normalized to actin and glyceraldehyde 3-phosphate dehydrogenase transcripts. Data shown are fold difference relative to unchallenged ticks. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$). Control: unchallenged tick, Ra: *R. amblyommii*-challenged ticks, Rm: *R. montanensis*-challenged ticks, Rr: *R. rickettsii*-challenged ticks, RaRm: combined *R. amblyommii* and *R. montanensis*-challenged ticks.

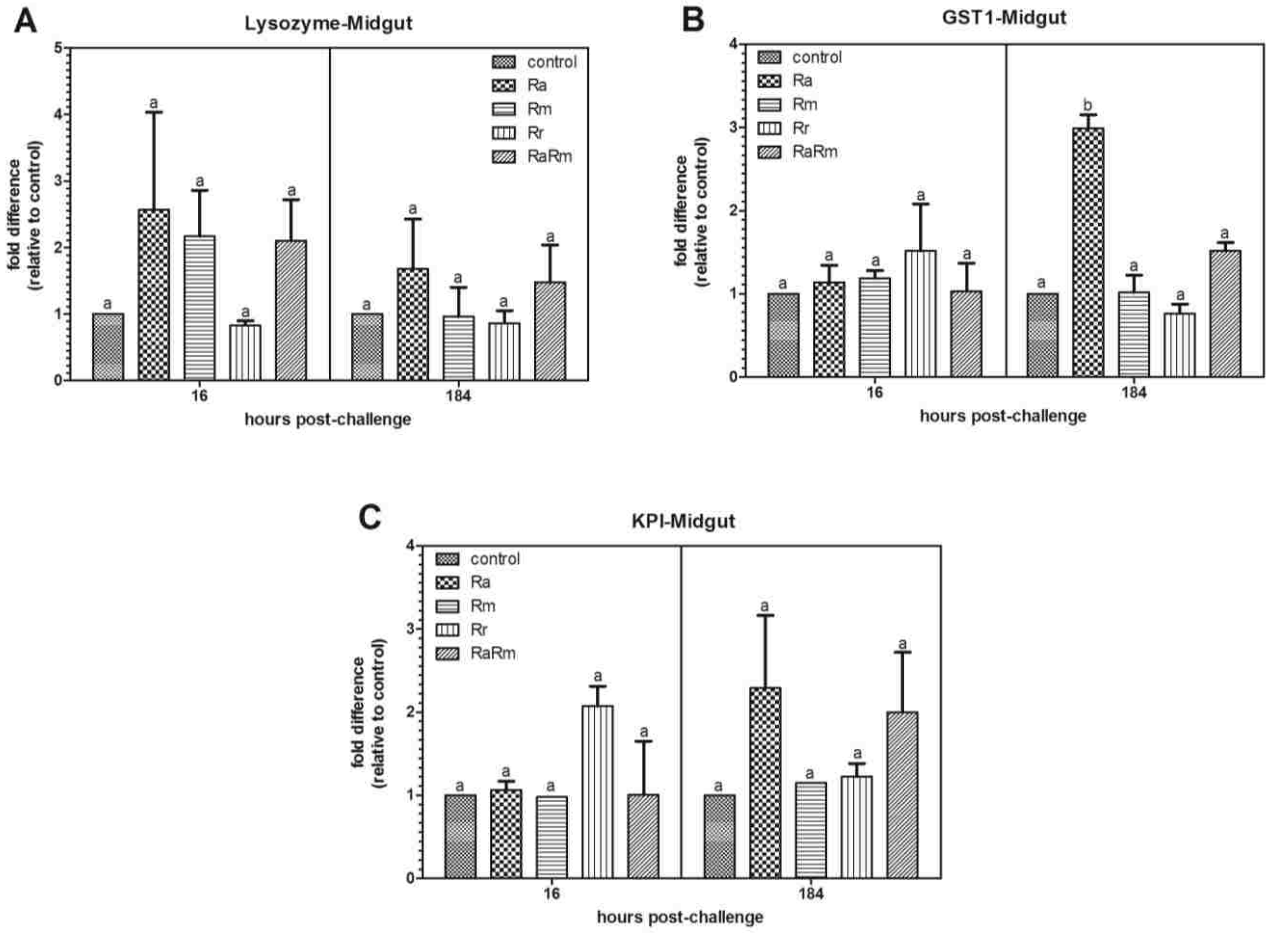

Figure 3.7. Gene expression of tick immune genes in tick midguts in response to rickettsial challenge (A: lysozyme, B: glutathione S-transferase1, C: Kunitz protease inhibitor). Total RNA was extracted from tick hemolymph and performed qRT-PCR assay. Transcription level of tick immune genes was normalized to actin and glyceraldehyde 3-phosphate dehydrogenase transcripts. Data shown are fold difference relative to unchallenged ticks. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$). Control: unchallenged tick, Ra: *R. amblyommii*-challenged ticks, Rm: *R. montanensis*-challenged ticks, Rr: *R. rickettsii*-challenged ticks, RaRm: combined *R. amblyommii* and *R. montanensis*-challenged ticks.

however, there was no significant difference in gene expression (Figure 3.7C).

3.4. Discussion

During long-term bloodfeeding, ticks may encounter a number of pathogens present in host blood. In addition to blood digestion related molecules, many defense molecules are needed to prevent ticks from succumbing to pathogen challenge. The majority of tick molecules identified is from tick salivary glands and are responsive for the regulation of blood flow and host immune response factors (Aljamali et al. 2009). However, during blood feeding, the tick midgut is the first site of contact with numerous microbes and ingested blood. Recently, a cDNA library of *D. variabilis* midgut has been generated from fed ticks (Anderson et al. 2008). Compared to proteins identified from salivary glands, most midgut proteins are involved in bloodmeal digestion, including oxidative stress reduction enzymatic regulation, antimicrobial activity, detoxification enzymes, peptidase inhibitor, protein digestion, mucins, and iron/heme metabolism and transport factors (Anderson et al. 2008). In the current study, defensin1, KPI, and GST1 are significantly upregulated in response to bloodfeeding. In contrast, lysozyme gene expression is significantly downregulated in the tick midgut during bloodfeeding.

This study is the first that measured the prevalence of tick consumption of ticks via CFT using fluorescent marker. Rhodamine B was used as a biomarker to evaluate tick feeding. Using Rhodamine B-feeding medium has shown that seventy percent of ticks ingested the feeding medium. The current study is also the first examination of the dissemination of typical and atypical *Rickettsia* in the tick vector. Ticks were challenged with atypical nonpathogenic (*R. amblyommii, Amblyomma*-associated *Rickettsia*), typical nonpathogenic (*R. montanensis, Dermacentor*-associated *Rickettsia*), and typical pathogenic (*R. rickettsii, Dermacentor*-associated *Rickettsia*) *Rickettsia* using CFT. Both typical rickettsial species, *R. montanensis* and

R. rickettsii, are able to persistently infect *D. variabilis*, and only *R. montanensis* is able to disseminate to tick ovaries supporting field and laboratory evidence of transovarial transmission of *R. montanensis* in *D. variabilis*. This may suggest that *D. variabilis* favors typical nonpathogenic *Rickettsia*, *R. montanensis*, more than typical pathogenic *Rickettsia*. It has been demonstrated previously that *D. andersoni* experimentally infected with *R. rickettsii* has reduced survival and fecundity of female ticks (Niebylski et al. 1999). Variation of volume of ingested media, ranging between 0.06 and 6.77 µl using CFT has been previously described where dual rickettsial challenge was designed to control the rickettsial organisms of two rickettsial species taken up by individual ticks (Macaluso et al. 2001). The results demonstrated that only *R. montanensis,* the typical *Rickettsia,* persistently infected *D. variabilis* and the infection level of *R. montanensis* is higher compared to *R. amblyommii.* Although, rickettsial replication in ticks was not observed in this study, the data suggested that there is a specific association between ticks and SFG *Rickettsia,* favoring particular species.

Additionally, the present study compared the specific response of tick immune genes during typical and atypical rickettsial infection. The gene expression of four tick immune genes, which were identified and characterized from *R. montanensis*-infected *D. variabilis* (Ceraul et al. 2007, 2008; Macaluso et al. 2003; Mulenga et al. 2003), was determined using qRT-PCR assay. Most of the findings were variable; however, similar results demonstrated that defensin1, lysozyme, GST1, and KPI are upregulated in *R. montanensis*-infected *D. variabilis*. In *D. variabilis,* there are two forms of defensin; defensin1 (Varisin) and defensin2. Defensin1 is mainly found in tick hemolymph, and defensin2 is highly expressed in tick ovaries (Ceraul et al. 2007). Defensin2 gene expression was also determined in this study (data not shown); however, tick ovaries were not persistently infected with rickettsiae; therefore, there was no difference in

gene expression. Lysozyme is highly expressed in the midgut and hemolymph (Ceraul et al. 2007; Simser et al. 2004), and a previous study by Ceraul et al (2007) demonstrated that lysozyme gene expression was upregulated in response to *R. montanensis* challenge at 24 and 72 hours post-experimental feeding. In this study, significant difference in lysozyme gene expression was not observed in *Rickettsia*-challenged ticks; however, the tick gene expression was only assessed at 184 hpc, which may be after expression peaks. A novel tick antimicrobial molecule, KPI, has been characterized from *R. montanensis*-challenged *D. variabilis* (Ceraul et al. 2008). It was demonstrated that in ticks, *R. montanensis* challenge induced DvKPI gene expression, and recombinant exogenously expressed DvKPI controled rickettsial infection in DvKPI-expressed L929 cells (Ceraul et al. 2008). Additionally, recent studies have shown that DvKPI potentially associates with rickettsiae and limits rickettsial invasion (Ceraul et al. 2011). However, in the current study, at 16 and 184 hpc there was no significant difference in KPI gene expression when challenged with *Rickettsia*. Another molecule potentially associated with tick response to rickettsial infection is GST, which is a major enzyme important in detoxification of xenobiotic compounds in both mammals and invertebrates (Torres-Rivera and Landa 2008). In ticks, GSTs are known to associate with acaricide resistance (Rosario-Cruz et al. 2009), blood feeding (Anderson et al. 2008), and stress response (Mulenga et al. 2003). Additionally, in *D. variabilis* infection with *Anaplasma marginale* (de la Fuente et al. 2007) and *R. montanensis* (Mulenga et al. 2003) resulted in upregulated GST gene expression. Similar to defensin, *D. variabilis* GSTs have two isoforms; GST1 which is highly expressed in tick midguts, and GST2 which is mainly expressed in tick ovaries (Dreher-Lesnick et al. 2006). In this study, only challenge with *R. amblyommii* significantly upregulated GST1 in the tick midguts, suggesting a specific tick response to atypical *Rickettsia*. Little is known about the role of GST during

rickettsial infection, and more studies on the direct interactions between GST and *Rickettsia* are needed to verify the specificity of the expression.

