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An Insight Into the Microbial Diversity and Expression of Cysteine Protease Inhibitors (Cystatin) in *Rickettsia parkeri* Infected *Amblyomma maculatum*

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AN INSIGHT INTO THE MICROBIAL DIVERSITY AND EXPRESSION OF CYSTEINE PROTEASE INHIBITORS (CYSTATIN) IN *RICKETTSIA PARKERI* INFECTED *AMBLYOMMA MACULATUM*

by

Khem Raj B.C.

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ABSTRACT

AN INSIGHT INTO THE MICROBIAL DIVERSITY AND EXPRESSION OF CYSTEINE PROTEASE INHIBITORS (CYSTATIN) IN *RICKETTSIA PARKERI* INFECTED *AMBLYOMMA MACULATUM*

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Amblyomma maculatum (Gulf Coast tick) is an emerging tick species of public health significance in United States. It is a competent vector of *Rickettsia parkeri*, an etiological agent of a human rickettsiosis. In this study, we investigated the spotted fever group of rickettsial diversity in *A. maculatum* based on rickettsial *ompA* gene PCR. Our results showed *A. maculatum* harbors *R. parkeri, R. amblyommii,* and *R. endosymbiont of A. maculatum.* While only *R. parkeri* was detected in female salivary glands which suggest its ability to traffic from midgut to salivary glands via hemocoel. The presence of *R. parkeri* was further confirmed by probe based qPCR assay. We found *R. parkeri* infection rate ranged 12-40% in field collected ticks. We also provided evidence of *R. parkeri* infection transovarially and transstadially transmitted in *A. maculatum*. We used a pyrosequencing approach to further study all possible bacterial diversity residing in field collected *A. maculatum*. The huge bacterial profiling in *A. maculatum* provided the basis of Amblyomma-bacterial interactions particularly in relation to *R. parkeri*. On the other side, we observed cystatins temporal transcriptional expression in *A. maculatum* across the blood meal cycle and our finding suggested their importance during blood feeding. Further, we saw

R. parkeri differentially regulates gene expressions of cystatins in *A. maculatum,* suggesting a possible role of cystatins in *R. parkeri* infection in ticks. This study encourages further study to assess the exact relationship of *R. parkeri* with bacterial diversity in *A. maculatum* and cystatins role during tick blood feeding and *R. parkeri* transmission.

DEDICATION

TO MY PARENTS

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LIST OF ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool

BP: Base pair

bTETAP: Bacterial tag-encoded Titanium amplicon pyrosequencing

cDNA: Complementary DNA

Ct: Cyclic threshold in qPCR assay

Cystatin: Cysteine protease inhibitor

DESeq: R package based differential expression study in RNAseq

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

GCT: Gulf Coast Tick

gltA: Rickettsial Citrate synthase gene

IACUC: Institutional Animal Care and Use Committee

IgG: Immunoglobulin G

KDa: Kilo Dalton

Log: Logarithmic value

MG: Midgut

MIF: Micro-Immunofluorescence

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

M-MLV RT: Moloney Murine Leukemia Virus Reverse Transcriptase

MOPS: 3-(N-morpholino) propanesulfonic acid buffer

mRNA: messenger RNA

NCBI: National Center for Biotechnology Information

ng/µL: Nanogram per microliter

OmpA: rickettsial outer membrane protein A gene

OmpB: rickettsial outer membrane protein B gene

OTU: Operational Taxonomic Unit

PCR: Polymerase Chain Reaction

QPCR/qRT-PCR: Quantitative real time polymerase chain reaction

ReAm: *Rickettsia endosymbiont of Amblyomma maculatum*

RMSF: Rocky Mountain spotted fever

RNA: Ribonucleic Acid

RNAseq: RNA or transcriptome sequencing

rrs: 16S rRNA bacterial gene

Sca4: Bacterial surface cell antigen 4

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFGR: Spotted Fever Group of Rickettsia

SG: Salivary Gland

Tot: Transovarial and transstadial transmission

USEARCH: Ultrafast Sequence analysis software

WB: Western Blotting

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Ticks

Ticks are highly specialized obligate, bloodsucking, nonpermanent ectoparasitic arthropods that feed on mammals, birds, reptiles, and amphibians in all regions of the earth (Keirans and Durden 2005).Ticks serve as the vector of greatest variety of pathogens to humans and veterinary species of any arthropod vector second to the mosquito (Sonenshine 1991). Ticks are well adapted in all weather conditions from tropical, temperate, and even subarctic habitats but are found with great density in tropical and subtropical areas (Keirans and Durden 2005). They have several morphological and physiological mechanisms for host selection, ingestion of host blood, mating, survival, and reproduction (Anderson and Magnarelli 2008).

Classification of Ticks

Ticks are classified in class Arachnida, subclass Acari, order Parasitifomes, and sub-order Ixodida. There are four families of ticks comprising 878 species. The tick families are Ixodidae (hard ticks), Argasidae (soft ticks), Nuttalliellidae, and Laelaptidae (Anderson and Magnarelli 2008). The latter two families are mono specific and are less important in disease and public health (Sonenshine 1991). Only two tick families are discussed here.

Family Ixodidae (hard ticks). Ixodidae are further divided into Prostriata, and Metastriata constituting 80% (601 species) of all the tick species described (Horak et al. 2002). Prostriata is characterized by a distinctive anal groove that encircles the anus anteriorly. The Prostriata group has only genus *Ixodes* but is the largest tick genus, consisting of 245 species of ticks. The most important tick species are black legged tick (*Ixodes scapularis*) found in north eastern America, *I. ricinus* in Europe and western Asia, *Ixodes persulcatus* in north eastern Europe and northern Asia, and western black legged tick (*I. pacificus*).

The metastriata ticks are characterized by a distinctive anal groove encircling the anus posteriorly. They include tick species of genus *Dermacentor, Rhipicephalus, Haemaphysalis, Hyalomma* and *Amblyomma*. In North America important tick species are of *Dermacentor* and *Amblyomma* genus including: American dog tick (*Dermacentor variablis*), Rocky mountain wood tick (*D. andersoni*), pacific coast tick (*D. occidentalis*), winter tick (*D. albipictus*), *Amblyomma americanum* (lone star tick) and *Amblyomma maculatum* (Gulf coast tick). The *Rhipicephalus, Hyalomma, Haemaphysalis* genera of tick are found in various regions of the world and are of medical and veterinary importance.

Argasidae (soft ticks). The Argasidae family of soft ticks is comprised of four genera having 184 species (Horak et al. 2002). Soft ticks comprise the species of genera *Argas*, *Carios*, *Ornithodoros* and *Otobius* and are found mostly in dry caves and xeric environments of African countries, parasitizing a range of birds and bats.

Morphological Features of Ticks

The tick body has two parts: capitulum (gnathosoma) and the body (idiosoma), the latter bear legs. The larval ticks have six legs, while nymphs and adults have eight legs. The body length of ticks at the unfed stage is 2 mm to 20 mm, while the blood engorged tick may be 25 to 30 mm and weigh up to 100 times their unfed weights (Anderson and Magnarelli 2008). The description of mouth parts is important. The mouth parts are found on capitulum. They include palps (two in number, four segments); ixodid ticks have chemosensillary sensillae in the last segment of palp. The role of palps is to hold the ticks horizontally and laterally during feeding. The next important structure is chelicerae (two segmented tubular) having highly moveable and sharp structure in extremities. The third structure is hypostome (with denticles backward pointing and ventral) used to holdfast on host and contain food canal inside (Sonenshine 1991).

The sexual dimorphism is seen in adult ixodid ticks. The female ticks have a small portion of scutum at dorsum while the male has a scutum covering whole dorsum. The biological function of female ticks having small scutum is to let her engorge and imbibe more blood during feeding. While the soft ticks are inornate and leathery in appearance, oval shaped and anterior surface rounded mouth parts are difficult to see from the dorsal surface. The body lacks scutum, instead of which leathery cuticle is found, while eyes may or may not present.

Life Cycle and Ecology of Hard-Ticks (Ixodidae)

The developmental stages of ticks are eggs, larvae, nymphs, and adults (male or female). Except eggs, all three stages require the blood meal for their survival and development. The larva imbibes the blood meal and molts into a sexually indistinguishable nymphal stage; the nymphs require blood meal to molt into sexually distinct male or female adults. Hematophagy is necessary for growth, development to next stage, and reproduction. The blood meal acquisition and development into the next developmental stage differs with species according to number of hosts used for blood meal. Based on the number of hosts used, tick are classified as single host life cycle, two host life cycle, and three or multiple host lifecycle. In *Amblyomma* and *Ixodes,* larvae, nymphs and adults require three different hosts for blood meal which is described as having three-host life cycle as with each blood meal they drop off to ground and molt. While in cases of genus *Rhipicephalus* larvae attach on the host and blood feeding and molting of larvae and nymphs occurs on the same host, which is single host life cycle. Ticks can be nest dwelling (nidiculous) parasites or the field dwelling (non nidiculous). All the soft ticks and some Ixodes ticks are nidiculous. The ticks reside in caves, nests, rock ledges, crevices, or burrows, or hide in soil or cracks or crevices of tree bark or wood nearby host-occupied sheltered sites where temperature, relative humidity, and wind more uniform throughout the year than those in open fields and forest (Balashov 1972).

Blood Feeding in Ticks

Blood feeding in Ixodid ticks has nine different steps including Appetence (hunting or seeking a host), Engagement (adherence to the skin or fur of the host), and Exploration (searching on the skin for a suitable attachment site), the steps before the attachments (Anderson and Magnarelli 2008). The tick uses tactile stimuli, odor, vibration, shadowing, and visual appearance as cues for questing host and searching for the right place for insertion. The insertion of the mouthparts into the host’s epidermis and dermis and successful attachment requires the salivary proteins modulating the host immune and blood coagulation cascade and cement cone formation. After the feeding site is established, blood pool formed and ingestion of blood occurs slowly for the first few days and rapidly during later stages of the blood meal cycle. The engorged Ixodid tick becomes 100 times bigger in weight than unfed after complete detachment and drop off from host (Anderson and Magnarelli 2008).

Soft ticks imbibe blood almost immediately after attachment to the host, they do not secrete cement and do not form new cuticle. The secretion of excess water occurs through their coxal pores. It has been reported that larval ticks complete feeding within 20 minutes while adults take 35 to 70 minutes (Sonenshine 1993).

Tick Blood Acquisition and Digestion

The tick first attaches on host skin, and the slow feeding of stage starts and the mating occurs in ticks, which induces tick feeding and a rapid engorgement stage occurs before one day of detachment stage (Franta et al. 2010). Based on total blood meal cycle, length of slow or fast feeding stage varies, in a representative eight day feeding cycle of *I. ricinus* female for the first day tick attaches on host and slow feeding starts one day post attachment up to day six, while the fast feeding stage occurs for last 24-48 hr before engorged tick drop off the host and during this period tick ingests major portion of blood meal (two-third) (Franta et al. 2010). The blood ingestion is the important phenomena involving important role of various anti-coagulants, analgesics and immunomodulatory molecules secreted in tick saliva, as discussed on tick saliva section. The blood ingestion increases size and shape of tick from small large and 100 fold weight gain compared to pre-fed stage. The large amount of blood feeding occurs only in female ticks while male ticks remain similar in size and weight. During the blood feeding, gut pumps water and electrolytes into hemocoel, which go back to the host via saliva, resulting concentrated meal in the midguts (Sauer 1977, Bowman and Sauer 2004). It has been observed that tick alternate blood ingestion and salivation with each cycle lasting for 5-20 min at a time (Gregson 1967, Waladde et al. 1979). The recent findings showed that tick blood digestion takes place intracellular in gut cell with the involvement of the cathepsins B, C and D and aspartic endopeptidases (Horn et al. 2009). The blood digestion (haemoglobinolysis) and activities of the cathepsins reaches their maximum activities during the rapid engorgement stage (Franta et al. 2010). But, the digestion of the blood meal after detachment has been unclear with synthesis of digestive enzymes whether it employs the enzymes synthesized during the slow feeding stage or newly synthesized late isoenzymes are responsible for post-feeding enzyme activities (Sojka et al. 2013).

The midgut is also the primary source of pathogen acquisition during the blood meal from the infected host. The fate of the ingested pathogens and micro flora has been affected by the blood digestion going on in guts. The pathogens have to overcome hemocidins, antimicrobial peptides, and protease inhibitors and oxidative burden caused by reactive oxygen species (ROS) generated by release of heme (Sojka et al. 2013). The ticks have hemosomes for accumulation of the digested hemoglobin, various antioxidant enzymes and ROS scavengers

(Anderson et al. 2008). The dynamic interaction between the antioxidant and ROS scavengers during the digestion in gut tissues has important role in the determination of the fate of the pathogen which further characterizes the success of pathogen in ticks (Sojka et al. 2013).

Tick Salivary Glands

The tick salivary glands are important organ with respect to hematophagy and pathogen development and transmissions. The salivary glands have three different acini viz. acini I, acini II and acini III. The sequential changes has been observed among the different cells in salivary acinus during the blood meal cycle (Binnington 1978). The pharmacological control of fluid secretion in salivary gland has been given described (Figure 1) in pictorial representation. The neurotransmitter dopamine acts via the G protein (Gs) coupled receptor which leads to release of cyclic AMP (cAMP) into cytosol. The cAMP activates protein kinase which leads to phosphorylation of numerous proteins; one of important is family of aquaporins (AQP) or water channel proteins that became inserted into cell membrane which promotes the fluid transport. The dopamine stimulates the uptake of extracellular ca2+ into cytosol via voltage gated ion channel. The ca2+ ion stimulates the phospholipase A2 (cPLA2) which releases the arachidonic acid (AA). The AA converted into prostaglandins, PGE_2 via cyclooxygenase (COX). PGE_2 when secreted into saliva plays role in antihaemostatic, vasodilatory, immunosuppressive and anti-inflammatory functions. Whereas PGE_2 has paracrine role as well which activate phospholipase C (PLC) and release of inositol triphosphates (IP3), and diacyglycerol (DAG). The IP3 cascade initiates the release of the ca2+ from endoplasmic reticulum (ER). Ca2+ ion mediates the exocytosis of secretory vesicles which the intracellular pathogen utilizes in transmitting to saliva towards the host (Karim and Adamson 2012).

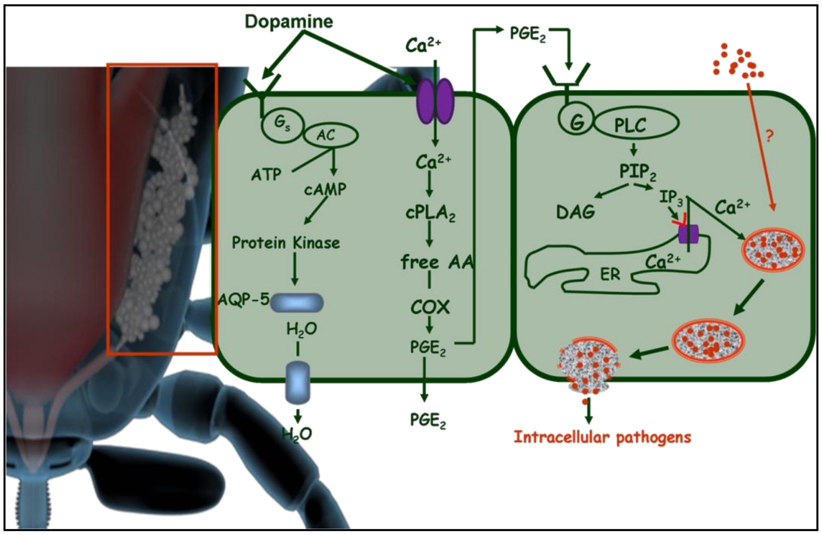


Figure 1. The intracellular signaling pathways mediating salivary gland secretory processes in Ixodid ticks (Karim and Adamson 2012).

The salivary glands play important role in the pathogen transmission. The fate of any successful pathogen after getting in gut tissues moves towards hemocoel to salivary glands and transmit to host via salivation. The blood meal affects the migration of pathogen residing in midgut tissues to salivary glands. The etiological agent of Lyme disease, *Borrelia burgdorferi*, develops in the midgut of the tick and it migrates to the salivary glands when the tick starts taking blood meal and injected into host via saliva (Ribeiro et al. 1987). In some cases, the pathogen may migrate to salivary gland without start of blood feeding to the salivary glands and reside throughout the molt as in *Anaplasma phagocytophilum,* but in other cases ingested pathogens remains in the tick guts without trafficking to other tissues (Foley and Nieto 2007).

Tick Saliva

In a tick not attached to host, the hygroscopic saliva secreted on the surface of hypostome and atmospheric moisture is absorbed and sucked back to tick which helps tick survival during dehydration. But, during blood feeding saliva production is the mechanism of excess water excretion. The role of saliva in tick feeding has been elaborately explained by Francischetti et al. (2009). The tick saliva acts against vertebrates' advanced blood coagulation cascade, platelet aggregation, and vasoconstriction to steal blood (Francischetti et al. 2009). The host cellular and humoral immune systems are acting against tick feeding but against these barriers tick salivary glands secreted hundreds of the different proteins (Francischetti et al. 2009, Karim et al. 2011) which act to overcome host responses. The tick salivary glands secrete to maintain constant supply of antihaemostatic, anti-inflammatory, analgesic and immunomodulatory proteins throughout tick attachment on host. Francischetti et al. (2009) further explained the ticks' countermeasures in saliva favoring it stealing vertebrate blood. The tick saliva has platelet aggregation inhibition factors; products interfering with or mimicking antithrombin, protein S, protein C, heparin or thrombomodulin and abundant metalloprotease activity with fibrin and fibrinogenolytic activities as well as anti-angiogenesis factor against the host clotting mechanism. The tick saliva is abundant in molecules which act against the host immune and pain responses. The tick saliva has found reducing the C3b deposition preventing host showing inflammatory responses, antagonist of anaphylatoxin counteracting the acute inflammation, neutrolphil chemotaxis and mast cell activation surviving the tick from acute responses. Similarly, the macrophage inhibition (MIF) factor in tick saliva has role in inhibiting NK cell mediated lysis and delayed type hypersensitivity. The tick responds histamine and serotonin action of host which causes reduction in blood sucking and salivation by binding with tick lipocalins. With the cumulative efforts of all the molecules secreted in the tick saliva, tick infestation is less likely of developing the antibodies that could neutralize the saliva proteins essential for successful blood feeding (Francischetti et al. 2009).

Tick Salivary Cysteine Protease Inhibitors (Cystatins)

Cystatins are the cysteine protease inhibitors, which have been found in both hard and soft tick sialotranscriptome and their activity was demonstrated in tick saliva as well (Kotsyfakis et al. 2006). The cystatins were first described in chicken egg white in late 1960s and later discovered to be present in vertebrates, invertebrates, plants, and protozoa (Fossum and Whitaker 1968). Cystatins, a protein superfamily is subdivided into families 1, 2 and 3. Family 1 members are cytosolic molecules with neither disulfide bonds nor carbohydrates, Family 2 consists of all the secreted cystatins found in biologic fluids (two disulfide bridges, and they do not bear sugars). These family 1 and 2 cystatins possess cystatin-like domain and has 11-14kDa weight. The family 3 (also kininogens) of several cystatin modules, thus being relatively larger molecules (60-120 kDa) (Vray et al. 2002).

The cystatins mediate the cysteine protease activity which play role in antigen presentations, immune system development, epidermal homeostasis, neovascularization, extracellular matrix degradation and neutrolphil chemotaxis during inflammation, apoptosis and proliferation of malignant cells and subsequent invasion into healthy cells (Kotsyfakis et al. 2006). The two tick salivary glands cystatins were characterized as inhibitor of cathepsins L and S has role in inflammation inhibition and dendritic cell maturation and these molecules are potential vaccine targets for controlling tick and tick borne diseases in *Ixodes scapularis* (Kotsyfakis et al. 2006, Kotsyfakis et al. 2008). The cystatins in ticks has been described in recent paper (Schwarz et al. 2012), the reported cystatins were from *A. americanum*, *A. variegatum*, *D. variabilis*, *Haemaphysalis longicornis*, *I. ricinus*, *I. scapularis*, *Rhipicephalus microplus* and *R. sanguineus* as well as from soft ticks *Ornithodoros moubata*, *O. coriaceus*, *O. parkeri* and they have role in tick different tick physiology and blood feeding. Recently, the sialotranscriptome of Gulf coast tick salivary glands has revealed 25 putative coding sequences (CDS) for cystatins and among them 15 have signal peptide indicative of their secretory nature (Karim et al. 2011).

The Gulf Coast Tick (*Amblyomma maculatum*)

The Gulf Coast tick (*Amblyomma maculatum*) is emerging arthropod of increasing public health significance. *A. maculatum* is a vector of *Rickettsia parkeri* found in coastal areas across Atlantic to Gulf coast region of the southern United States (Bishopp and Hixson 1936) with inland range extensions as far as Oklahoma and Kansas (Teel et al. 2010) (Figure 3). The range of Gulf coast tick (GCT) has, however, expanded most likely due to transportation and movement of infested livestock and migratory birds (Goddard and Norment 1983). It is also found in regions of several Central and South American countries that border the

Gulf of Mexico and the Caribbean Sea (Estrada-Pena et al. 2005). The life cycle consists of four different developmental stages viz. eggs, larva, nymphs and adults (male or female). The larvae and nymphs feed on small animals and birds while adults generally found infested on large vertebrate hosts. The adult Gulf coast tick feeds on vertebrate host for about two weeks, engorged female in average takes 3-4 days for pre-oviposition period and lays continuously up to 25 days in average producing about 1000 eggs/day/female and gravid female spent off (Drummond and Whetstone 1970). The ticks can be managed in lab condition at 27^{0}C temperature, 70-98% relative humidity and photoperiod of 12/12 light hours(Drummond and Whetstone 1970). The pictorial diagram of *A. maculatum* life cycle has been shown in Figure 2.

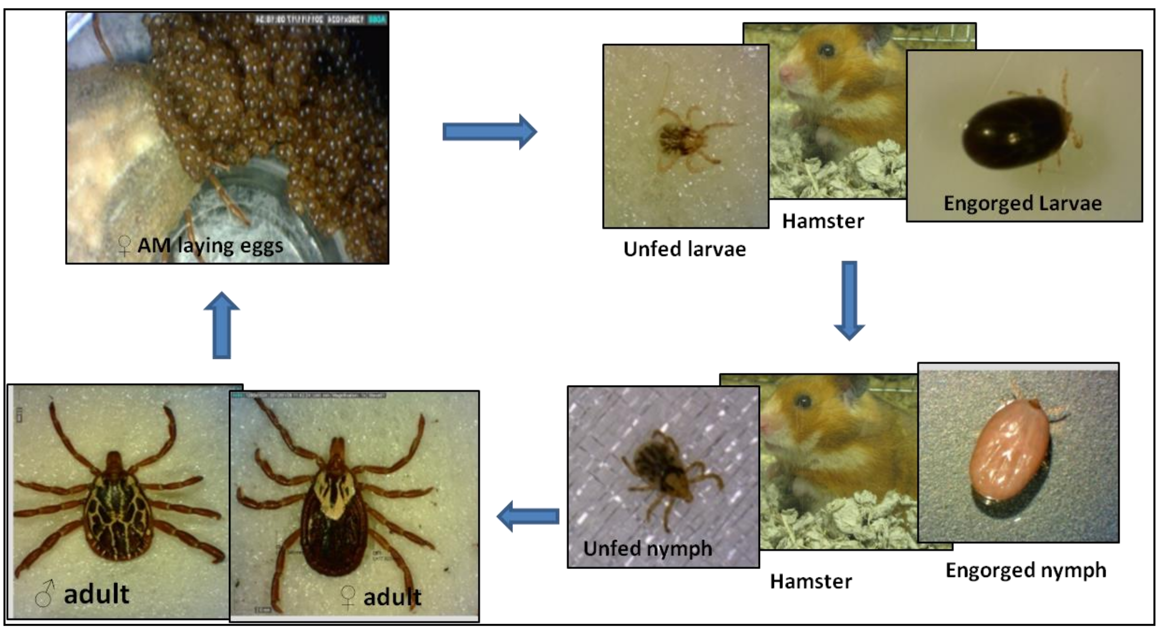


Figure 2. Life cycle of *Amblyomma maculatum,* Gulf coast tick. The collected adults male and female infested on Rabbit ear in sock stockinet. The engorged ticks (only female shown in figure) incubate and lay eggs. The larva will hatch after a month of incubation of eggs. Larva (6-legged) requires blood meal and engorged larvae molts into nymph (8-legged). After blood meal nymphs molts into sexually separate male and female.