The limited dissemination and increased immune response associated with atypical *Rickettsia* suggest a molecular basis for vector competence. Typical *Rickettsia*, *R. montanensis* and *R. rickettsii* persistently infected *D. variabilis* compared to *R. amblyommii*, atypical *Rickettsia*, and GST1 may play a role in control of the infection of atypical *Rickettsia* which may contribute to the vector competence of ticks for *Rickettsia*. However, the molecular function of GST1 during rickettsial infection requires further characterization. Studying vector competence of ticks for individual *Rickettsia* will enhance our understanding of ecology and epidemiology of tick-borne rickettsioses in nature.

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CHAPTER 4
FUNCTIONAL CHARACTERIZATION OF *DERMACENTOR VARIABILIS* GLUTATHIONE S-TRANSFERASE IN RESPONSE TO RICKETTSIAL INFECTION

4.1. Introduction

Ticks are the only vectors of spotted fever group (SFG) *Rickettsia* which are obligate gram negative intracellular bacteria belonging to the genus *Rickettsia*. In nature, ticks are not only disease agent vectors, but also reservoir hosts which can maintain rickettsial infection via horizontal and vertical transmission. However, the capability of an individual tick species to sustain a particular rickettsial species is known to be limited. In order to understand such a specific interaction, many studies have identified tick molecules which were differentially expressed during rickettsial infection (Macaluso et al. 2003a, 2003b, 2006). Tick immune-like molecules have primarily been examined due to their direct effect and immediate response during microbe challenge (Ceraul et al. 2003, 2008, 2011; Johns et al. 2001a, 2001b; Simser et al. 2004); however, the specificity of tick immune-like molecules in response to rickettsial infection has not been studied.

Data presented in Chapter 3, described a specific tick immune-like molecule, glutathione S-transferase1 (DvGST1) that only responds to infection with atypical *Rickettsia*, *Rickettsia amblyommii*, in *Dermacentor variabilis* midgut. The significant upregulation of DvGST1 gene expression is observed at 184 hours post-challenge in *R. amblyommii*-challenged ticks compared to unchallenged and *R. montanensis*-challenged ticks; nevertheless, the function of DvGST1 during rickettsial challenge has not been characterized. Additionally, in the same experiment, the results of rickettsial dissemination demonstrated that only typical *Rickettsia* (i.e. *R. montanensis* and *R. rickettsii*) persistently infects *D. variabilis*.

Glutathione S-transferases (GST; EC 2.5.1.18) are known as a multifunctional enzyme

family (He et al. 1999) that is involved mainly with detoxification of both endogenous and xenobiotic compounds (Enayati et al. 2005a, 2005b; Wilce et al. 1995). In arthropods, GST is one of the enzymes associated with pesticide resistance (Sharp et al. 1991) and oxidative stress (Kim et al. 2011), and a number of studies have focused on the role of GST in particular insecticide resistance (Low et al. 2010; Wang et al. 2008). Increasing of GST activity in arthropods has been shown to be related to acaricide and insecticide resistance (Enayati et al. 2005). In *D. variabilis*, GST was first identified during comparative transcriptional analysis of *R. montanensis*-infected and uninfected tick ovaries using subtractive hybridization (Mulenga et al. 2003). Subsequent examination identified the second isoform of DvGST, and demonstrated that DvGST isoform-1 is present in the tick midgut, while DvGST isoform-2 (DvGST2) is found in both tick midguts and ovaries (Dreher-Lesnick et al. 2006). The gene expression of both DvGST isoforms is upregulated during bloodfeeding and downregulated when challenged with *Escherichia coli* (Dreher-Lesnick et al. 2006). Amino acid sequence analysis demonstrated that DvGST1 and DvGST2 are similar to mammalian class theta and insect class delta GSTs. DvGST1 falls into the same clade as *Ixodes pacificus* GST, while DvGST2 falls in a separate clade (Dreher-Lesnick et al. 2006); however, little is known about the role of DvGST2 during rickettsial infection.

The objectives of this study are to 1) examine effect of DvGST1 on infection of *R. amblyommii*, atypical *Rickettsia*, in the tick midgut; and, 2) examine DvGST2 gene expression in response to atypical and typical rickettsial challenge. The hypothesis being test is that silencing of DvGST1 gene results in increased *R. amblyommii* infection in *D. variabilis*, and DvGST2 differently responses to atypical or typical *Rickettsia* in tick ovaries. Studying of the specific immune mechanism by ticks to rickettsial infection will provide insight into vector competence

of ticks for *Rickettsia*. Ultimately, deciphering the basis of vector competence will lead to a better understanding of the ecology and epidemiology of tick-borne rickettsioses.

4.2. Materials and Methods

4.2.1. Tick and *Rickettsia*

Rickettsia-free *D. variabilis* (Say) were initially provided by Dr. Daniel Sonenshine, Old Dominion University. The tick life cycle is routinely maintained using mice for larval feeding, rats for nymphal feeding, and guinea pigs for adult feeding at the School of Veterinary Medicine, Louisiana State University. All tick life cycle stages were maintained in an environmental chamber at 27±1°C, 87±2% relative humidity (RH), and a 16: 8 (light: dark) cycle. Prior to rickettsial challenge, female ticks were allowed to feed on Hartley guinea pigs (*Cavia porcellus*) for 5 days. Fed female ticks were forcibly removed and cleaned with 1% bleach, 70% ethanol, and distilled H_2O. For RNA-interference mediated gene silencing (RNAi) experiments, ticks were first subjected to double-stranded RNA (dsRNA) injection and then allowed to feed on Hartley guinea pigs for 5 days. All animals were handled according to Louisiana State University's Institutional Animal Care and Use Committee regulations.

Rickettsia amblyommii (Darkwater) provided by Dr. Christopher Paddock and *R. montanensis* (M5/6; Microtus isolate) were maintained and propagated in Vero E6 cells in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone) at 34°C and 5% CO_2. For rickettsial challenge, infected Vero E6 cells were detached and transferred to Erlenmeyer flasks containing sterile 3-mm borosilicate glass beads (Sigma), and vortexed at high speed for 3 min. The cell lysate was filtered through a 2 micron (pore size) syringe filter (Millipore). Viability and enumeration of *Rickettsia* were assessed by staining with a *Bac*Light viability stain kit (Invitrogen), and rickettsiae were counted in a Petroff-

Hausser bacteria counting chamber (Sunyakumthorn et al. 2008) using a Leica microscope. The rickettsial pellets were resuspended in 1:125 diluted heat-inactivated bovine blood and diluted to 10^9 rickettsiae/ml.

4.2.2. Experimental Design

Objective 1: examine effect of DvGST1 on the infection of *R. amblyommii*, atypical *Rickettsia,* in tick midgut. *D. variabilis* was used as a tick model for this study. In order to silence the DvGST1 gene in ticks, ticks were injected with DvGST1 dsRNA or negative control dsRNA (targeting GFPuv gene). Gene silencing was confirmed using qRT-PCR and GST enzymatic assays. GST1 silencing ticks were challenged with *R. amblyommii*. The diagram of experimental design is shown in Figure 4.1. Two separate experiments were performed.

Objective 2: examine DvGST2 gene expression in response to atypical and typical rickettsial challenge. In order to determine the response of DvGST2 gene expression to rickettsial dissemination during atypical and typical rickettsial infection, three groups of partially fed female *D. variabilis* were capillary fed with *R. amblyommii* (atypical *Rickettsia*), *R. montanensis* (typical *Rickettsia*) and GST2 gene expression in tick tissues was quantified by quantitative reverse transcription-PCR (qRT-PCR) assay. The diagram of experimental design is shown in Figure 4.2. Two separate experiments were performed.

4.2.3. Synthesis of dsRNA for RNA Interference

Total RNA was extracted from tick midguts dissected from three partially fed female *D. variabilis* using RNeasy kit (QIAGEN). Total RNA was treated with 2 units of DNase (Ambion) for 1 h and subjected to cDNA synthesis using an iScript reverse transcription kit (Bio-Rad) according to the manufacturer's protocol. The cDNA was used for amplification of the target gene to generate a template for dsRNA synthesis. Briefly, in 250 µl reaction volume,

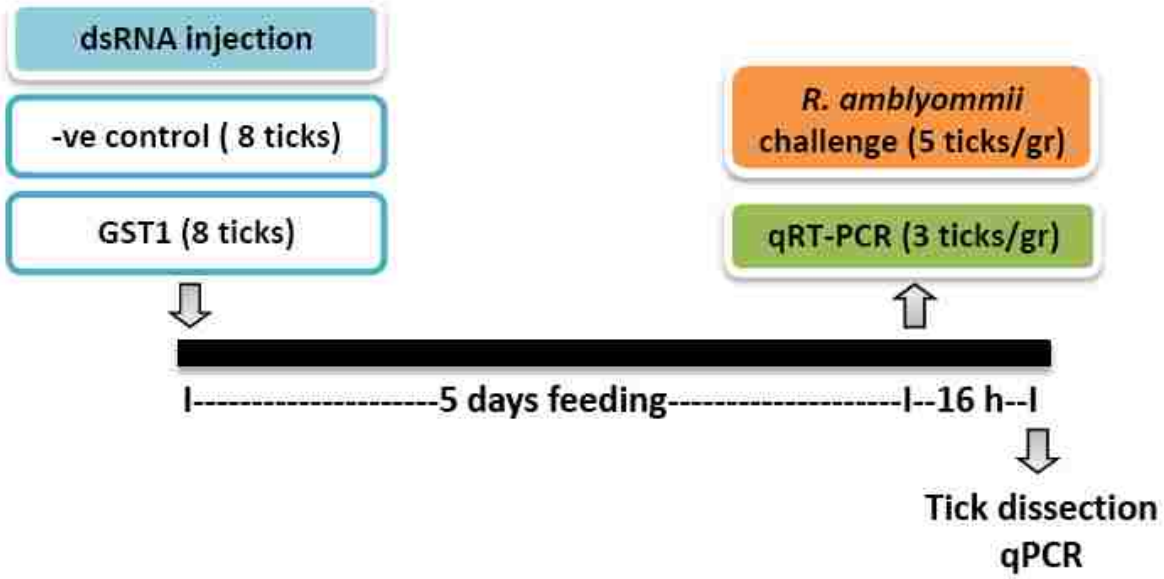

Figure 4.1. Diagram of experimental design of objective 1: examine effect of DvGST1 on the infection of *R. amblyommii*, atypical *Rickettsia* in tick midguts.

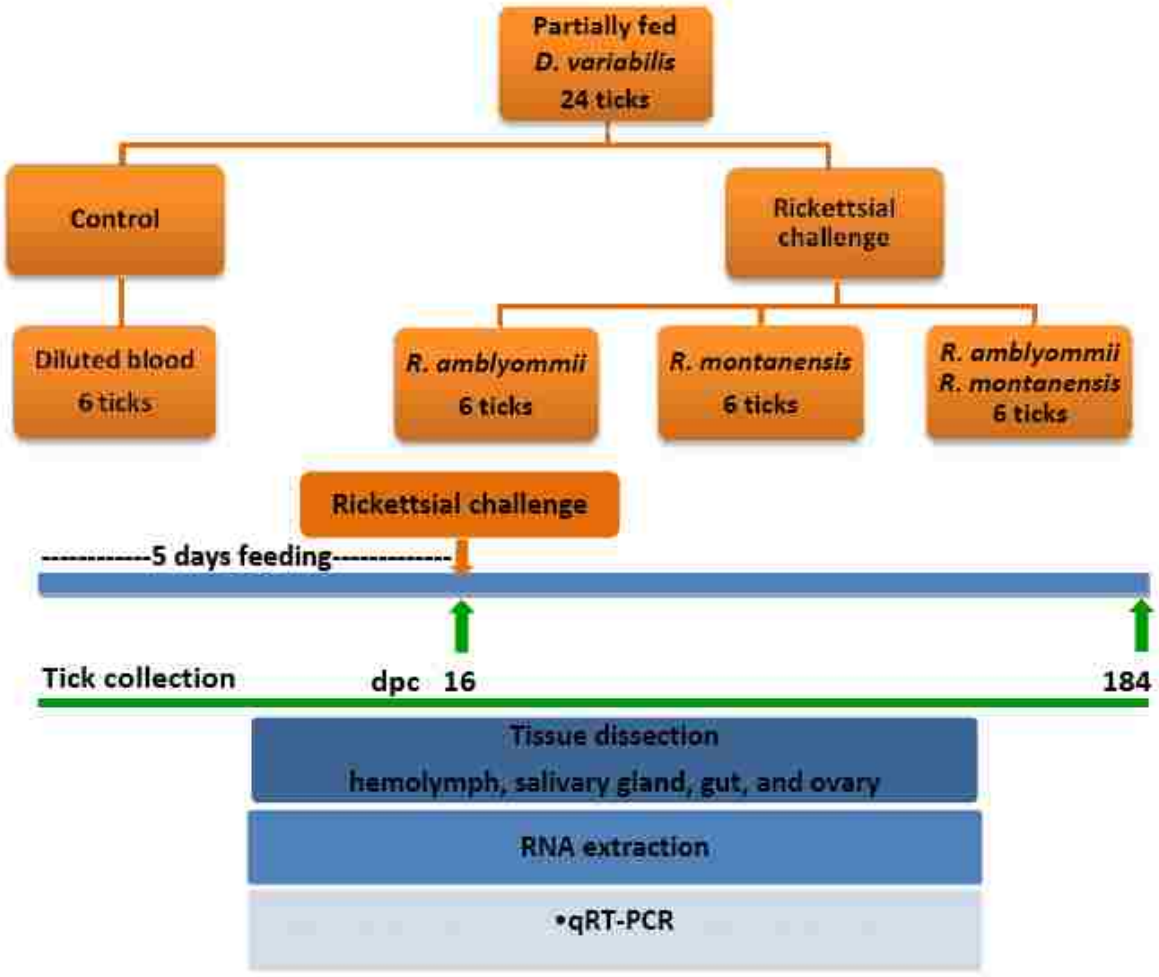

Figure 4.2. Diagram of experimental design of objective 2: examine DvGST2 gene expression in response to atypical (*R. amblyommii*) and typical (*R. montanensis*) rickettsial challenge.

10 µl of a cDNA template was used as a template with DvGSTT7For and DvGST1T7Rev primers containing T7 promoter sequence at 5' end. The plasmid containing the green fluorescent protein (GFPuv) sequence was used as a template for negative control dsRNA using GFPuvT7For and GFPuvT7Rev primers. The dsRNA constructs and primers were designed using E-RNAi web application (Arziman 2005; Horn 2010). All primers are shown in Table 4.1. The reaction mixtures were subjected to 1 cycle at 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final cycle at 72°C for 5 min. The 537 bp and 540 bp PCR products of GST1 and GFPuv, respectively were electrophoresed on 2% agarose gel and purified using a Wizard® SV Gel and PCR Clean-Up kit (Promega). For dsRNA synthesis, two micrograms of PCR product were used for each 40-µl reaction using the MegaScript RNAi kit (Ambion) according to the manufacturer's protocol. After 16 h of incubation at 37°C, the dsRNA reaction mixture was treated with 2 units of Turbo DNase (Ambion) at 37°C for 15 min. The dsRNA was then purified using an RNeasy kit (QIAGEN). Briefly, 60 µl of H_2O was added to 40 µl of dsRNA reaction mixture, and 350 µl of RLT buffer and 250 µl of 100% ethanol were added, respectively. The solution was then applied to a spin column and subjected to centrifugation at 12,000 ×*g* for 15 s. The column was washed twice with 500 µl of RPE buffer, and dsRNA was eluted with 80 µl TE buffer. The dsRNA concentration was measured using the NanoDrop 100 Spectrometer (Thermo Scientific) and adjusted to 2 µg RNA in 1 µl using TE buffer prior to the injection.

4.2.4. RNA Interference-mediated Gene Silencing in Ticks

Unfed female *D. variabilis* were cleaned with 70% ethanol and rinsed twice with water, prior to the injection with dsRNA. Three groups (20 ticks/group) of female ticks were restrained ventrally on sticky tape and injected with approximately 1 µl of TE buffer (group 1), negative

Table 4.1. Primers used in RNA interference, qRT-PCR, and qPCR assays

Primer	Sequence (5'-3')	Experiment
DvGST1-416For	TATTTCCGGCCAAAGTGGTT	qRT-PCR
DvGST1-590Rev	CCCAATCGCTACTCCCAGAG	
DvGST2-484For	AAGGCTGGAGCTCCTCATTG	qRT-PCR
DvGST2-600Rev	ACAGGGTCCGCTGCAGTATT	
DvGAPDH-926For	ACTCCCACAGCAGCATCTTT	qRT-PCR
DvGAPDH-1024Rev	TGCTGTAGCCGTACTCGTTG	
DvActin-1424For	CTTTGTTTTCCCGAGCAGAG	qRT-PCR
DvActin-1572Rev	CCAGGGCAGTAGAAGACGAG	
DvGST1T7For	taatacgactcactatagggTCACGTTGCACGACCTTAAC	RNAi (dsRNA)
DvGST1T7Rev	taatacgactcactatagggGCCTTCTTGAAACGCTGGTA	
GFPuvT7For	taatacgactcactatagggCATGCCATGTGTAATCCCAG	RNAi (dsRNA)
GFPuvT7Rev	taatacgactcactatagggGTGTTCAATGCTTTTCCCGT	
OmpB*Ra*477F	GGTGCTGCGGCTTCTACATTAG	qPCR
OmpB*Ra*618R	CTGAAACTTGAATAAATCCATTAGTAACAT	
*Ra*OmpB_FAM	FAM/CGCGATCTCCTCTTACACTTGGACAGAAT GCTTATCGCG/BHQ_1	
CRT*Dv*321F	AGGAGAAAAGCAAGGGACTG	qPCR
CRT*Dv* 452R	CAATGTTCTGCTCGTGCTTG	
*Dv*CRT_TYE665	TYE665/TGGAGAAGGGCTCGAACTTGGC/IAbRQ Sp	

control dsRNA (2 µg/µl) (group 2), or GST1 dsRNA (2 µg/µl) (group 3) at the area between Coxa I and basis capituli using a 5 µl glass syringe attached an 1-inch 33-gauge needle (Hamilton). The injected ticks were kept in the environmental chamber overnight, and surviving ticks were allowed to feed on guinea pigs the next day.

4.2.5. Gene Expression of DvGST1-silenced Ticks

In order to evaluate whether the dsRNA injection decreased the mRNA expression of GST1 gene, at 2 days post-feeding (dpf), 5 ticks form each group were forcibly removed from guinea pigs. The tick midguts were dissected and kept in RLT buffer (QIAGEN) at -80°C until used for RNA isolation. At 5 dpf, all ticks were forcibly detached from guinea pigs, and five ticks from each group were dissected. The remaining ticks were kept in the environmental chamber, and at 9, and 13 dpf, 5 ticks were dissected and midguts collected. Total RNA was extracted from tick midguts using the RNeasy kit (QIAGEN) and subjected to qRT-PCR assay using GST1 primers as shown in Table 4.1.

4.2.6. Glutathione S-transferase Enzymatic Assay

In order to validate whether DvGST1 protein was impacted in the midgut of DvGST1 dsRNA-injected ticks, 30 unfed ticks were injected with negative control dsRNA (15 ticks) or GST1 dsRNA (15 ticks) and fed on guinea pigs as described above. At 5 dpf, midguts were dissected from each tick and homogenized in 40 µl of GST sample buffer (BioVision) using plastic pestles. The tick midgut lysate was sonicated in an ice-bath sonicator (Crest Ultrasonic) for 30 min and centrifuged at 16,000 ×g at 4 °C for 30 min. The supernatant was transferred to a new tube, and protein concentration was determined using the Bradford protein assay (Bio-Rad). The tick midgut supernatant was diluted 1:10, and protein concentration was calculated using a BSA protein standard curve.