Tick Borne Rickettsial Diseases

Tick-borne rickettsial diseases are caused by two groups of intracellular bacteria belonging to the order Rickettsiales and including (a) bacteria belonging to spotted fever group of the genus Rickettsia within the family Rickettsiaceae (Raoult and Roux 1997), and (b) bacteria within the family Anaplasmataceae including several genera such as Anaplasma and Ehrlichia (Dumler et al. 2001). Traditionally, rickettsial agents has been divided into three groups based on immunological cross-reactivity and vector species viz. Spotted fever group (SFG), typhus group (TG), and the scrub typhus group (STG). It has been described that SFG Rickettsiae (SFGR) optimal growth temperature of 32° C, a guanosine plus cytosine (G+C) content between 32% and 33%, can polymerize actin and thereby enter the nuclei of host cells (Teysseire et al. 1992, Heinzen et al. 1993, Teysseire et al. 1995, Merhej and Raoult 2011) cause spotted fever in humans. While the typhus group (TG) Rickettsiae are associated with body lice (*R. prowazekii*) or fleas (*R. typhi*) and have optimal growth temperature of 35°C, a G+C content of 29%, were only found in cytoplasm of host cells (Teysseire et al. 1992, Heinzen et al. 1993, Merhej and Raoult 2011) and cause typhus in humans. Now a days there is genetic guidelines for the classification of Rickettsial isolates at genus, species, group levels using the rickettsial genes including 16S rRNA (*rrs*) gene, *gltA*, *ompA*, *ompB*, and *gene D* (Fournier et al. 2003). According to this module any rickettsial isolate to be a new species should not have nucleotide homology with validated species ≥ 99.8% and ≥ 99.9% for the *rrs* and *gltA* genes respectively and should not have ≥98.8%, ≥99.2%, and ≥

99.3% nucleotide similarity for the *ompA* and *ompB* gene and gene D, respectively (Fournier et al. 2003). Importantly, all the tick associated *Rickettsia* belong to the spotted fever group Rickettsia with the exception of *Rickettsia bellii* and *Rickettsia canadensis* of Rickettsiaceae (Parola et al. 2005).

There were few known rickettsial infection before 1984 and between the 1984 and 2004 many rickettsial pathogens were identified with the utilization of cell culture and molecular based techniques. The increased number of rickettsial agents detection removed the old concept that only one tick-borne rickettsiosis is prevalent in one geographical area with the detection of rickettsial pathogen where no species have been identified, typical rickettsiosis have been found to be caused by other rickettsial species (Renvoise et al. 2009). The reviews of different spotted fever group of Rickettsia (SFGR) are provided in tabulated forms (Tables 1, 2, and 3) modified from previous report (Parola et al. 2005). Table 1 shows the tick associated non-pathogenic spotted fever group of rickettsia (SFGR), associated tick vector and geographical distribution. The SFGR species which are pathogenic to human and its first identification in tick has been presented in Table 2. The use of modern diagnosis tools has facilitated the detection of the various other rickettsiae but the pathogenicity has not been completely known yet. The potential disease causing SFGR of ticks are grouped as potential pathogen (Table 3).

Transovarial Transmission of Rickettsia

The transmission of rickettsial agent from adult female tick to eggs (transovarial) and successively to larvae and nymphs and adults (transstadial transmission) has been observed in rickettsial agents. It has been reported in *Rickettsia conorii conorii* in naturally infected *Rhipicephalus sanguineus* (Socolovschi et al. 2012); *Dermacentor variablis* with artificial capillary feeding of *Rickettsia montana* and *R. rhipicephali* (Macaluso et al. 2001); Similarly, *Rickettsia africae* in *Amblyomma variegatum* (Socolovschi et al. 2009) and *Rickettsia rickettsii* in naturally or artificially infected *Dermacentor andersoni* (Burgdorfer 1963). The *R. parkeri* has been experimentally infected to lone star tick (*Amblyomma americanum*) and shown that it could be viable up to two generations (Goddard 2003) in lab conditions while it has not been shown in naturally infected *A. maculatum* ticks.

Table 1

Tick Associated Non-Pathogenic Spotted Fever Group Rickettsia

Rickettsia	Vector associated	Distribution	References
R. peacockii	*Dermacentor andersoni*	US	(Baldridge et al. 2004)
R. montanensis	*Dermacentor variablis, Dermacentor andersoni*	US	(Ammerman et al. 2004)
R. bellii	*Demacentor, Argas haemaphysalis, Amblyomma, Ornithodoros*	US, Brazil	(Gage et al. 1994)
R. rhipicephali	*Rhipicephalus sanguineus, Dermacentor occidentalis*	US, Europe and Africa	(Hayes and Burgdorfer 1979, Duh et al. 2003)
R. monacensis	*Ixodes ricinus*	Europe	(Christova et al. 2003)
R. tamurae	*Amblyomma testudinarium*	Western Japan	(Fournier et al. 2002)
R. asiatica	*Ixodes ovatus*	Central Japan	(Fournier et al. 2002, Blair et al. 2004)
Candidates R. andeanae	*Amblyomma maculatum, Ixodes boliviensis*	Peru	(Blair et al. 2004)

Table 2

Tick Associated Pathogenic SFG Rickettsia

Rickettsia	Vector associated	Disease/yr identified in tick	Distribution	References
R. rickettsii	*Dermacentor andersoni Dermacentor variabilis Rhipicephalus sanguineus Amblyomma cajennense, Amblyomma arueolatum*	Rocky mountain Spotted Fever (1906)	US, Western hemisphere	(Treadwell et al. 2000)
R. conorii conorii	*Rhipicephalus sanguineus*	Mediterranean spotted fever (1932)	Mediterranean area, Northern Africa and southern Europe	(Zhu et al. 2005)
R. conorii israelensis R. sibirica sibirica	*Rhipicephalus sanguineus Dermacentor nuttallii, Dermacentor marginatus Dermacentor Silvarum Haemaphysalis Concinna*	Israeli spotted fever (1974) Siberian tick typhus (unknown)	Israel Portugal Russia, China and Pakistan	(Raoult and Roux 1997) (Robertson and Wisseman 1973, Balayeva et al. 1996)
	Dermacentor sinicus	North Asian tick typhus (1974)		
R. australis	*Ixodes holocyclus Ixodes tasmani*	Queensland tick typhus (1974)	Queensland, south costal New South Wales, eastern Victoria, and Tasmania	(Graves et al. 1993)

Table 2 (continued).

Rickettsia	Vector associated	Disease/yr identified in tick	Distribution	References
R. japonica	*Ixodes ovatus, Dermacentor taiwanensis, Haemaphysalis longicornis, Haemaphysalis flava*	Oriental or Japanese spotted fever (1996)	Southwestern and central Japan	(Uchida et al. 1992, Mahara 1997)
R. conorii caspia	*Rhipicephalus sanguineus, Rhipicephalus pumilio*	Astrakhan fever (1992)	Astrakhan, Russia, Africa	(Tarasevich et al. 1991)
R. africae	*Amblyomma hebraeum, Amblyomma variegatum*	African tick bite (1990)	South Africa	(Kelly et al. 1991)
R. honei	*Aponomma hydrosauri, Amblyomma cajennense, Ixodes granulatus*	Flinders Island spotted fever (1993)	Tasmania	(Graves et al. 1993)
R. slovaca	*Dermacentor marginatus Dermacentor reticulatus*	Tick-borne lymphadeno mpathy (1968), Dermacentor borne necrosis and lymphadeno mpathy (1968)	France, Switzerland, Slovakia, Ukraine, Yugoslavia, Armenia, and Portugal	(Sekeyova et al. 1998)
R. sibirica mongolitimon ae	*Hyalomma asiaticum Hyalomma truncatum*	Lymphangitis associated Rickettsiosis (1991)	Mongolia, China	(Fournier et al. 2005)
R. heilongjiange nsis	*Dermacentor silvarum*	Far eastern spotted fever (1982)	China,	(Zhang et al. 2000)

Table 2 (continued).

Rickettsia	Vector associated	Disease/yr identified in tick	Distribution	References
R. aeschlimannii	*Hyalomma marginatum marginatum, Hyalomma marginatum rufipes Rhipicephalus appendiculatus*	Unnamed (1997)	Morocco, Croatia	(Matsumoto et al. 2004)
R. parkeri	*Amblyomma maculatum, Amblyomma americanum,* Amblyomma triste	1939	US	(Parker et al. 1939)
R. massiliae	*Rhipicephalus sanguineus, Rhipicephalus turanicus, Rhipicephalus muhsamae, Rhipicephalus lunulatus, Rhipicephalus sulcatus*	Unnamed (1992)	Massiliae, Portugal	(Bacellar et al. 1995)
R. marmionii	*Haemaphysalis novaeguineae*	Australian spotted fever (2003/5)	Queensland, Tasmania, and South Australia	(Parola et al. 2005)

Rickettsiosis as a Human Disease in the United States

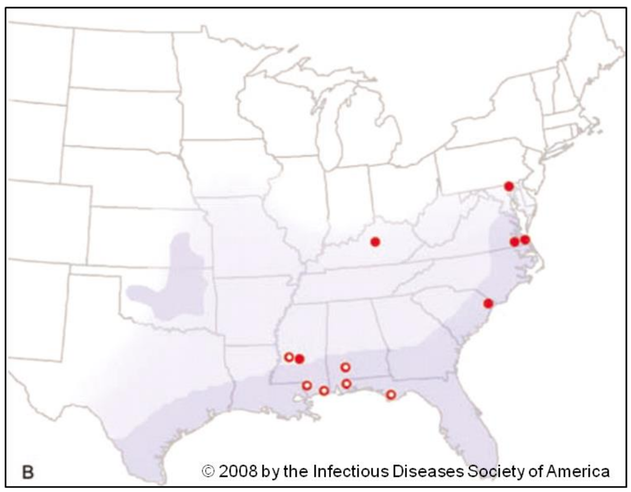
Rickettsial diseases are caused by infection with obligate intracellular Gram-negative Alphaproteobacteria transmitted by arthropod vectors and may affect an estimated one billion people worldwide (Parola et al. 2005, Walker and Ismail 2008). Even today, the majority of the rickettsial disease cases are not diagnosed and reported. In the United states, five rickettsial agents have been reported causing diseases in humans: Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii* (Niebylski et al. 1999), Rickettsialpox by infection of *Rickettsia akari* (Krusell et al. 2002), *Rickettsia felis* causing typhus like rickettsiosis (Azad et al. 1992), *Rickettsia parkeri* rickettsiosis (Paddock et al. 2004), and *Rickettsia species 364D* (Shapiro et al. 2010) infections.

Table 3

Tick Associated Potential Pathogenic SFG Rickettsia

Rickettsia	Vector associated	Disease/yr identified in tick	Distribution	References
R. conorii indica	*Rhipicephalus sanguineus*	Indian tick typhus;1950	India	(Parola et al. 2001)
R. canadensis	*Haemaphysalis leporispalustris*	1967	California and Texas	(McKiel et al. 1967)
R. amblyommii	*Amblyomma americanum, Amblyomma cajennense, Amblyomma coelebs*	1974	US and south America	(Jiang et al. 2010)
R. texiana	*Amblyomma americanum*	Bullis fever (1943)		(Anigstein and Anigstein 1975)
R. helvetica	*Ixodes ricinus Ixodes ovatus Ixodes persulcatus Ixodes monospinus*	1979	Europe	(Beati et al. 1993)
R. strain 364-D	*Dermacentor occidentalis*		California	(Shapiro et al. 2010)

Rickettsia parkeri, a member of SFGR, was initially identified in *A. maculatum* in 1937 as a maculatum agent (Parker et al. 1939). The first human *R. parkeri* infection was reported in 2004 as the cause of a new spotted fever rickettsiosis (Paddock et al. 2004). It took 65 years to be *maculatum agent* to be *Rickettsia parkeri* Rickettsiosis. *R. parkeri* is now recognized as a human pathogen of increasing public health concern (Paddock et al. 2004, Cragun et al. 2010) across the southern United States (Raoult and Paddock 2005, Finely et al. 2006, Whitman et al. 2007), with relatively high infection prevalence reported in several areas (Sumner et al. 2007, Paddock et al. 2010, Wright et al. 2011). The distribution of tick vector and the *R. parkeri* rickettsiosis cases overlap the geographical distributions (Figure 3).



Paddock C D et al. Clin Infect Dis. 2008;47:1188-1196

Figure 3. The *Rickettsia parkeri* Rickettsiosis in the United States**.** Classic range (dark blue) of *A. maculatum* in the United States, based on historical and contemporary records (Sumner et al. 2007); locations of confirmed (shaded circles) and probable (unshaded circles) cases of *R. parkeri* Rickettsiosis in human (Paddock et al. 2008).

Rickettsia parkeri Rickettsiosis Study Animal Model

Animal models have been used to characterize pathology, test vaccine efficiency, and examine transmission parameters of Rickettsial agents. The guinea pigs were tried to study *Rickettsia parkeri* but guinea pigs showed infection without clinical signs (Jordan et al. 2009). The *R. parkeri* animal model has been proposed recently for the transmission characterization and to further characterize the pathology associated with *R. parkeri* infection. They showed C3H/HeJ mice are the promising animal model to study tick transmission, dissemination, and pathology of *R. parkeri* Rickettsiosis (Grasperge et al. 2012).

Molecular Detection of SFG Rickettsia

Serological and Immunological Detection of Rickettsia

The detection of the rickettsia starts with the Weil-Felix test, oldest test and this test has been used for initial screening or rule out of rickettsial infection still in developing countries (Isaac et al. 2004). Next tools used for diagnosis is Micro-immunofluorescescence (MIF), though it is less sensitive assay due to the cross-reactivity that often exists among antigens of pathogens within the same genus and occasionally in different genera (Brouqui et al. 2004). The immunoassays can be used as initial assay but cannot be solely depend on this due to cross reactivity with many likely Rickettsial agents, but cross-absorption (CA) techniques and Western blotting (WB) combined together is suggested to differentiate rickettsial infections by antibody evaluation (La Scola and Raoult 1997).

PCR Based SFGR Identification

The Rickettsia are classified in the α-Proteobacteria with the study of 16S rRNA but the interspecies identification with evolutionary relationship is successfully performed with rickettsial outer membrane protein (*ompA*) gene (Fournier et al. 1998) and citrate synthase encoding gene (*gltA*) (Roux et al.

1997). Nested PCRs based diagnosis are performed to diagnose *Rickettsia rickettsii* in the clinical samples (Tzianabos et al. 1989) and in the *Amblyomma maculatum* ticks for identification of *Rickettsia* by *ompA* amplicon sequencing (Blair et al. 2004).

The *Rickettsia prowazekii*, a causative agent of epidemic typhus, detected in infected lice and blood of experimentally infected mice 3 or 6 day post infection used probe based *gltA* gene primers (Svraka et al. 2006). Similarly, *Rickettsia felis* was quantified in cat fleas by quantifying *R. felis* 17kDa gene copies by qPCR (Reif et al. 2008) and *Rickettisia amblyommii* by *ompB* lone star ticks (Jiang et al. 2010).

Rickettsia parkeri Infection in Field Collected Amblyomma maculatum

In North Carolina, *R. parkeri* infection rate was identified as 20-30% ticks sampled (Varela-Stokes et al. 2011), while in Virginia *A. maculatum* have been found to be 43% (Wright et al. 2011). It's prevalece in field collected Gulf coast tick was also reported from Arkansas with 30% (Trout et al. 2010) and has been also reported from different other places of the US: Florida, Georgia, Kentucky, Mississippi, Oklahoma, South Carolina, and Tennessee (Sumner et al. 2007, Cohen et al. 2009). The *Rickettsia parkeri* were found to be 28% in field collected ticks from Florida and Mississippi (Paddock et al. 2010). Cumulatively the *R. parkeri* infection rate among field collected *A. maculatum* ticks ranges from 28-43.1% (Cohen et al. 2009, Paddock et al. 2010, Trout et al. 2010, Varela-Stokes et al. 2011, Wright et al. 2011) in United States. The infection rate study has been performed in ticks as a whole and the *R. parkeri* in tick tissues has not been reported by any studies yet.

Microbial Diversity in Ticks

Metagenomic Approach

The 16S ribosomal RNA is the important 30S ribosomal subunit used by prokaryotes for the translation of RNA into proteins. There are nine hyper variable regions that demonstrate considerable sequence diversity and can be used for the identification of the bacteria (Chakravorty et al. 2007). The differentiation of bacterial species can be performed sequencing only with portion of the 16S rDNA or a single hyper variable region rather than the full length of 16S rDNA (Chakravorty et al. 2007). The sequencing of hyper variable region extending from V1 and V3 ribosomal region is common in different studies (Dowd et al. 2008a, Dowd et al. 2008b). Unlike the traditional sequencing, 454 Titanium platform use parallel sequencing, generating over 1 million reads per 454 run. This platform has average read length of 400 bp, one sequencing can generate 400-600 billion base reads per run (Rogers and Venter 2005). The traditional Sanger based sequencing employs one template-one sequence reads using dideoxyneucleotide chain termination method, dominated the DNA sequencing since it was first introduced in 1977 (Sanger et al. 1977). The use of the metagenomic approach identified the many different bacteria not amenable to culture in regular media and this method has been successfully used to reveal bacterial diversity in environment samples.

Microbial Diversity in Ticks

The study of bacterial communities associated with ticks that transmit pathogenic agents has revealed new microbial associations including previously unknown tick-borne pathogens or vector competencies (Burgdorfer et al. 1973, Clay et al. 2008, Vilcins et al. 2009). Microbial diversity in the *Amblyomma americanum*, lone star ticks has been assessed (Heise et al. 2010) in unfed and fed colony reared and field collected lone star ticks from all the internal structure except exoskeleton by PCR amplification, cloning and sequencing methods. They found the almost 90% of *Coxiella endosymbionts of A. americanum* in colony reared and about 95% in field collected ticks irrespective of place and sex but they noticed the significant increase of Rickettsiales (2 to 46%) after blood feeding and reduced *Coxiella endosymbiont of A. americanum* to 20% even in colony reared ticks. The increased bacteria associated with Enterobactericeae are associated with bacterial anti-stress capability. They also, showed presence of *R. rickettsii,* which had not been reported previously along with *R. amblyommii* and *R. massiliae*. The bacterial diversity study provided the presence of huge bacterial species but the biological significance of which has to be determined yet though recent studies are focusing on assessing the importance of bacterial communities and seeking individual or communities role in different physiological or presence of pathogen (Wang et al. 2011). In tick species: *Ixodes ricinus* and *Rhipicephalus microplus,* metagenomic study has further provided the tick bacterial diversity, though the biological significance has not known but they proposed the presence of core microbiome in ticks (Andreotti et al. 2011, Carpi et al. 2011).

Pathogen Induced Differential Gene Expression

There are studies with the differential gene expression in ticks with respect to the pathogen infections. In *Dermacentor variabilis* infected with rickettsia differentially express nine genes in midgut, salivary glands and ovary and are confirmed by semi quantitative RT-PCR and northern blotting (Macaluso et al. 2003). The manipulation of the host cytoskeletal molecules by microorganism for the successful invasion in host is discovered in many bacterial species including *Listeria, Shigella, Rickettsia, Burkholderia, and Mycobacterium* (Dramsi and Cossart 1998, Gouin et al. 2004, Hamaguchi et al. 2008). It has been found that surface cell antigen 4 (*Sca 4*) of *Rickettsia prowazekii* binds and activates vinculin before binding host cytoskeleton (Park et al. 2011). Similarly, Salp16 was induced in *I. scapularis* in salivary gland and gut tissue blood feeding on either the host or the tick is infected with *A. phagocytophilum* which has been further verified by RNA interference of *Salp16*. RNA interference of *Salp16* in *I. scapularis* reduced the ability of tick to acquire *A. phagocytophilum* but it has not role in pathogen transmission (Sukumaran et al. 2006). The pathogen induced differential expression of tick genes in *Anaplasma marginale* infected *Boophilus microplus* where they found 279 differentially expressed genes (Zivkovic et al. 2010). The screening of differentially regulated tick genes with the pathogen infection is the survey of important tick molecule for control of tick’s ability to harbor and transmission of pathogens.

Rationale of Study

The *Rickettsia parkeri* has been detected in field collected whole ticks. While the specific identification and quantification of *R. parkeri* in tick tissues has not been reported yet. It is important to identify infection rate of *R. parkeri* in wild *A. maculatum*. The *R. parkeri* acquisition, propagation in tick vector and progression to tick progenies would be of great interest. The increasing knowledge of microbial diversity with the advent of next generation sequencing approach would be equally important to study in *A. maculatum* tick tissues. This will further provide the base line microbial community structures and propose for the possible interaction between them in relation to pathogen, *R. parkeri.* The *R. parkeri* interacts with the tick vector by inducing different tick genes. Cysteine protease inhibitors (cystatins) are important protein superfamily having role in tick's successful blood feeding and facilitating pathogen transmission. The importance of cystatins during the *A. maculatum* blood meal cycle by studying the mRNA expression profiling of selected *A. maculatum* cystatins would be our interest. Further, the cystatins differential gene regulation with the *R. parkeri* infection at different time points further provide the importance of tick cystatin gene with the pathogen infection. This study seeks the interaction of *R. parkeri* with microbial diversity in its vector (*A. maculatum*) and importance of tick cystatins in tick physiology and pathogen transmission.

CHAPTER II

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

We hypothesized that *Rickettsia parkeri* differentially regulates cystatins in tick tissues.

Specific Aims

Aim 1

Determine microbial diversity in field collected *Amblyomma maculatum* ticks tissues.

Aim 2

Determine *R. parkeri* induced cystatins expression in *A. maculatum* tissues.

Experimental Design

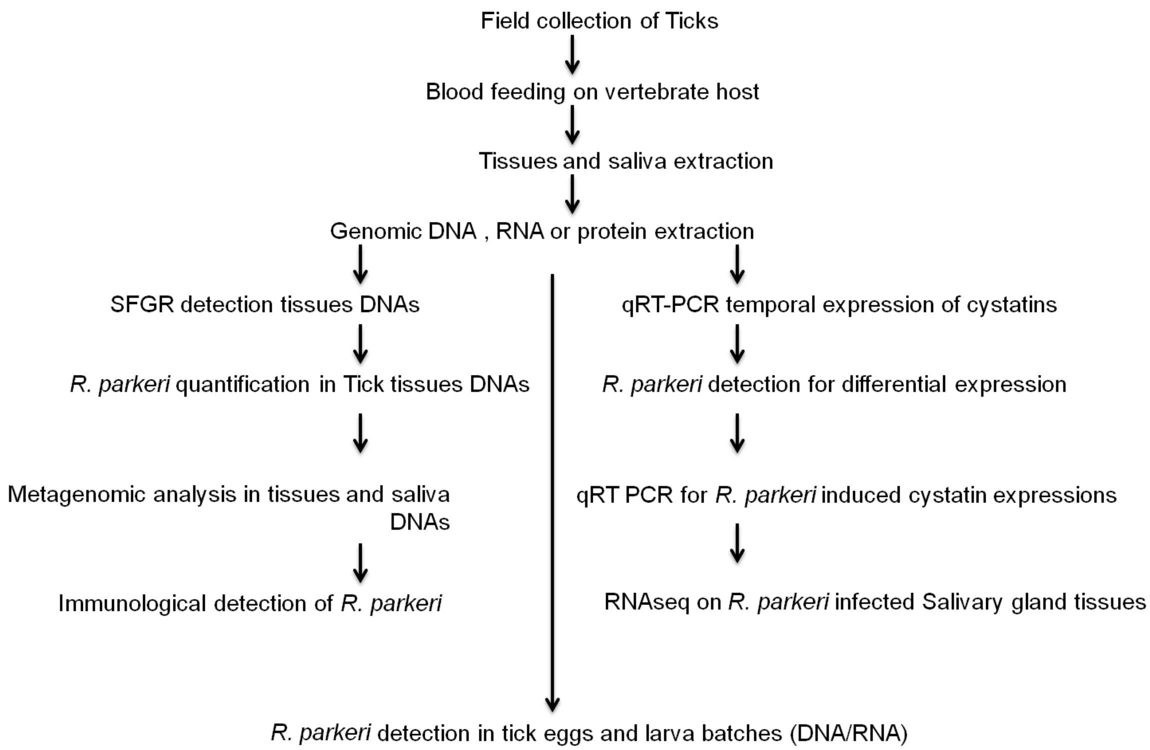


Figure 4. The experimental design. The flow diagram showing collection of ticks to processing and all the methods applied to test the research question.