Protein (100 µg) from each sample was subjected to an enzymatic assay using a GST colorimatric activity assay kit (BioVision) according to the manufacturer's protocol. Reaction components including 1-Chloro-2, 4-dinirobenzone (CDNB), L-glutathione reduced (G-SH), and GST samples were prepared in 96-well plates, mixed, and gently shaken for 10 sec (Habig et al. 1974). The increased rate of a reaction product, glutathione-2, 4-Dinitorbenzene (G-SDNB), was determined by measuring absorbance at 340 nm every minute for 6 time points using a SpectraMax M2 microplate reader (Molecular Devices). The calculation of DvGST1 Vmax was calculated from the slope of the steepest line segment using SoftMax Pro software (Molecular Devices).

4.2.7. Rickettsial Challenge of DvGST1-silenced Ticks

To determine the effect of DvGST1 during rickettsial infection, DvGST1-silenced ticks were challenged with *R. amblyommii*. Two groups (16 ticks/group) of unfed female ticks were injected with negative control dsRNA (group 1, 8 ticks) or DvGST1 dsRNA (group 2, 8 ticks) and allowed to feed on guinea pigs for five days.

At 5 dpf, 3 ticks from each group were dissected and midguts were collected for qRT-PCR assay in order to confirm gene silencing of the DvGST1 gene in the tick population. Ticks (n = 5) were then capillary fed with *R. amblyommii* (10^9rickettsiae/ml) through a 50-µl microcapillary tube (KIMBLE) and stored in an environmental chamber at 27±1°C with 87±2% RH. After 16 h, ticks were washed with 1% bleach for 5 min and 70% ethanol for 5 min and rinsed with distilled H_2O and PBS. Tick tissues were recovered for DNA extraction and assessed with rickettsial dissemination. Two individual experiments were preformed.

4.2.8. Rickettsial Burden in Tick Tissues Using a qPCR Assay

In order to determine the rickettsial dissemination and burden in DvGST1-silenced ticks

after rickettsial challenge, ticks were dissected and specific tissues (hemolymph, salivary glands, midguts, ovaries) were recovered. Tick tissues were kept in ATL buffer (QIAGEN) for DNA extraction. gDNA from tick tissues was extracted using the DNeasy kit (QIAGEN) and utilized in *R. amblyommii* species-specific (Jiang et al. 2009) and *D. variabilis* calreticulin qPCR assays using a LightCycler® 480 II (Roche). The PCR reaction reagents were mixed in 96-well plates containing 2X LightCycler® 480 Probe Master (Roche), 0.2 µM OmpB*Ra*477F and OmpB*Ra*618R primers, 0.3 µM *Ra*OmpB_FAM probes, and 10 µl of cDNA template in a total reaction volume of 35 µl. Ten microlitres of each reaction mixture were transferred into 3 wells of a 384-well plate. All reactions were run with the following program: a pre-incubation step of 95 °C for 10 min, 45 amplification cycles of 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 1 sec. All primers and probes are shown in Table 4.1.

4.2.9. DvGST2 Gene Expression in Response to Rickettsial Challenge

To examine the gene expression of DvGST2 during rickettsial challenge, partially fed female ticks were divided to four feeding groups (Group1: 1:125 diluted bovine blood, Group 2: *R. amblyommii*, Group 3: *R. montanensis* and Group 4: combined *R. amblyommii* and *R. montanensis*). Ticks were restrained on double-sided adhesive foam in a glass Petri dish and allowed to imbibe the feeding medium through a 50-µl microcapillary tube (KIMBLE) for 16 h. All ticks were detached from the adhesive form, sterilized by 1% bleach and 70% ethanol, and rinsed with distilled H_2O and PBS. Three ticks from each group were dissected to collect hemolymph and remove salivary gland, midgut, and ovary tissues (Macaluso et al. 2001). Tick salivary glands, midguts, and ovaries were rinsed in PBS three times to remove hemocytes. Dissected tissues from three ticks were pooled into the same tube containing 600 µl of RLT plus buffer (QIAGEN) and stored at -80°C until used for nucleic acid isolation.

4.2.10. Quantitative Reverse Transcription-PCR (qRT-PCR) of Tick Gene Expression

Total RNA and gDNA were extracted from dissected tick tissues (hemolymph, salivary gland, midgut, ovary) using the ALLPrep DNA/RNA Mini kit (QIAGEN) according to the manufacturer's protocol, treated with 2 units of DNase (Ambion) for 1 h, and purified using the RNA cleanup kit (Zymo Research). Two hundred microgram of DNase-treated RNA was used for cDNA synthesis using the iScript reverse transcription kit (Bio-Rad) according to the manufacturer's protocol.

The PCR component including 2 µl of cDNA template, 2X LightCycler® 480 SYBR Green I master (Roche) and 0.1 µM forward and reverse primers in 35-µl final volume was mixed in 96-well plates and transferred in triplicate 10 µl reactions on 384-well plates. The qPCR was subjected to 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 5 s; and a final cycle at 95°C for 5 sec and 65°C for 1 min and performed with a LightCycler® 480 II (Roche). Gene expression for actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were used as reference genes in order to normalize the cDNA template. Gene expression analysis was done using LightCycler® 480 (1.5.0) software (Roche) using advanced relative quantification analysis (Efficiency method, a modified $\Delta\Delta C_T$ method, Roche). The relative gene expression of control unchallenged ticks was used to normalize those of *Rickettsia*-challenged ticks to generate the fold difference. Tick gene specific primers are listed in Table 4.1.

4.2.11. Statistical Analysis

Prism GraphPad software (version 5) was used to examine the differences in gene expression and GST enzymatic activity. Analysis of variance was used to examine potential differences between the four groups of ticks (unchallenged, *R. amblyommii*-challenged, *R.*

montanensis-challenged, and *R. amblyommii* and *R. montanensis*-challenged) and three groups of ticks used in RNAi (TE buffer-, negative control dsRNA-, and DvGST1 dsRNA-injected ticks). When overall significance was found, Tukey's multiple comparison post-hoc test was used to determine pair-wise differences. The unpaired *t*-test was used to analyze significant differences between negative control dsRNA- and DvGST1-injected ticks. All bioassays were done two times and all results are presented as the mean ± SEM, and a P value of ≤ 0.05 was considered significantly difference.

4.3. Results

4.3.1. DvGST1 Silencing in *Dermacentor variabilis*

To knockdown DvGST1 gene expression, DvGST1 dsRNA was injected into female *D. variabilis*, and injected ticks were allowed to feed on guinea pigs. At 2 dpf, 5 ticks were forcibly detached from the guinea pigs and midguts were recovered and pooled for transcriptional analysis. The remaining ticks were allowed to feed until day 5. Five ticks from each group were dissected at 5, 9, and 13 dpi, and qRT-PCR assay was used to determine the DvGST1 mRNA expression. The results demonstrated that mRNA expression of DvGST1 in DvGST1 dsRNA-injected tick began to significantly decrease at 5 dpf compared to TE and negative dsRNA–injected ticks, and stayed to that level until day 13 (Figure 4.3). At 9 dpf decreasing of DvGST1 mRNA level in DvGST1 dsRNA-injected ticks was significantly different from negative dsRNA- injected ticks but not TE-injected ticks. There was no significant difference of DvGST1 mRNA expression among injected ticks at 13 dpf.

4.3.2. GST Activity in GST1-silenced *Dermacentor variabilis*

In order to verify if the GST1 protein expression was modified in GST1-silenced ticks, tick midgut protein from GST1-silenced ticks were extracted and a GST enzymatic assay was

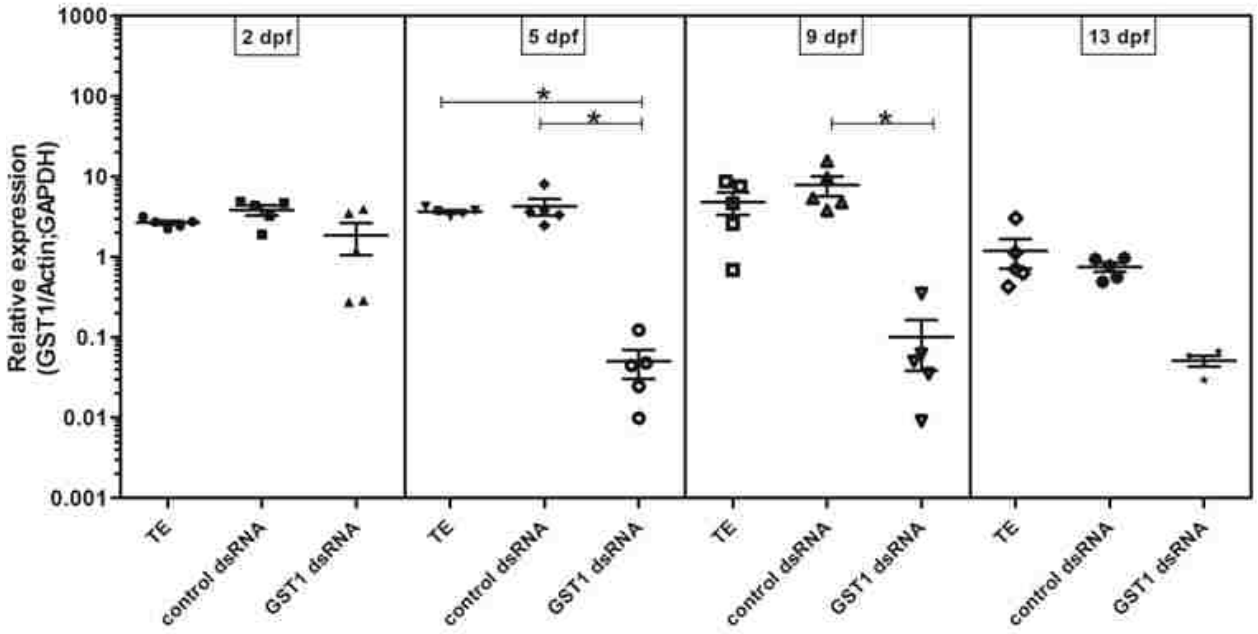

Figure 4.3. Silencing of *Dermacentor variabilis* GST1 gene in tick midguts. Unfed female *D. variabilis* was injected with negative control (GFPuv) and DvGST1 dsRNAs and allowed to feed on guinea pigs for 2-5 days. At 2, 5, 9, and 13 days post-feeding (dpf), tick midguts were recovered and performed qRT-PCR assay to determine the DvGST1gene expression. Data represent mean ± SEM. Each symbol represents one tick (n = 5). The asterisk indicates significant difference ($P \leq 0.05$).

performed. A significant decrease of Vmax values indicating G-SDNB, a GST-conjugated product, was observed in DvGST1 dsRNA-injected ticks, compared to negative control dsRNA-injected ticks, confirmed that the silencing of GST1 gene using dsRNA injection resulted in decreased GST1 function in tick midgut (Figure 4.4).