CHAPTER III

MATERIALS AND METHODS

Sources of Ticks

The adult Gulf Coast ticks were collected using drag-cloth method from Sand Hill Crane National Wildlife Refuge, Gautier, Mississippi as described previously (Falco and Fish 1988) in early fall of 2011 and 2012. The collected adult ticks were identified based on morphological characteristics (Keirans and Litwak 1989).

We purchased *A. maculatum* tick from Oklahoma State University tick rearing facility on a regular basis. We called it lab colony *A. maculatum* and they were having *Rickettsial endosymbionts* infection. The Rickettsial infection free *A. maculatum* ticks were purchased from Texas A & M based on information discussed (Moraru et al. 2013) and we revalidate it based on *ompA* gene amplification were used in immunological study of *R. parkeri*.

Tick and Tick Tissues

Tick blood feeding. The field collected ticks were blood fed in vertebrate host (rabbit/sheep) according to approved Institutional Animal Care and Use Committee (IACUC) protocol. The 2011 collections were partially blood-fed on rabbit and were pulled after partial feeding, weighed and dissected to isolate midguts and salivary gland tissues from each female tick (Karim et al. 2002)**.** Tick saliva from partially fed *A. maculatum* was collected as described previously (Ribeiro et al. 1992). Briefly, Dopamine and Theophylline (1mM each) in 20 mM MOPS buffered saline with 3% DMSO, pH 7.0 were injected as stimulant for salivation (Needham and Sauer 1979). The collected tissues were kept in tissue storage buffer/ RNA later and the collected saliva was used immediately after collection or stored at -80°C freezer.

Tick tissues for temporal gene expression. The lab based female *A. maculatum* ticks (Oklahoma State rearing facility) were infested on sheep and were pulled at different time points on host (hours): 12, 18, 24, 36, 48, 72, 96, 120, 144, 168, 192, and 216 (replete ticks). The ticks were dissected and midgut and salivary gland tissues were kept in RNA later solution in freezer. The unfed ticks (0 hour) were dissected and tissues were kept similarly as other time points.

Tick tissues for differential gene expressions. The field collected ticks (2012 collection) along with similar number of lab colony ticks were infested on separate sheep and were pulled at different time points as two-day, three-day, and five-day, six-day, eight-day or tenth day and few ticks from both groups were kept until they dropped off. The pulled ticks were dissected for isolation of midgut and salivary gland tissues and isolated tissues were placed in RNA later solution and kept in freezer with while the individual tick carcasses (female tick body minus tick midgut and salivary gland tissues) and some male ticks were kept in ATL buffer for DNA extraction. Initially, individual tick carcasses were tested for the presence of *R. parkeri* by *ompB* qPCR method and based on carcass infection; the corresponding tick midgut and salivary glands were pooled together as infected or uninfected tick tissues. The pooled midgut and salivary glands tissues were checked for the infection before differential gene expression studies (Table 7).

Tick rearing and eggs and larvae. The dropped off ticks both from lab and field collection were placed in incubation at 28°C and 14/10 photo period for laying. After 25 day of lay-period, small chunk of freshly laid eggs were taken out from individual tick vial for total RNA extraction while rests were kept for hatching to larvae. From the hatched larvae, a small fraction of unfed larvae were taken out for the DNA extraction (Qiagen, CA), and subsequently checked for *Rickettsia parkeri* infection.

Microbial Diversity Study in *A. maculatum*

Rickettsial diversity. The infection of Spotted Fever Group rickettsia (SFGR) was tested by using an outer membrane protein A (*ompA*) gene specific primers in a nested PCR reaction (Blair et al. 2004). The primer RR 190-70 (F-5'-ATGGCGAATATTTCTCCAAAA-3') and RR 190-701(R- 5'-GTTCCGTTAATGGCAGCATCT-3') were used for the primary reaction, and 190-FN1 (F-5'-AAGCAATACAACAAGGTC-3') and 190-RN1 (R-5'-TGACAGTTATTATACCTC-3') for the nested reaction. In the primary reaction 2.5 µL of DNA template, 12.5 µL of 2X Master Mix (Promega, Madison, WI), 8 µL of nuclease free water, and 1 µL of each primer. In the nested reaction, the same mixture was used except with the nested primers and 2.5 µL of DNA from the primary reaction. The PCR reactions were performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories, USA) as follows: 1 cycle at 95°C for 3 min, 35 cycles of 95°C for 20 s, 46°C for 30 s, and 63°C for 60 s, and 1 cycle at 72°C for 7 min. The purified DNA samples were sequenced at Eurofins MWG Operon (Huntsville, AL). The sequences were submitted to BLAST analysis at National

Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) to determine homologous species.

Identification and Quantification of Rickettsia parkeri

The real time qPCR assay for detection of *Rickettsia parkeri* was performed to validate and quantify in tick tissues with naturally infected with SFGR screened from *ompA* gene nested PCR method (Jiang et al. 2012). The *ompB* gene was amplified by PCR using gene specific primers Rpa129F (5'-CAAATGTTGCAGTTCCTCTAAATG-3') and Rpa224R (5'-AAAACAAACCGTTAAAACTACCG-3'). Serial ten-fold dilutions (from 2 X 10^8 to 2X10^1) of the purified *ompB* PCR product were used for standard curve preparation (Figure 6). The standard curve preparation along with all unknown tick sample genomic DNA were prepared in a 25µL of reaction volume containing 2µL of (50ng/µL) of template DNA or cDNA, 0.7µM of forward and reverse primers, 0.4µM of probe (Rpa188p) (6-FAM-CGCGAAATTAATACCCTTATGAGCAGCAGTCGCG-BHQ-1), and 8 mM of MgSO4. The qPCR reactions were performed in a Thermal Cycler (CFX96 Real time detection system, Bio-Rad Laboratories, CA) as follows: 1 cycle each of 50°C for 2 min and 95°C for 2 min, 45 cycles of 95°C for 15s and 60°C for 30s. The non-template control (nuclease free water was used instead of template DNA or cDNA) and a positive control (a known *Rickettsia parkeri* infected sample) had been included in each qPCR run.

Immunological Detection of Rickettsia parkeri

The tick midgut and salivary glands tissues were mixed in 100 µL of extraction buffer (0.15M Tris-HCl, 0.3M NaCl, 10% glycerol containing protease inhibitors) followed by sonication for three times with 5 seconds pulse. The samples were centrifuged at ~20,000 x g for 10 minutes at 4ºC. The protein concentrations of the supernatants were estimated using the Bradford assay. The extracted midgut and salivary glands proteins were separated by 4-20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred onto nitrocellulose membranes in a transblot Cell (Bio-Rad) following the manufacturers' instructions. The *Rickettsia parkeri* grown in vero cell (Whole cells) and its supernatant were used as positive control while the *Amblyomma maculatum* (Texas A & M; *Rickettsia* free tissues) as negative control. The duplicate gel was stained with Coommassie Brilliant Blue for visualization. Nonspecific binding sites were blocked with 5% skim milk and mouse pre-immune sera (1:10,000). The membranes were incubated with mouse *R. parkeri* polyclonal antibody (1:500 dilutions). The antigen-antibody complexes were visualized with horseradish peroxidase-conjugated anti-mouse IgG (KPL) at a dilution of 1:10,000 and detected with Super Signal chemiluminescent substrate (Pierce, IL) using Bio-Rad ChemiDox XRS. The same blot was reused for probing actin using the monoclonal Anti-β-Actin-peroxidase (1:25000) (Sigma) for reference to *R. parkeri* cross re-activities (Figure 7).

Microbial Biodiversity in Field Collected Tick tissues and Saliva

The field collected *A. maculatum* ticks were screened with 16S RNA pyrosequencing for assessing the microbial diversity associated with SFGR infected tick tissues and secreted microbial communities in saliva. Bacterial tag-encoded Titanium amplicon pyrosequencing (bTETAP) was performed as described (Dowd et al. 2008a, Dowd et al. 2008b). The metagenomic sequencing was curated to obtain Q25 sequence data, which were processed using proprietary analysis pipeline (www.mrdnalab.com). All the sequences were trimmed to remove barcodes, primers and short sequences less than 200 base pairs. Further, the sequences with ambiguous base calls and homopolymer runs exceeding 6 base pairs were depleted. The sequences were than denoised and chimeras removed before operational taxonomic units (OTU) clustering performed using USEARCH (Drive5, WA). OTUs were defined after removal of singleton sequences, clustering at 97% similarity (Dowd et al. 2008a, Dowd et al. 2008b, Edgar 2010, Capone et al. 2011, Dowd et al. 2011, Eren et al. 2011, Swanson et al. 2011). The taxonomic level of classification of OTUs were performed using BLASTn against a curated GreenGenes database (DeSantis et al. 2006) and compiled into each taxonomic level into both *counts* and *percentage* files.

Cysteine Protease Inhibitors (Cystatins) Gene Expression

Selection of sialostatins. We selected cystatins based on the nucleotide similarity search from known cystatins of *Ixodes scapularis* with having signal peptides (AEO35364, AEO36092, AEO36722, AEO35688 and AEO35689), and without signal peptide (AEO35899). The primers from respective coding sequences were prepared for the expression studies (Table 4).

Total RNA extraction and cDNA preparation. Total RNA was extracted from the dissected tick midgut and salivary glands tissues and egg batches from both lab and field collected *A. maculatum* ticks. The RNA purification, cDNA synthesis and qRT PCR were performed with standard protocols as done previously (Browning et al. 2012). Briefly, RNA purification had been performed according to manufacturer's protocol (GE Health, Germany). Reverse transcription of total RNA was done using Moloney murine leukemia virus (M-MLV) reverse transcriptase according to the manufacturer's instructions (Invitrogen).

Real time polymerase chain reaction. First strand cDNA was used to measure mRNA levels with BIORAD CFX96 Real Time System. The Maxima SYBR Green qPCR Master Mix (Fermentas) was used according to manufacturer's recommendations; approximately 25 ng of cDNA and gene specific primers were used for each reaction mixture. The primers concentrations and reaction condition were standardized for each gene before qRT-PCR run. The primers for *cystatin-AEO35364* and *cystatin-AEO35689* worked well as150nM concentration and primers for cystatin-AEO36092, *cystatin-AEO36722*, *cystatin-AEO35688* and *cystatin-AEO35899* worked well at 300nM concentration. The differential gene expressions had been performed with 12ng of cDNA for allowing total RNA excess enough for all the experiments. The amplification of the target gene had been performed in C1000 Thermal Cycler using program, 2 min at 50°C, 10 min at 95°C followed by 36 cycles of 10 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C and plate read followed by melting curve 65°C to 95°C with increment of 0.5°C for 0.05 sec. All protocols for qRT-PCR experiments are in line with MIQE guidelines.