4.3.3. Effect of Tick GST1 Silencing in Response to Rickettsial Infection in *Dermacentor variabilis*

In order to determine the effect of DvGST1 during atypical rickettsial infection, unfed female *D. variabilis* was injected with negative control dsRNA or DvGST1 dsRNA and challenged with *R. amblyommii*. Prior to rickettsial challenge, injected ticks were allowed to feed on guinea pigs for five days. The ticks were detached from guinea pigs and the silencing of DvGST1 gene expression in tick midguts was determined using a qRT-PCR assay. A significant decrease in DvGST1 mRNA level of DvGST1 dsRNA-injected ticks was identified, and compared to negative control dsRNA-injected ticks (Figure 4.5).

Negative control and DvGST1 dsRNA-silenced ticks were challenged with *R. amblyommii* for 16 h in environmental chamber. Challenged ticks were dissected, and rickettsial burden in tick tissues was determined using a qPCR assay. In experiment one, 80% (4/5) of the ticks from the negative control dsRNA-injected group were infected with *R. amblyommii* and 60% (3/5) of the DvGST1 dsRNA-injected ticks were infected with *R. amblyommii*. In experiment two, 40% (2/5) of the negative control dsRNA-injected ticks and 80% (4/5) of the DvGST1 dsRNA-injected ticks were infected with *R. amblyommii* (Table 4.2). The infection of specific tick tissues was independent of the treatment as tick ovaries from negative control dsRNA-injected ticks but not DvGST1 dsRNA-injected ticks in both experiment 1 and experiment 2 were infected with *R. amblyommii*. Conversely, *R. amblyommii* infection was

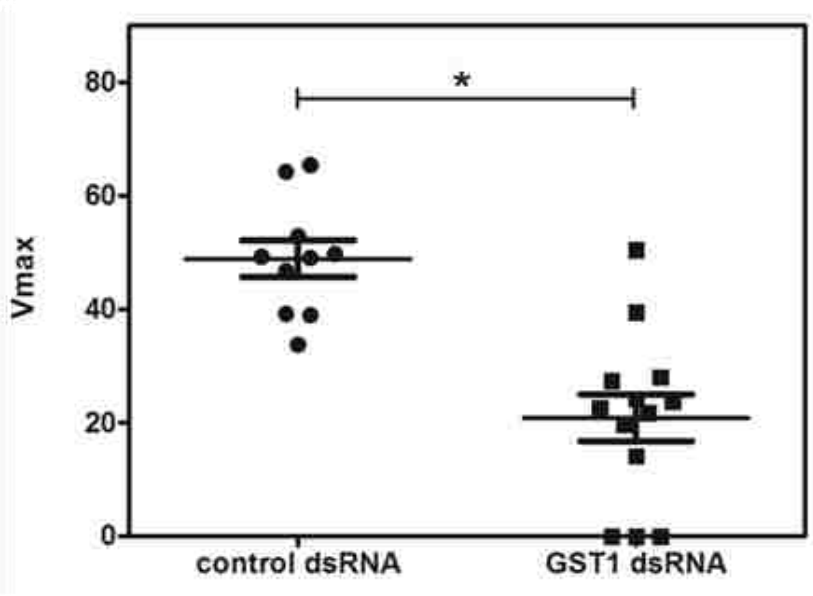

Figure 4.4. GST enzymatic assay of GST1-silenced *Dermacentor variabilis*. Unfed female *D. variabilis* was injected with negative control (GFPuv), n = 10 and DvGST1 dsRNAs, n = 13 and allowed to feed on guinea pigs for 5 days. Tick midguts were dissected and performed protein extraction. One hundred microgram of tick midgut protein extract was subjected to GST enzymatic assay to determine DvGST1 activity. Data represent mean ± SEM. Each symbol represents one tick. The asterisk indicates significant difference ($P \leq 0.05$).

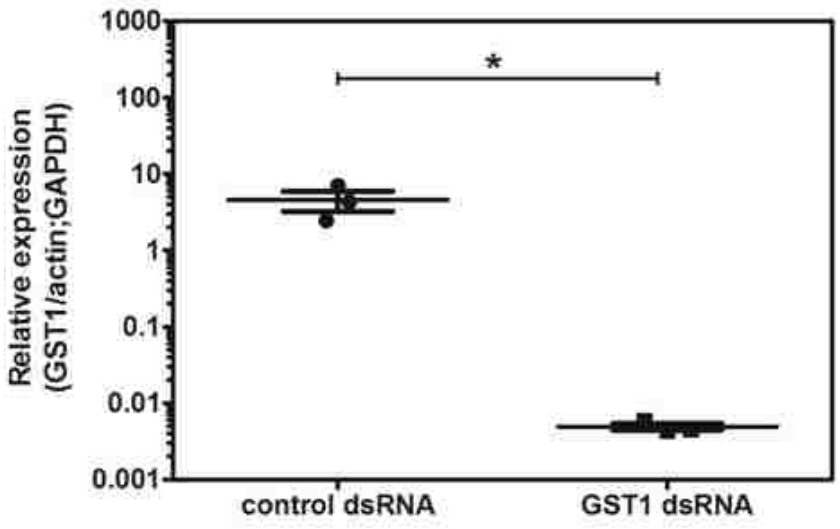

Figure 4.5. Confirmation of DvGST1 silencing in dsRNA-injected ticks. Silencing of DvGST1 genes was confirmed in DvGST1dsRNA-injected ticks using a qRT-PCR assay prior to rickettsial challenge. Data represent mean ± SEM. Each symbol represents one tick (n = 3). The asterisk indicates significant difference ($P \leq 0.05$).

Table 4.2. Copy number of rickettsial outer membrane protein B gene relative to 10^8 copies of tick calreticulin gene in DvGST1-silienced ticks. DvGST1-silenced ticks or the negative control dsRNA-injected ticks were challenged with *R. amblyommii* for 16 h. Tick tissues were recovered and subjected to a qRT-PCR assay (shade boxed represent infected ticks)

Gene silencing ticks		Group 1 Negative control dsRNA-injected ticks					Group 2 GST1 dsRNA-injected ticks				
Experiment one	Tick tissue	1	2	3	4	5	6	7	8	9	10
	HL		1.0E+07	2.4E+06	4.0E+06	3.2E+06		1.0E+06	3.1E+06	1.5E+06	
	SG										
	MG				1.3E+05						
	OV				3.7E+05						
Experiment two	Tissue	1	2	3	4	5	6	7	8	9	10
	HL				2.0E+07	1.8E+06	1.6E+06				4.5E+06
	SG								7.9E+04		
	MG				2.0E+05					1.2E+06	
	OV				1.6E+07						

HL = hemolymph
SG = salivary gland
MG = midgut
OV = ovary

detected in salivary glands of DvGST1 dsRNA-injected ticks, but not negative control dsRNA-injected ticks.

4.3.4. DvGST2 Gene Expression in Response to Rickettsial Challenge

In order to determine whether DvGST2 is a specifically response to *R. amblyommii* challenge, similar to DvGST1, partially fed female ticks were challenged with *R. amblyommi, R. montanensis,* or combined *R. amblyommii* and *R. montanensis.* Total RNA was extracted from tick tissues and subjected to qRT-PCR assay using DvGST2 primers. The results demonstrated that DvGST2 is constitutively expressed in tick ovaries, and the DvGST2 gene expression was low in tick salivary glands and undetectable in tick hemolymph and midgut. Consequently, data shown is only from tick ovaries, and there is no difference of DvGST2 expression during rickettsial challenge (Figure 4.6).

4.4. Discussion

A number of tick-derived immune-like molecules have been identified from *Rickettsia*-infected ticks using differential expression analysis. In Chapter 3, a specific association of tick molecules and atypical rickettsial infection was identified. DvGST1 was shown to be responsive to infection with *R. amblyommii*, an atypical *Rickettsia* for that tick host species. In order to gain insight into specific mechanism of GSTs in *D. variabilis* during rickettsial infection, the present study examines the functional activity of DvGSTs. The gene expression of DvGST2, the second isoform in tick ovaries, in *D. variabilis* was not altered during rickettsial challenge. RNAi-mediated gene silencing of DvGST1 resulted in a significant decrease in DvGST1 transcript in *D. variabilis* midguts. Additionally, GST activity in tick midguts was also decreased significantly, compared to negative control ticks when the gene was silenced. However, using RNAi-mediated DvGST1 silencing demonstrated no difference in rickettsial infection between