Normalization and statistical analysis. The transcriptional expression was normalized with *A. maculatum β-actin* based on $2^{-\Delta\Delta Ct}$ method as performed earlier (Browning et al. 2012) with the CFX96 BioRad real time system. The graphical representation had been performed in Sigma Plot software package. We observed differential regulations of the selected cystatins with the *R. parkeri* infection. The regulation threshold was considered two fold and statistical significance was observed at P-value <0.05 (Figures 12 and 13).

Table 4

The Primers used for the Cysteine Protease Inhibitors (Cystatins) Gene Expression Studies

Cystatins	Amplicon (bp)	qRT-PCR primers (5'-3')
AEO35364	105	Am35703-F 5'- CACCAACTACCGGATCACTTT-3' Am35703-R 5'- GTACTCTTCACATCCCGTGTTT-3'
AEO36092	111	Am41423-F 5'-GCCGTTCTGATTGTTGCTTG-3' Am41423-R 5'-GCGTTGAGAGACAGCATAGT-3'
AEO36722	126	Am47084-F 5'- GTTGACACCCGTGTTTCTTTG-3' Am47084-R 5'-GTTTGCTGGGAAACTGCATAG-3'
AEO35688	115	Am18992-F 5'-CTTGAGGTCATCGATGCAGAG-3' Am18992-R 5'- GACAGAGCTCCTTGGTGTATG-3'
AEO35899	122	Am65804-F 5- ACTTCAAGCCCGTCAACTAC-3 Am65804-F 5- GATATCGCCTCCGATGCTTT-3
AEO35689	144	Am94776-F 5'- GACGCCGATCCCATCTATAAC-3' Am94776-R 5'- GGTGAATCTCAGTCGGTAGTTG-3'
AEO33855	169	AmActin-F 5'-TGG CTC CTT CCA CCA TGA AGA TCA -3' AmActin-R 5'-TAG AAG CAC TTG CGG TGC ACA ATG-3'

RNAseq: Next Generation Sequencing

The total RNAs from *R. parkeri* infected three-day and five-day fed field collected *A. maculatum* salivary glands tissues along with the lab colony *A. maculatum* salivary glands tissues were submitted to Otogenetics Corporation (Norcross, GA USA) for RNA-Seq assays. Briefly, total RNA integrity and purity were performed with Agilent Bioanalyzer and OD260/280. The globin mRNA which may result from any recent blood meal was depleted with treating 5 µg of total RNA using the Ambion GLOBINclear-Human Kit and subsequently 1-2 µg of cDNA was generated from the depleted sample using the Clontech SmartPCR cDNA kit (Clontech Laboratories, Inc., Mountain View, CA USA, catalog# 634925) from 100ng of total RNA. The adaptor sequences were removed by restriction digest and the resulting cDNA was fragmented using Covaris (Covaris, Inc., Woburn, MA USA), profiled using Agilent Bioanalyzer, and subjected to Illumina library preparation using NEBNext reagents (New England Biolabs, Ipswich, MA USA, catalog# E6040). Before the sequencing the quality, quantity and the size distribution of the Illumina libraries were determined using an Agilent Bioanalyzer 2100. The libraries were then submitted for Illumina HiSeq2000 sequencing according to the standard operation. Paired-end 90 or 100 nucleotide reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK).

Estimation of differential gene expression. The differential gene expression was calculated between three-day and five-day fed *A. maculatum* (field collection) salivary glands infected with *R. parkeri* and *R. parkeri* free salivary glands from *A. maculatum* (lab colony). The one differential expression test (DESeq) was used for negative binomial distribution for the modeling variation (Anders and Huber 2010). The results indicated the fold change of each transcript between the groups as well as p-value significance. The adjusted p-values were employed to identify false discovery rate at which each transcript is significant. This DESeq is not suitable when there is substantial biological variation.

The three-day and five-day fed *A. maculatum* salivary gland infected with *R. parkeri* were studied for the differential tick gene expressions with comparing to same days fed lab colony *A. maculatum* salivary glands. The differentially expressed salivary glands genes were made in logarithmic of fold change difference from the lab colony *R. parkeri* free tick salivary gland. The overall calculation of the lab versus infected salivary glands performed too (Figure 9).

CHAPTER IV

RESULTS

Screening of Spotted Fever Group of Rickettsia (SFGR) in *A. maculatum*

The prevalence of SFGR infection in the collected ticks tissues DNAs were determined using *ompA* gene-specific primers in nested PCR (Figure 5). The PCR products obtained from each sample was sequenced and the nucleotide homology was assessed by searching the non-redundant nucleotide collection at NCBI.

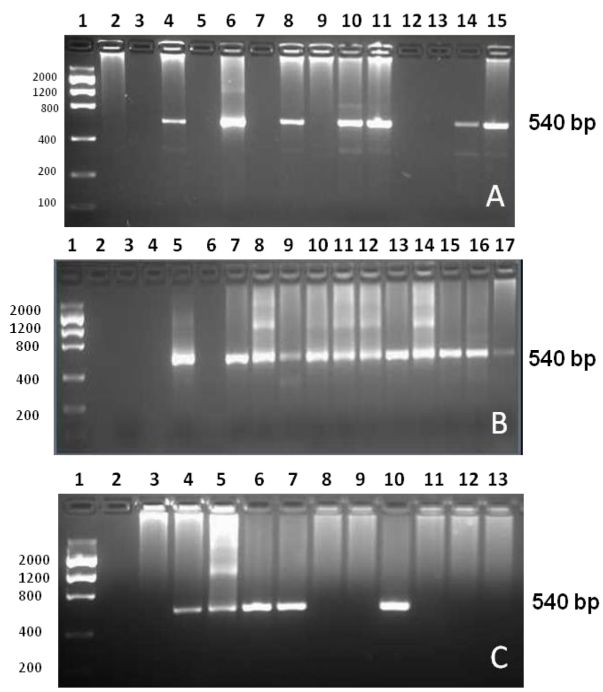


Figure 5. Molecular detection of Spotted Fever Group of Rickettsia in field collected *Amblyomma maculatum.* Tick tissues were tested for the presence of SFGR using the *ompA* nested PCR assay. (A) ♂ ticks: 1: DNA ladder, 2: non template control, 3: non primer control, 4: positive rickettsial template, lanes 5 to 15 DNA: templates of male tick samples. (B) ♀ midgut DNAs: Lane 1: DNA ladder, 2, 4 and 6: Blank, 3: non template control; 5: Positive Rickettsial template, lanes 7 to 17: DNA template. (C) ♀ salivary Glands DNAs. 1: DNA Ladder; 2: non template control; 3: non primer control; 4: positive rickettsial template; lanes 5 to 13: salivary gland DNA template.

Table 5

Spotted Fever Group Rickettsia (SFGR) Identification in A. maculatum Tissues

Tick	Sample ID	BLASTn (Closest homology)	% Identity	Gene Bank Acc. No.	*R. parkeri* Copies/μL
♀ MG	SH_Mg1	*R. parkeri*	100	JQ914757	-
	SH_Mg2	*R. parkeri*	96	JQ914758	-
	SH_Mg3	*R. parkeri*	92	JQ914759	-
	SH_Mg4	*R. parkeri*	98	JQ914760	-
	SH_Mg5	*R. endosymbiont of A. maculatum*	99	JQ914761	-
	SH_Mg7	*R. amblyommii*	99	JQ914762	-
	SH_B1	*R. parkeri*	100	JQ914763	-
	SH_B2	*R. parkeri*	100	JX134636	4000 ± 1106
	SH_B3	*R. parkeri*	100	JX134637	6 ± 2
	SH_B4	*R. endosymbiont of A. maculatum*	100	JX134638	-
	SH_B5	*R. endosymbiont of A. maculatum*	98	JX134639	-
	SH_B6	*R. endosymbiont of A. maculatum*	98	JX134640	-
	SH_B7	*R. parkeri*	100	JQ914764	-
	SH_B8	*R. parkeri*	99	JQ914765	755 ± 88
	SH_B9	*R. parkeri*	99	JQ914766	-
	SH_B10	*R. endosymbiont of A. maculatum*	100	JQ914767	-
	SH_D1	*R. endosymbiont of A. maculatum*	99	JQ914768	-
	SH_D2	*R. endosymbiont of A. maculatum*	100	JQ914769	-
	SH_D4	*R. endosymbiont of A. maculatum*	100	JQ914770	-
	SH_D5	*R. parkeri*	94	JQ914771	-
♀ SG	SH_SG1	*R. parkeri*	100	JQ914772	1794 ± 177
	SH_SG2	*R. parkeri*	100	JQ914773	-
	SH_SG3	*R. parkeri*	100	JQ914774	-
	SH_SG6	*R. parkeri*	100	JQ914775	-

Table 5 (continued).

Tick	Sample ID	BLASTn (Closest homology)	% identity	Gene Bank Acc. No.	*R. parkeri* Copies/µL
♂ tissues	SH_M2	*R. parkeri*	99	JX134641	-
	SH_M4	*R. amblyommii*	100	JQ914776	-
	SH_M6	*R. endosymbiont of A. maculatum*	99	JQ914777	-
	SH_M7	*R. amblyommii*	99	JQ914778	-
	SH_M10	*R. amblyommii*	100	JQ914779	-
	SH_M11	*R. amblyommii*	100	JQ914780	-

Of the 11 male ticks examined, 54% (6/11) were found to be SFGR positive with 99-100% homology to *R. parkeri*, *R. amblyommii*, or *R. endosymbiont of A. maculatum* (Table 5). We next examined midgut, salivary gland, and saliva SFGR infection in partially fed adult females. Analysis of the midguts revealed that 80% (20/25) of the tissues examined were SFGR positive and sequence homology revealed closest to *R. parkeri, R. amblyommii,* or *R. endosymbiont of A. maculatum*. Interestingly, salivary glands showed 50% (4/8) SFGRs and sequences closely related to *R. parkeri* (Table 5). The closest resemblances to the Gene Bank data bases of rickettsial *ompA* fragment could not able to confirm up to species level due to very small differences between different rickettsial *ompA* gene fragments. It is widely accepted that identification of Rickettsial species based on sequence homology to *gltA*, *ompB*, or *ompA* genes should exhibit at least 99.9%, 99.2%, and 98.8% similarity, respectively, for species validation (Fournier and Raoult 2009). The identification of *R. parkeri* in tick tissues (male and female) encouraged us to study further on *R. parkeri.*

Quantification of *R. parkeri* in *A. maculatum*

Standard Equation for R. parkeri Copy Number Calculation

The *R. parkeri* infections detected in *ompA* nested PCR screening were validated and quantified by *ompB* gene based qPCR assay. The *R. parkeri ompB* gene was amplified from the previously screened tick DNA samples. From the known *ompB* PCR products (concentrations or copy number) the qPCR run generated sets of specific cycle threshold (Ct) values for separate *ompB* PCR dilutions. From known ompB PCR products and respective Ct values a standard equation was generated (MS-excel) which were used for copy number calculation in rest of study (Figure 6).

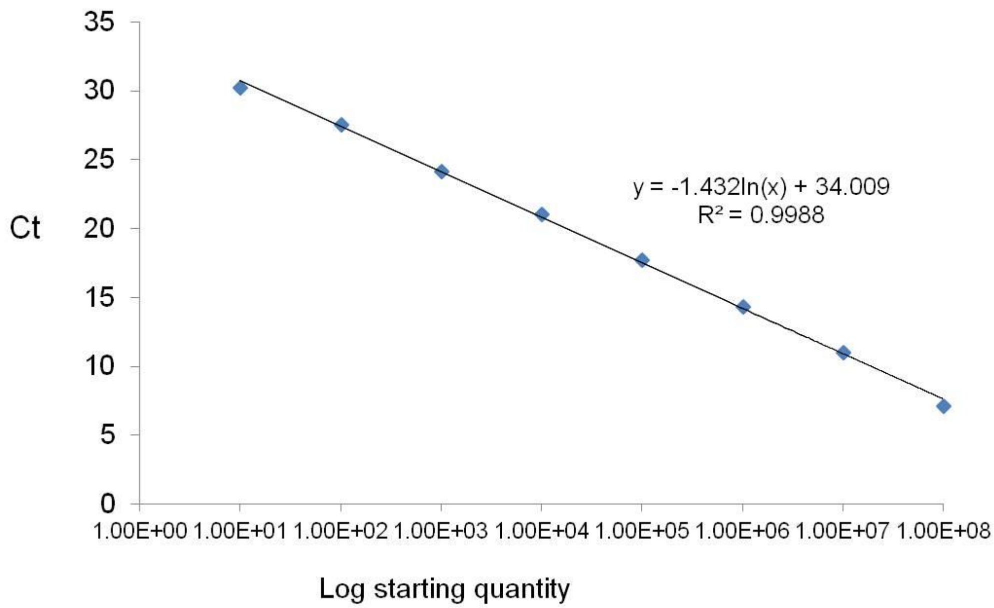


Figure 6. Quantification of the *Rickettsia parkeri* in *A. maculatum* tissues. The X-axis represents the known logarithmic of *R. parkeri ompB* gene and Y-axis represents the threshold cycle amplification of *R. parkeri ompB*. The standard equation used for the quantification of *R. parkeri* is shown in the figure.

Quantification of Rickettsia parkeri in Tick Tissues

The infection level of *R. parkeri* in collected ticks was observed as 12% (3/25) and to our surprise among the *R. parkeri* screened by *ompA* sequencing, only three out of 11 in midgut and one out of four in salivary glands tissues were validated for *R. parkeri* infection. The infection level of *R. parkeri* in the midgut samples of the partially fed adult female ticks ranges from 6 to 4000 copies/µL while the single salivary gland that tested positive had an infection load of 1794 copies/µL.

Table 6

Detection of Rickettsia parkeri in A. maculatum Tick Carcasses

Carcasses	Infections/ total tested	*R. parkeri* load ranges (copies/µL ± SD)
Unfed (n=8)	1/8	402.6 ± 54.2
2 day Fed (n=8)	4/8	1.4 ±1 .2 to 7201.1 ± 551.0
3 day Fed (n=10)	10/10	9.3 ± 3.9 to 6668.6 ± 1604.0
4 day Fed (n=11)	2/11	1871.0 ± 154.0 and 411.1 ± 41.7
5 day Fed (n=10)	7/10	1.2 ± 0.3 to 2389.0 ± 271.9
6 day Fed (n= 18)	5/18	3.3 ± 0.1 to 22793.2 ± 3797.7
8 day Fed (n=8)	1/8	815.0 ± 200.8
10 day Fed (n=10)	1/10	3683.4 ± 959.2
Male (n=5)	2/5	943.7 ± 137.2 and 2020.4 ± 47.4

Note: The whole tick minus tick midgut and salivary gland is tick carcasses. The copy numbers of *R. parkeri* were represented mean of technical triplicates and standard deviations.

The field collection of *A. maculatum* ticks (2012 collection), we were able to collect 82 adult female ticks and similar number of male ticks. The tick carcasses were checked for the *R. parkeri* infection before the pooling respective midgut and salivary glands tissues. The *R. parkeri* infection rate in carcasses was observed to be 35.8% (31/82) (Table 6). Similarly, the *R. parkeri* infection was observed in male ticks in group (three/group). We found 40% (2/5) *R. parkeri* infection rate in male *A. maculatum* ticks (Table 6).

Based on the infection of the carcasses the respective midgut and salivary glands were pooled and checked for *R. parkeri* infection from cDNA (50ng/µL). The three-day fed and five-day field *A. maculatum* salivary glands tissues were used for RNAseq analysis for global differential gene expression (Table 7). While the two-day and six-day fed *A. maculatum* tick midgut and salivary glands were used for differential cystatin gene expression studies.

Table 7

Detection of Rickettsia parkeri in Tick Tissues

Days on Host	Sample types	Midgut tissues	Salivary glands tissues	EGGs
Day 2	Lab Clean	0	0	
	SH Clean	0	0	
	SH infected	2543 ± 805	558 ± 41	
Day 3	Lab Clean	0	0	
	SH infected	279 ± 23	42 ± 14	
Day 5	Lab Clean	0	0	
	SH infected	352 ± 110	22 ± 9	
Day 6	Lab Clean	0	0	
	SH Clean	0	0	
	SH infected	729 ± 144	1964 ± 657	
After Incubation	Lab Clean batch			0
	SH Clean batch			0
	SH infected batch 1			114 ± 33
	SH infected batch 2			30 ± 1

Note: The field collected ticks are also referred SH. The tissues were pooled based on the carcasses infectivity. The *R. parkeri* were represented as copies/µL ± SD).

We observed there was three-fold *R. parkeri* copy number decrease from two-day to six-day fed midgut tissues while three-fold increase was observed in salivary glands tissues. The two infected egg batches were used for cystatin gene expression studies in the Table 7.

Transovarial and Transstadial Transmission of Rickettsia parkeri

The detection of the *R. parkeri* in eggs and larva from the field collected ticks was the proof of transovarial and transstadial transmission of the *R. parkeri* in naturally infected *A. maculatum*. We reported *R. parkeri* in *A. maculatum* eggs (cDNA) ranged 30-135 copies/µL and corresponding unfed larvae (DNA) with higher level of infection ranged 65-25240 copies/µL. The *R. parkeri* infection rate of naturally infected egg batches observed as 40% (4/10) and 50% (5/10) in corresponding larvae batches (Table 8).

Table 8

Detection of Rickettsia parkeri in Field Collected A. maculatum Egg and Larva Batches

Tick	*R. parkeri* (Eggs) Copies/µL ± SD	*R. parkeri* (larva) Copies/µL ± SD
1	135.4 ± 15.2	25240.3 ± 6814.2
2	119.5 ± 41.2	12146.4 ± 773.6
3	105.5 ± 7.5	13035.6 ± 5206.5
4	30.7 ± 1.4	1502.5 ± 275.1
5	ND	65.7 ± 10.2

Note: Field collected adults ticks were blood fed and allowed to lay eggs and hatch to larvae, the qPCR assay was applied in eggs (cDNA) and larvae (DNA) (N=10).

Immunological Detection of *R. parkeri* in *A. maculatum*

The mouse generated *R. parkeri* polyclonal antibody generously provided by Naval Medical Research Center (NMRC) was used in this assay. The polyclonal antibody (1:500 dilution) cross reacted with the *R. parkeri* grown vero cells and vero cell supernatants (positive controls) at ~30 and ~75kDa molecular weight though some faint band had been observed at ~100kDa (Figure 7). The Rickettsia free *A. maculatum* (Texas A & M) (negative controls) tick midgut and salivary gland did not show any prominent cross reactivity but the field collected tick tissues and lab *A. maculatum* (Oklahoma State University) showed the cross reactivity. The prominent cross-reacted bands in tick midgut tissues (~70kDa) and salivary gland tissues (~75kDa) lanes were lower than that observed at positive controls (Figure 7). The field collected ticks were infected with *R. parkeri* (verified in respective carcasses) while lab based ticks were infected with *R. endosymbiont of A. maculatum*.

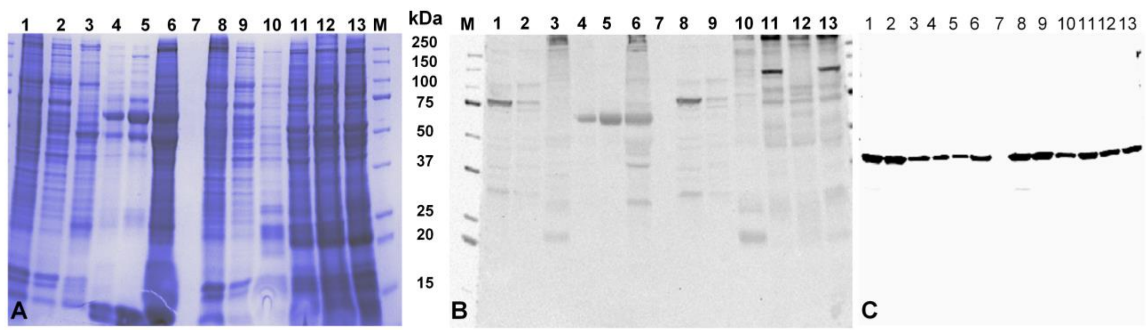


Figure 7. The immuno assay detection of *R. parkeri* in tick tissues. A 4-20% SDS-PAGE stained with Coomassie staining (A) and its corresponding immunoblot (B) demonstrating cross reactivity to Rickettsia parkeri antibody (B) and β-actin (C). Standard protein marker adjoining molecular size lane (M); Lanes: 1 and 2 were *Rickettsia parkeri* grown in verocells and supernatant of the same respectively and same order follows in lanes 8 and 9. Lane 7 was made blank. Lane 3 and Lane10 were *A. maculatum* (Texas A & M) midgut and salivary gland tissues respectively (Rickettsia free tissues); Lanes 4 and 5 (midgut tissues) and lanes 10 and 11(corresponding salivary glands) respectively from the ticks collected from field; Lanes 6 (midgut) and lane 13 (salivary gland) of lab colony *A. maculatum* (infected with *R. endosymbiont*).

The Microbiome of *A. maculatum*

Pyrosequencing offers an expedient and efficient opportunity to analyze bacterial communities, avoiding the need for intensive culture-based techniques. We utilized the bTETAP approach in 454-pyrosequencing platform for exploring bacterial species residing in blood-fed tissue from female *A. maculatum*. The midguts (n=4), salivary glands (n=2), and saliva (n=1) genomic DNA were set for pyrosequencing. In total, we obtained 27,691 sequence reads for the analysis after all the necessary trimming and removing all low quality sequences as described in methods and materials. The overall sequence reads were grouped according to the tissues. The 12,330 sequence reads from all from midgut tissues, 13,009 sequences reads from salivary glands and 2352 sequence reads from saliva were searched for BLASTn in GreenGenes databases for the similarity searches. The percentage of the sequences for a reference bacterial gene in overall tick tissues is considered here for the prevalence level in each tissue.

In all tested tissues and saliva bacterial phyla Proteobacteria, Actinobacteria and Firmicutes were common and that predominant covered all bacterial diversity in saliva sample and >95% in midgut and salivary gland bacterial diversities. Other phyla, Bacteroidetes, Spirochaetes, Cyanobacteria and Fusobacteria were detected in midgut tissues while salivary glands tissues revealed few reads from Bacteroidetes, Spirochaetes and Chloroflexi. At the bacterial family level, Francisellaceae, Enterobacteriaceae and Rickettsiaceae were important families detected in our study across the tick tissues and saliva which covered majority of bacterial species (>80-90%). The majority of endosymbiont in this study belongs to the Francisellaceae family where as the gut harbors bacteria belonging to Enterobacteriaceae family.

At the genera level, pyrosequencing revealed 54 different bacterial genera in midgut tissues; 23 bacterial genera in salivary gland tissues and 16 bacterial genera in saliva. The relative prevalence of the bacterial genera across the tick midgut, salivary glands and saliva were represented in heat map (Figure 8).