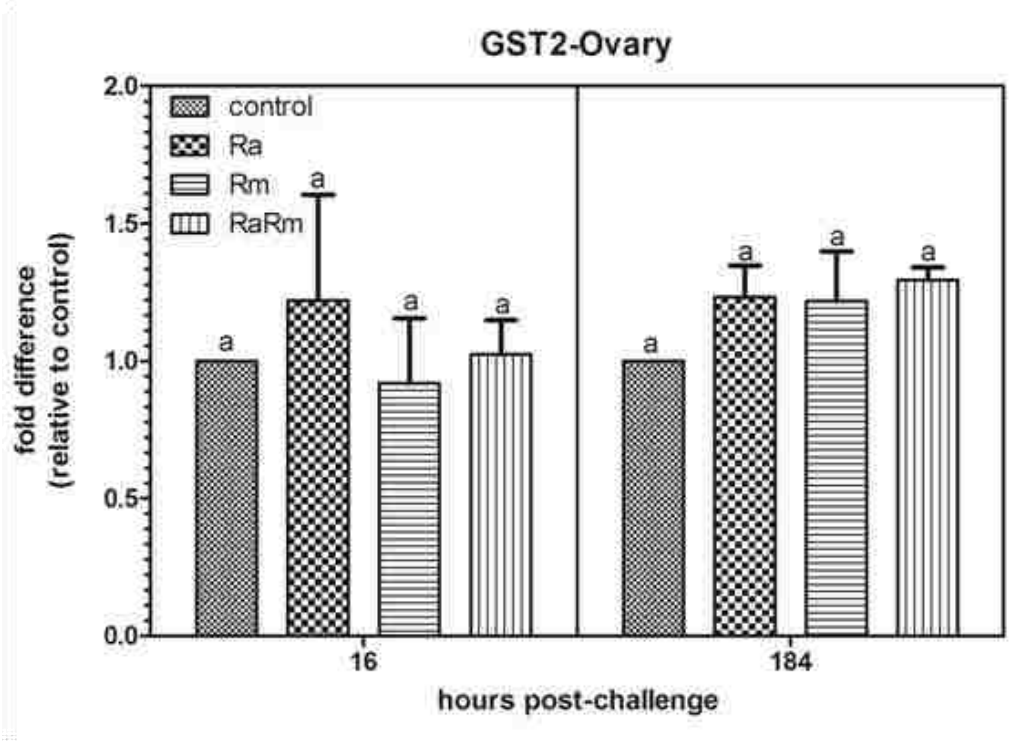

Figure 4.6. *Dermacentor variabilis* GST2 gene expression in response to rickettsial challenge. Total RNA was extracted from tick ovaries and assayed by qRT-PCR assay. Transcription level of tick immune genes was normalized to actin and glyceraldehyde 3-phosphate dehydrogenase transcripts. Data shown are fold difference relative to unchallenged ticks. Error bar represents standard error of means (SEM). The bars with same letter are not significantly different ($P \leq 0.05$). Control: unchallenged tick, Ra: *R. amblyommii*-challenged ticks, Rm: *R. montanensis*-challenged ticks, RaRm: combined *R. amblyommii* and *R. montanensis*-challenged ticks.

negative control and DvGST1 dsRNA-injected ticks. Surprisingly, *R. amblyommii* was detected in tick ovaries when ticks are injected with negative control dsRNA (GFPuv).

GSTs are multifunctional enzymes, and their isoforms have distinct function (Rosa de Lima et al. 2002) including intracellular transportation, digestive processes, and prostaglandin synthesis. Most ticks have more than one isoform of GST; however, their function has not been well-defined. One isoform of GST was identified from *Haemaphysalis longicornis* and *Rhipicephalus appendiculatus* (da Silva, Jr. et al. 2004), and there are two GSTs (He et al. 1999; Rosa de Lima et al. 2002) in *Boophilus microplus*. Additionally, tissue-specific isoforms have been described as a single isoform and were identified in *B. microplus* larvae and a second isoform was identified from adult salivary glands; however, their function has not been characterized (da Silva, Jr. et al. 2004). In *D. variabilis*, two isoforms were identified from midguts and ovaries (Dreher-Lesnick et al. 2006), and the current study demonstrated that both isoforms may have a different function. In Chapter 3, DvGST1 in tick midgut specifically responded to atypical *Rickettsia* and may play a role in controlling rickettsial infection, while DvGST2 in tick ovaries was not responsive to rickettsial infection and may be involved in tick oogenesis (Dreher-Lesnick et al. 2006).

In ticks, RNAi-mediated gene silencing has been used widely to functionally characterize the influence of genes of interest in many circumstances such as bloodfeeding (Mulenga and Khumthong 2010a, 2010b), pathogen invasion (Ceraul et al. 2011), and pathogen transmission (Dai et al. 2010). In this study, RNAi was used to silence DvGST1 gene expression in *D. variabilis*. The gene expression of DvGST1 began to decrease at 5 dpf and stayed to that level until day 13; however, only at 5 and 9 dpf was DvGST1 gene expression was significantly decreased compared to TE- and negative control dsRNA-injected ticks. Decrease of gene

transcription in those control ticks may be due to the fact that after 5 dpf all ticks were removed from guinea pigs and kept in an environmental chamber; therefore, there may have been a decrease in metabolism such as lipid and protein synthesis in order to maintain energy when food/nutrients are limited.

When the target and control dsRNA-injected ticks were challenged with *R. amblyommii* there was no difference in rickettsial infection between the two groups. In contrast, 20% of the tick ovaries from GFPuv dsRNAs-injected ticks were infected with *R. amblyommii*. This suggested that the injection of GFPuv, but not DvGST1, dsRNAs facilitated rickettsial infection in tick ovaries. This may be the off-target effect of dsRNA of GFPuv which generates many of small interfering RNAs (siRNA), and those siRNAs may non-specifically interfere with the gene expression of tick molecules that are related to rickettsial control; however, this requires further examination. The unexpected effect of pathogen infection in *D. variabilis* using RNAi has been shown previously in defensin1-silenced ticks (Kocan et al. 2008) in which it was expected that silencing of defensin1 gene expression would result in an increase of *Anaplasma marginale* infection in ticks. However, it was reported that *D. variabilis* males injected with defensin1 dsRNA and challenged with *A. marginale* had a significant reduction of *A. marginale* organisms in those defensin1 silenced ticks suggesting a role of defensin in *A. marginale* infection and replication (Kocan et al. 2008). The alternative explanation is that the off-target effect of dsRNA results in unexpected changes of the gene expression of off-target genes (Kocan et al. 2008). RNAi using long dsRNAs (300-800 bp) was commonly used previously in *Drosophila melanogaster* and *Caenorhabditis elegans* (Seinen et al. 2010), and is considered a very powerful method; however, using long dsRNAs increases the risk of off-target effects (Seinen et al. 2010). A number of RNAi studies report a drawback of RNAi by off-target effect

which cannot be ignored (Seinen et al. 2010); thus, this needs consideration in interpretation of data.

Characterization of *D. variabilis* GSTs in response to atypical rickettsial infection suggested a different role for the two GST isoforms. DvGST1, midgut isoform, specifically responded to atypical rickettsial infection while DvGST2, ovary isoform, did not. Functional study of DvGST1 using RNAi demonstrated that silencing of DvGST1 had no effect on atypical rickettsial infection compared to negative control ticks which appeared to have an off-target dsRNA effect. In order to verify and further characterize the function of DvGST1 in *D. variabilis*, alternative methods to fully understand this interaction are needed. Studying the molecular mechanisms of tick immune molecules and *Rickettsia* will lead to a better understanding of vector competence of ticks for *Rickettsia*.

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CHAPTER 5
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

5.1 Discussion of Results and Future Directions

Ticks are second only to mosquitoes as disease vectors, which can transmit a variety of pathogens to humans and animals, for example, viruses, bacteria, and fungi (Sonenshine 1993). In the United States, Lyme disease is the most common vector-borne disease; however, an increase of tick-borne rickettsial diseases has also been reported in North America, South America, and Europe (Dumler 2010). A recent report by the Center for Disease Control and Prevention documents a continuous increase in tick-borne rickettsioses since 1998, and up to 2,500 cases of Rocky Mountain spotted fever (RMSF) (Dumler 2010). However, field surveys for the disease agent, *Rickettsia rickettsii*, in arthropod vectors including *Dermacentor* ticks demonstrate very low prevalence of infection even in endemic areas (Ammerman et al. 2004; Stromdahl et al. 2010). Additionally, a recent outbreak of RMSF resulted in the identification of a previously unrecognized vector of *R. rickettsii*, *Rhipicephalus sanguineus*, suggesting a more complex transmission cycle of RMSF in the United States. The confounding factors of limited prevalence of *R. rickettsii* in foci of RMSF and recognition of new arthropod vectors demand a fresh look at the interaction between ticks and *Rickettsia*. Thus, in order to better understand the ecology and epidemiology of tick-borne rickettsioses the overall goal of this study was to delineate mechanisms of vector competence of ticks for *Rickettsia*. Towards this objective, experiments were designed to assess tick response to rickettsial infection (atypical and typical *Rickettsia*) and identify/characterize the key tick-derived molecules that mediate the specificity of the tick/*Rickettsia* interaction.

For spotted fever group (SFG) *Rickettsia*, ticks are not only horizontal transmission vectors but also reservoir hosts which can maintain *Rickettsia* via vertical transmission. In the

United States, the distribution of most SFG *Rickettsia* is limited to the distribution of the principle tick host; thus the vertical maintenance of SFG *Rickettsia* is thought to be specific. To elucidate the molecular mechanisms of the tick/*Rickettsia* relationship, many studies have identified tick-derived molecules that are differentially expressed during rickettsial infection. Identified tick molecules have been classified by their predicted functions, for example, receptor/adhesion, tick immune and stress response, and tick-host interaction proteins (Macaluso et al. 2003, 2006; Mulenga et al. 2003). Prior to the current study, functional characterization of these putative tick proteins, as they relate to vector competence, has not been identified.

Dermacentor variabilis α-catenin (Dvα-catenin) was previously demonstrated to be upregulated in ovaries of ticks chronically infected with *Rickettsia montanensis*, compared to uninfected ticks (Macaluso et al. 2003, 2006). Alpha-catenin is a known cytoskeleton protein that forms E-cadherin-dependent cell-cell adhesion complex via β-catenin and also binds actin filament (Hartsock and Nelson 2008). Therefore, it is speculated that Dvα-catenin may play a role in actin rearrangement during rickettsial infection. In the current study, to begin the characterization of the role of Dvα-catenin in rickettsial infection, full-length Dvα-catenin cDNA was cloned and expressed in a Baculovirus expression system. Comparative sequence analysis demonstrated that this novel sequence was most similar to *Ixodes scapularis* α-catenin and is conserved among many species. A portion of Dvα-catenin is homologous to the vinculin conserved domain containing a putative actin binding region at the C-terminus and a β-catenin binding and dimerization regions at the N-terminus. Quantitative RT-PCR and western blot analysis demonstrated that Dvα-catenin was predominantly expressed in tick ovaries compared to other tick tissues. In order to determine the specific response of Dvα-catenin gene expression to atypical (*R. amblyommii*) and typical (*R. montanensis*) *Rickettsia* in a tissue-specific manner,

an *ex vivo* study of tick tissues (backless tick culture) was developed due to a lack of specific tick tissue cell lines. Dvα-catenin gene expression was significantly downregulated at 12 hours post-inoculation in *R. montanensis*- but not in *R. amblyommii*-infected ovaries compared to control unexposed ticks, suggesting a specific response of the tick to specific rickettsial species. This study demonstrated that utilizing backless tick culture was viable for tissue-specific analysis in ticks; however, the incubation times are limited due to the nature of cell viability and contamination issues. Therefore, it is difficult to examine rickettsial multiplication and dissemination in tick tissues using backless tick culture; however, the acute response to rickettsial infection is measurable.

Ticks acquire rickettsial infection while taking bloodmeal, which can take several days for female ticks and other immature life cycle stages (Sonenshine 1993). During bloodmeal acquisition, tick midgut which is the first site of contact, encounters both the ingested bloodmeal and also any microbes in the host blood. Global gene expression analysis of *D. variabilis* midgut during bloodfeeding has demonstrated that the expression profile in tick midgut dramatically changes beginning at day 2 and extends through day 6 (Anderson et al. 2008). Most transcripts were identified as proteins likely related to bloodmeal digestion including oxidative stress reduction/detoxification enzymes, peptidase inhibitors, protein digestion enzyme, cell/lipid/protein binding proteins, and antimicrobial molecules (Anderson et al. 2008). Similarly, the current study also demonstrated that at 5 day post-feeding (5 dpf) selected tick immune gene expression was upregulated, with the exception of lysozyme. However, in this study there was only one time point collection (5 dpf); therefore, the dynamics of gene expression of tick midgut during bloodfeeding were not fully defined.

The current study is the first to examine the dissemination of atypical and typical

Rickettsia using the capillary feeding technique (CFT), an artificial infection system that is similar to the natural route of rickettsial infection acquired from vertebrate hosts. Using Rhodamine B as a biomarker to evaluate the efficacy of CFT, demonstrated that 70% of ticks ingested the feeding medium. Although the volume uptake by ticks was variable, ranging between 0.06 and 6.77 µl (Macaluso et al. 2001); CFT has provided an effective artificial feeding system for a number of studies examining tick and *Rickettsia* interactions (Ceraul et al. 2007, 2008, 2011; Macaluso et al. 2003). *Rickettsia*-free female *D. variabilis* were challenged with *R. amblyommii, R. montanensis, R. rickettsii*, or a combination of *R. amblyommii* and *R. montanensis* using CFT for 16 h. Persistent infection of typical *Rickettsia* (i.e. *R. montanensis* and *R. rickettsii*) but not atypical *Rickettsia* (i.e. *R. amblyommii*), was observed and only *R. montanensi*s disseminated to tick ovaries, a prerequisite for vertical transmission. Interestingly, the data suggested that at16 hours post-challenge (hpc), the tick ovaries were infected with *R. montanensis*; however, the rickettsial infection of tick ovaries was not persistent as no rickettsial infection was detected in tick ovaries at later time points through 184 hours. The biological significance of these findings is not clear and the mixed results may be due to the limitation of tick artificial feeding. In nature, female ixodid ticks take several days to complete their bloodmeal, but in this study ticks were allowed to feed through capillary glass tube for only 16 h. The differences in feeding duration between natural feeding and CFT, including physiological differences associated with ovarian development, make the comparison between rickettsial dissemination *ex vivo* and rickettsial infection of ticks in nature difficult. Nevertheless, the aim of this study was to compare rickettsial dissemination between atypical and typical *Rickettsia*, and there are no laboratory animal models for nonpathogenic *Rickettsia* (i.e. *R. amblyommii*, and *R. montanensis*) available. Therefore, CFT serves as an appropriate tool to capture short

windows of rickettsial infection. Inactivation and reactivation of *Rickettsia* have been previously demonstrated in *R. rickettsii*-infected *D. andersoni* (Hayes and Burgdorfer 1982) and rickettsial activity is likely correlated to tick metabolism (Munderloh and Kurtti 1995); therefore, another explanation for non-persistent rickettsial infection in tick ovaries may be due to decreased tick metabolism as a means to reserve nutrients during off-host periods (Needham and Teel 1991). Thus, the decrease in metabolism in the tick may directly result in decreased rickettsial replication and successful dissemination. Subsequent studies allowing ticks to feed on hosts after rickettsial challenging are needed in order to assess rickettsial dissemination and amplification during the active feeding period.

The results of the current study suggest that *R. montanensis,* a typical nonpathogenic *Rickettsia,* and *R. rickettsii,* a typical pathogenic *Rickettsia,* are able to persistently infect *D. variabilis,* compared to atypical *Rickettsia*. However, the comparison of rickettsial dissemination between typical nonpathogenic and typical pathogenic *Rickettsia* was not determined in the current study. It has been demonstrated that the infection of nonpathogenic SFG *Rickettsia, R. peacockii*, in *D. andersoni* blocks transovarial transmission of pathogenic *Rickettsia, R. rickettsii* (Burgdorfer et al. 1981). However, it is not known whether nonpathogenic *Rickettsia* disseminates better in ticks compared to pathogenic *Rickettsia*; therefore, dual rickettsial challenge with combined *R. montanensis* and *R. rickettsii* needs to be examined.

In addition to rickettsial dissemination, the current study is also the first demonstration of specific tick response to atypical and typical rickettsial infection. Tick transcription of immune-related molecules was assessed simultaneously with rickettsial dissemination analysis. Comparative analysis of four target tick immune factors including defensin1, lysozyme,

glutathione S-transferase1 (DvGST1), and Kunitz protease inhibitor (KPI) were examined in *R. amblyommii*-, *R. montanensis*-, *R. rickettsii*-, and combined *R. amblyommii* and *R. montanensis*-challenged ticks compared to unchallenged ticks. The results demonstrated that only DvGST1 gene expression was specifically upregulated in response to atypical rickettsial challenge in the tick midgut at 184 hpc. It would be interesting to examine the DvGST1 expression in response to other atypical rickettsial species, for example, *R. parkeri*. Ticks may or may not respond in the same way, and it is not known how a tick recognizes atypical *Rickettsia*. In order to fully understand the specific interaction between ticks and *Rickettsia*, rickettsial factors facilitating tick infection should be identified and it should be determined if these factors are SFG species-specific. Likewise, it is realized that the current study examined a limited set of target molecules. While there is a logical progression towards the selection of target genes in the current study, global gene expression via transcriptional analysis using a mRNA microarray is required to better characterize the specificity of the tick response. However, due to the ongoing *D. variabilis* genome project (Pagel Van et al. 2007) it is not possible to do mRNA microarray for *D. variabilis* at this time.

Glutathione S-transferases are multifunctional enzymes that play a role in endogenous and xenobiotic detoxification (Enayati et al. 2005; Wilce et al. 1995). In arthropods, their association with pesticide resistance is well-studied (Mounsey et al. 2010; Sharp et al. 1991). In *D. variabilis*, GST (DvGST1) activation in response to atypical rickettsial infection was observed in the current study. However, a second isoform, DvGST2, which is highly expressed in tick ovaries was not responsive to rickettsial challenge. In the current study, the functional characterization of DvGST1 was examined using RNA inference (RNAi). In treated ticks, mRNA expression analysis and enzymatic assays demonstrated significant reduction of DvGST1

transcription and enzymatic activity, compared to negative control ticks. However, when dsRNA-injected ticks were challenged with *R. amblyommii,* 60% and 70% of negative control and DvGST1-silenced ticks are infected with, respectively, making it difficult to unequivocally implicate this molecule in rickettsial infection. Interestingly, in the negative control group, *R. amblyommii* was able to disseminate to tick ovaries which might be due to the off-target effect of GFPuv dsRNA which was used as a negative dsRNA. Therefore, a new negative control dsRNA, not displaying an off-target effect, is needed and the bioassays repeated. Alternatively, in order to further examine the effect of DvGST1 during atypical rickettsial infection, a DvGST1-expressing cell line can be utilized to assess the influence of DvGST1 on survival and growth of atypical *Rickettsia*.

The increasing reports of RMSF cases (Dumler 2010) is in contrast to the very low prevalence of *R. rickettsii*-infected *Dermacentor* ticks in nature (Ammerman et al. 2004; Stromdahl et al. 2010), thus, the confounding principles of tick-borne rickettsial diseases should be better defined. Likewise, the study of tick/*Rickettsia* relationships is needed in order to better understand ecology and epidemiology of tick-borne rickettsioses. The research in this dissertation sought to identify and delineate some specific mechanisms of the tick during rickettsial infection that may contribute to vector competence for *Rickettsia*. Using novel or modified bioassays, several aspects of the relationship were examined. Results of the current study suggest that Dvα-catenin may play a role in controlling rickettsial infection in tick ovaries. Also, rickettsial dissemination in ticks favors typical *Rickettsia* (i.e. *R. montanensis* and *R. rickettsii*) resulting in persistent infection of *D. variabilis*. During rickettsial challenge, DvGST1 was specifically upregulated in response to atypical *Rickettsia* suggesting a role of DvGST1 in controlling atypical rickettsial infection; however, the function of DvGST1 has to be further

characterized. This current study has identified and characterized tick molecular candidates that may play an important role in rickettsial infection and contribute to vector competence of the tick for SFG *Rickettsia*.

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APPENDIX A
COMMONLY USED ABBREVIATIONS

AG – Ancestral group

AMP – Antimicrobial peptide

Arp2/3 – Actin related protein 2/3

cDNA – Complementary DNA

CDNB – 1-Chloro-2, 4-dinitrobenzene

CFT – Capillary feeding technique

CRT – Calreticulin

C_T – Threshold cycle

Da - Dalton

DEPC – Diethylpyrocarbonate

DNA – Deoxyribonucleic acid

dpf – Days post-feeding

dsRNA – Double-stranded RNA

EspB – *E. coli* secreted protein B

F-actin – Filamentous actin

FAK – Focal adhesion kinase

FAM – Fluorescein amidite

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

gDNA – Genomic DNA

GFP – Green fluorescent protein

G-SDNB – Glutathione-2, 4-Dinitrobenzene

G-SH – L-glutathione reduced

GSP – Gene specific primer

GST – Glutathione s-transferase

HEX – Hexachloro-fluorescein

HGA – Human granulocytic anaplasmosis

HME – Human monocytic ehrlichiosis

hpc – Hours post-challenge

hpi – Hours post-inoculation

HSD – Honestly significant difference

IAFGP – Ixodes scapularis antifreeze glycoprotein

ISE6 – Ixodes scapularis cell line

KPI – Kunitz protease inhibitor

Ku70 - subunit of DNA-dependent protein kinase

LPS - lipopolysaccharide

LSU – Louisiana State University

MAPK – Mitogen activated protein kinase

MOI – Multiplicity of infection

mRNA – Messenger RNA

MSF – Mediterranean spotted fever

NP-40 – Nonidet P40

N-terminus – Amino-terminus

ODU – Old Dominion University

OmpA – Outer membrane protein A

OmpB – Outer membrane protein B

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PGRP – Peptidoglycan recognition protein

PIC – Protease inhibitor cocktail

PM – Peritrophic membrane

PPR – Pattern recognition receptor

PVDF – Polyvinylidene fluoride

PWV – Powassan virus

qRT-PCR – Quantitative reverse transcriptase PCR

RACE – Rapid amplification of cDNA ends

RH – Relative humidity

RMSF – Rocky Mountain spotted fever

RNA – Ribonucleic acid

RNAi – RNA interference

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of means

SFG – Spotted fever group

TBRD – Tick-borne rickettsial diseases

TBST – Tris-buffered saline containing Tween-20

TE – Tris-EDTA

TG – Typhus group

TRG – Transitional group

W/V – Weight/volume

APPENDIX B
REAGENTS AND PROTOCOLS

1. Cell culture media and reagents

1.1 Mineral stock A (100 ml)

Dissolve the following component in 90 ml Milli-Q water, bring final volume to 100 ml, aliquot and store at -20°C.

Mineral Stock A component	Amount
$CoCl_2.6H_2O$	0.020 g
$CuSO_4.5H_2O$	0.020 g
$MnSO_4.H_2O$	0.160 g
$ZnSO_4.7H_2O$	0.200 g

1.2 Mineral stock B (100 ml)

Dissolve 0.02 gram of $NaMoO_4.2H_2O$ in 100 ml Milli-Q water, aliquot and store at -20°C.

1.3 Mineral stock C (100 ml)

Dissolve 0.02 gram of Na_2SeO_3 in 100 ml Milli-Q water, aliquot and store at -20°C.

1.4 Mineral stock D (50 ml)

Dissolve the following in 30 ml Milli-Q water, adjust to50 ml and sterile using filter unit.

Mineral Stock D component	**Amount**
L-Ascorbic acid	0.501 g
L-Glutathione acid	0.512 g
$FeSO_4.7H_2O$	0.028 g
Mineral stock A	0.5 ml
Mineral stock B	0.5 ml
Mineral stock C	0.5 ml

1.5 Vitamin stock (50 ml)

Dissolve the following in 30 ml Milli-Q water, adjust to50 ml and sterile using filter unit.

Mineral Stock D component	Amount
p-aminobenzoic acid	0.100 g
Cyanocobalamin (B12)	0.050 g
d-Biotin (SUPELCO)	0.10 g

1.6 L15B medium (2 liters)

- The following ingredients are added to a 2 liters volumetric flask containing approximately 1600 ml of Milli-Q water.

L15 medium component	Amount
L15 powder (2 L)	27.84 g
L-aspartic acid	0.596 g
L-glutamine	0.584 g
L-Proline	0.6 g
L-Glutamic acid	1.0 g
α-ketoglutaric acid	0.598 g
D-(+)-glucose	28.821 g
Mineral solution D	2 ml
Vitamin solution	2 ml

* All chemicals are cell culture grade (Sigma)

- Bring final volume to 2 liters, stir gently for 1.5 h protected from light at room temperature.
- Filter sterile using 0.22 m filter unit (Millipore) and store at 4°C until used.

1.7 Complete L15B medium for ISE6 cells

- Dissolve 1.475 g of Tryptone phosphate broth (BD) in 50 ml of Milli-Q water and sterile by autoclaving.
- Add 10 ml of heat-inactivated FBS (HyClone) and 10 ml of Tryptone phosphate broth (BD) in 80 ml of L15B medium described above.
- Adjust the pH to 7 with 0.6 ml 1 N NaOH
- Store medium at 4°C. Do not store for more than one month.

2. *Rickettsia* purification using glass beads

2.1 *Rickettsia*-infected cells are harvested using a cell scraper.

2.2 Cell suspension is transferred to an Erlenmeyer flask containing sterile 3-mm borosilicate glass beads (Sigma) for 3 min.

2.3 Cell lystaes are transferred to 50 ml centrifuge tube and centrifuge at 4°C and 300 × *g* for 5 min to pellet cellular debris.

2.4 The supernatants are filtered through a 2-μm-pore-size (Whatman)

3. *Rickettsia* counting

3.1 lyse cells using a 5CC syringe attached 27 g needle (at least 5 times)

3.2 Centrifuge at 275 x *g* at 4^{o}C for 10 min.

3.3 Filter with sterile 2 micron filter (Whatman *PURADISC 25 G).*

3.4 Take 100 µl, and transfer to 1.5 ml tube and spin at 16,000 ×*g* at 4^{o}C for 10 min.

3.5 Resuspend *Rickettsia* pellet with 500 µl 0.85% NaCl, and spin at 16,000 ×*g* at 4^{o}C for 10 min.

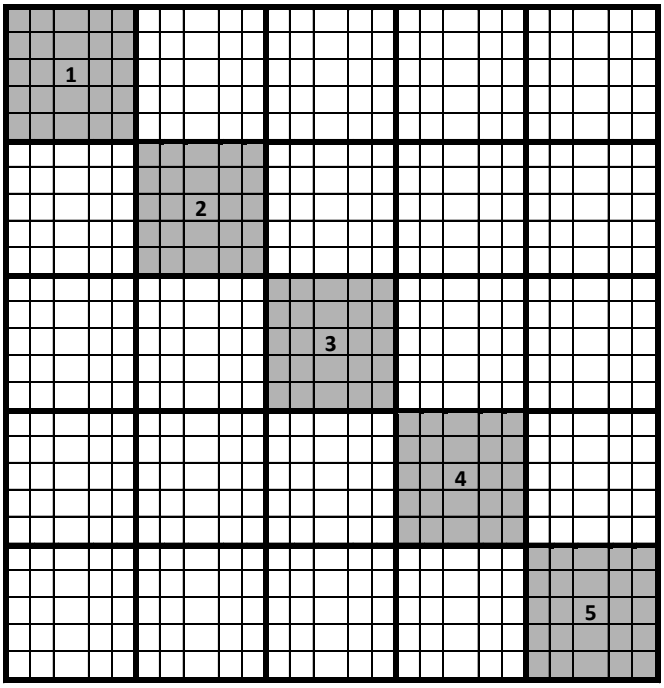
3.6 Resuspend in 100 µl 0.85% NaCl and dilute 1:50 and mix 100 µl of cell suspension with 0.3 µl of dye mixture (LIVE/DEAD *Bac*Light Bacterial Viability Kit, Invitrogen)

3.7 Incubate in dark for 15 min.

3.8 Pipet 10 µl to count using Bacteria counting chamber (try to fill whole 10 ul in the chamber).

Calculation

Rickettsia/ml = (Rickettsial organisms in 5 squares) x 5 x 0.05 x 10^6 x dilution factor (50)



4. Chemiluminescent immunodetection

4.1 Wash membrane with dH_2O at RT on orbital shaker for 10 min.

4.2 Transfer membrane to 3% BSA in TTBS (0.1 % TWEEN in TBST) at room temperature for 1 h.

4.3 Briefly rinse in TBST. Wash membrane 2 times with excess TBST for 10 min/wash.

4.4 Incubate membrane with 10 ml of a 1:5000 dilution of primary antibody (Mouse polyclonal antibody against Dvα-catenin) in TBST for 1 h at room temperature.

4.5 Briefly rinse in TTBS. Wash membrane 2 times with excess TBST for 10 min/wash.

4.6 Rinse in dH_2O for 2 min, twice.

4.7 Incubate membrane with 10 ml of goat anti-mouse HRP-conjugated antibody.

4.8 Briefly rinse in TBST. Wash membrane 3 times with excess TBST for 10 min/wash.

4.9 Rinse the membrane with dH_2O for 2 min twice.

4.10 Drained excess reagent. Covered blot with clear plastic wrap for 5 min. (SuperSignal® West Pico Mouse IgG Detection Kit, PIERCE

4.11 Exposed blot to X-ray Film.

VITA

Piyanate Sunyakumthorn was born in Chonburi, Thailand. She is the daughter of Mr. Sompong Sunyakumthorn and Mrs. Siriluk Sunyakumthorn, and she is the older sister of Salisa Sunyakumthorn and Paweena Sunyakumthorn. Piyanate graduated with a bachelor of sciences in biochemistry at Kasetsart University in 2000, and enrolled in the master program in biochemistry at Mahidol University. Piyanate finished her master degree in 2003 and joined Armed Forced Research Institute of Medical Sciences, working with COL Jariyanart Gaywee on a project involving the identification and characterization of rickettsial isolates from humans, arthropod vectors, and reservoir hosts in Thailand. Piyanate became interested in the biology of vector-borne diseases, and she wanted to pursue a research career in vector-borne diseases. In 2006, Piyanate moved to Baton Rouge, Louisiana to join Dr. Kevin Macaluso's laboratory where she learned about the pivotal role of arthropods as disease reservoirs and vectors, and in the spring of 2008 Piyanate began her doctorate degree in the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University under the guidance of Dr. Kevin Macaluso. Piyanate finished her dissertation research and will graduate in May, 2011. After graduation Piyanate will continue to pursue her research interests in the field of vector-borne diseases in Thailand.