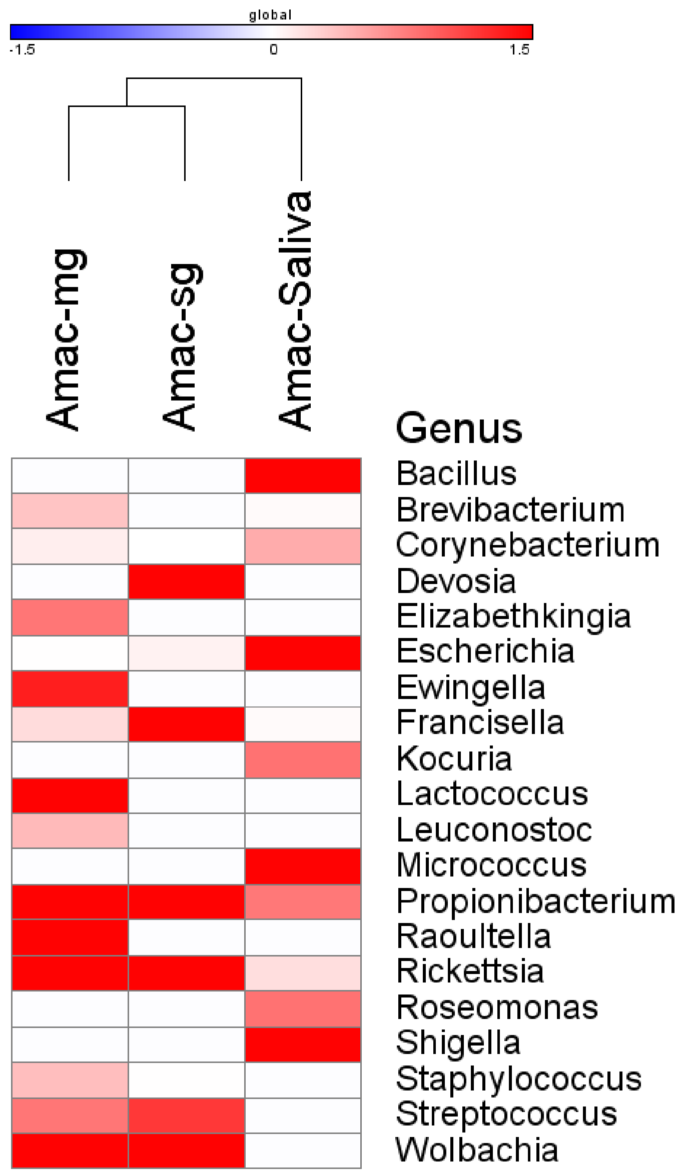


Figure 8. The relative prevalence of top 20 bacterial genera in *A. maculatum. A. maculatum* tissues labeled as midgut tissues (Amac_mg); Salivary gland tissues (Amac_sg) and saliva (Amac_Saliva). The sequence percentage reads were used for visualization. The data visualization of was performed in GENE-E version 3.0.26 (Broad Institute, Inc.).

The top 20 bacterial genera of *A. maculatum* revealed included *Bacillus, Brevibacterium, Clostridium, Coprococcus, Corynebacterium, Devosia, Elizabethkingia, Escherichia, Francisella, Kocuria, Lactococcus, Leuconostoc, Micrococcus, Pedomicrobium, Propionibacterium, Raoultella, Rickettsia, Roseomonas, Shigella, Skermanella, Staphylococcus, Streptococcus* and *Wobachia*. There were six genera common across the tick tissues viz. *Francisella, Propionibacterium, Rickettsia, Pseudomonas*, *Corynebacterium*, and *Escherichia* (Figure 9). We observed dominant level of *Shigella* (88.7%) in *A. maculatum* saliva. We further saw the differential level of bacterial genera in tick tissues in heat map.

The Rickettsiae were the important genera observed in pyrosequencing of *A. maculatum*. We observed the rickettsia dynamically present in tick midgut and salivary glands. The few rickettsial reads in saliva (Figure 8) suggested rickettsial secretion in saliva which was not observed with *ompA* PCR assay. In the figure 9, we presented the more than 1% sequences representing particular bacterial genera of midgut and salivary glands tissues rendering less than 1% as others. We observed *Rickettsia*, *Wolbachia* and *Propionibacterium* presence in both tissues (higher in midgut and lower at salivary glands). While *Francisella*, *Devosia* (both endosymbionts), and *Streptococcus* in only salivary glands (>1% reads) while *Raoultella*, *Ewingella* (both Enterobacteriaceae), and *Lactococcus* (Streptococcaceae) only in midgut tissues (Figure 9).

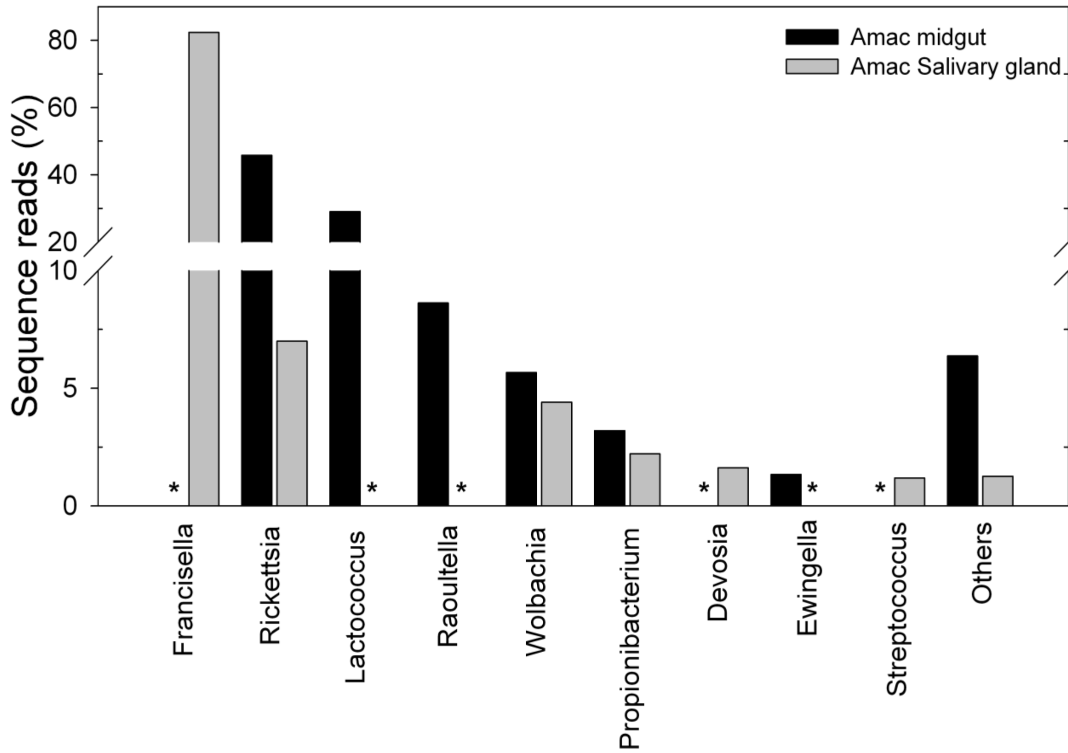


Figure 9. Relative prevalence of bacterial genera across the *A. maculatum* tissues. The bacterial diversity in the tissues from field collected female *A. maculatum* tissues based on 454- pyrosequencing approach. The asterisk sign (*) denotes the no or less than 1% reads for that genera. Values below 1% were grouped as "Others" for all three samples.

Temporal Gene Expression of Cystatins in *A. maculatum*

We selected the total six cysteine protease inhibitors (cystatins) from the *A. maculatum* sialotranscriptome project. The transcripts information available in Gene Bank with protein accession numbers AEO35364, AEO35688, AEO35689, AEO36092, AEO36722 all being having putative secretory signal peptide and AEO35899 without putative secretory peptide as determined in SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (data not shown) were selected based on the divergence and functional characterized known cystatins of *Ixodes scapularis.* From the selected cystatins protein corresponding mRNA transcripts were used for primer design. The each pair (forward and reverse) of primers from each cystatins were checked for specific amplification and validated by DNA sequencing from amplified products before using them for qRT-PCR assays.

The transcriptional expressions of the selected cystatins were observed in tick midgut and salivary glands tissues at different time points of feeding on host (Figures 10 and 11). The *cystatin-AEO35364* (Figure 10A) gene expression increased towards repletion in midgut while in salivary gland it peaks at 12 hour on host and decreased towards repletion stage.

The gene expressions of the *cystatin-AEO35688* (Figure 10B) gradually increase towards the engorgement stage in midgut and salivary glands tissues. While the expression *cystatin-AEO35689* (Figure 10C) observed to be higher unfed (0hr) and early feeding stage (two day) while it gradually decreased after two day and remains constantly expressed in both tissues onwards.

We observed the cyclic expressions of *cystatin-AEO36092* (Figure 11A) in midgut and salivary glands tissues. The gene expressions increased up to first-day and decreased gradually up to second-day and again increased at six-day in midgut but at four-day in salivary glands. The expression decreased in both tissues onwards but increased at repletion (nine-day).

The *cystatin-AEO36722* (Figure 11B) gene expressions remained fairly higher at unfed stage in both tissues and remained constant towards entire feeding cycle except enormously expressed (up to 400 fold) in midgut during repletion.

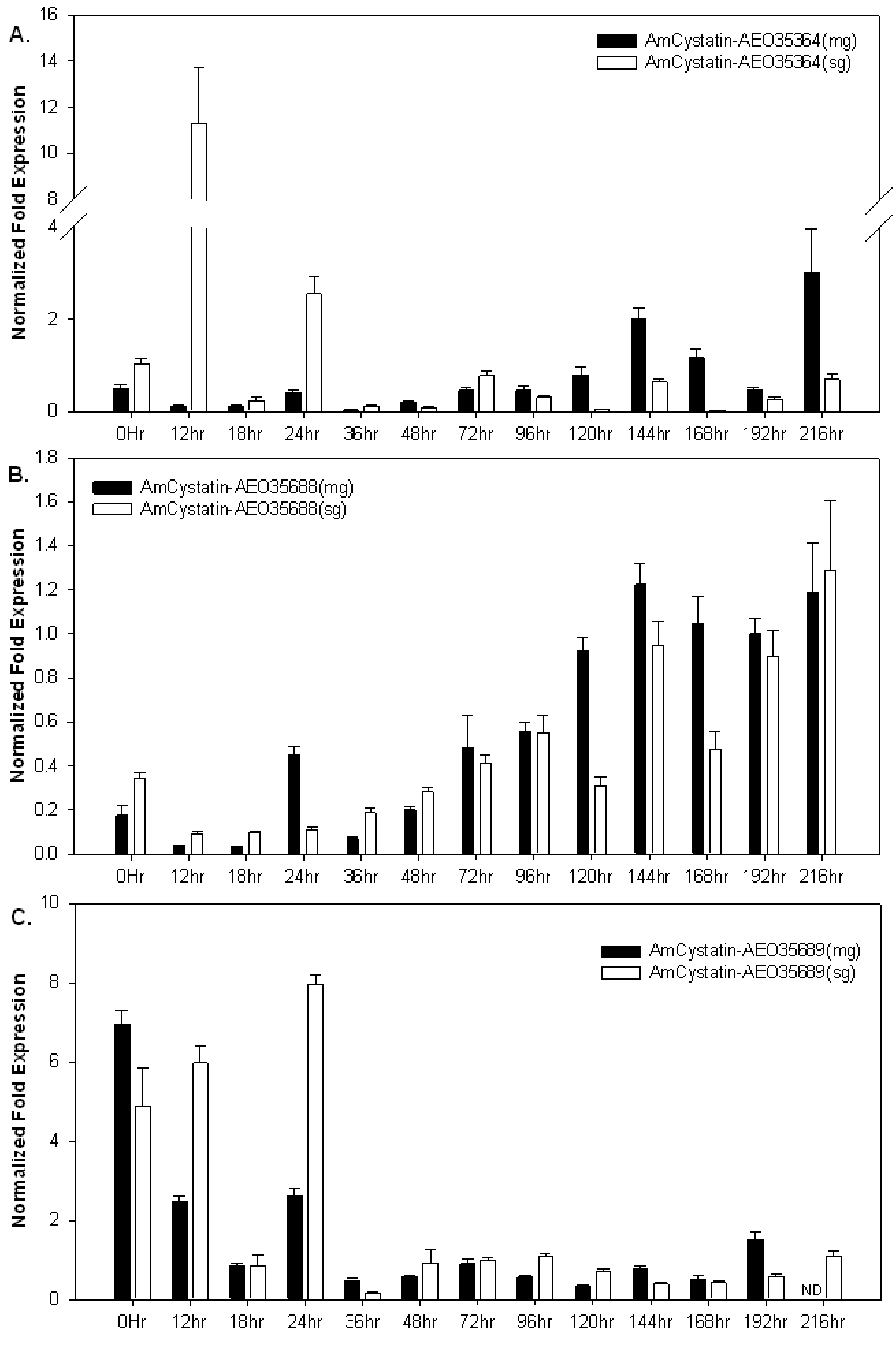


Figure 10. The temporal transcriptional expressions of cystatins in *A. maculatum.* The *A. maculatum* cystatins *cystatin-AEO35364*, *cystatin-AEO35688* and *cystatin-AEO35689* transcript level were determined in (A) midgut tissues and (B) salivary gland tissues across the blood meal cycle of nine days.

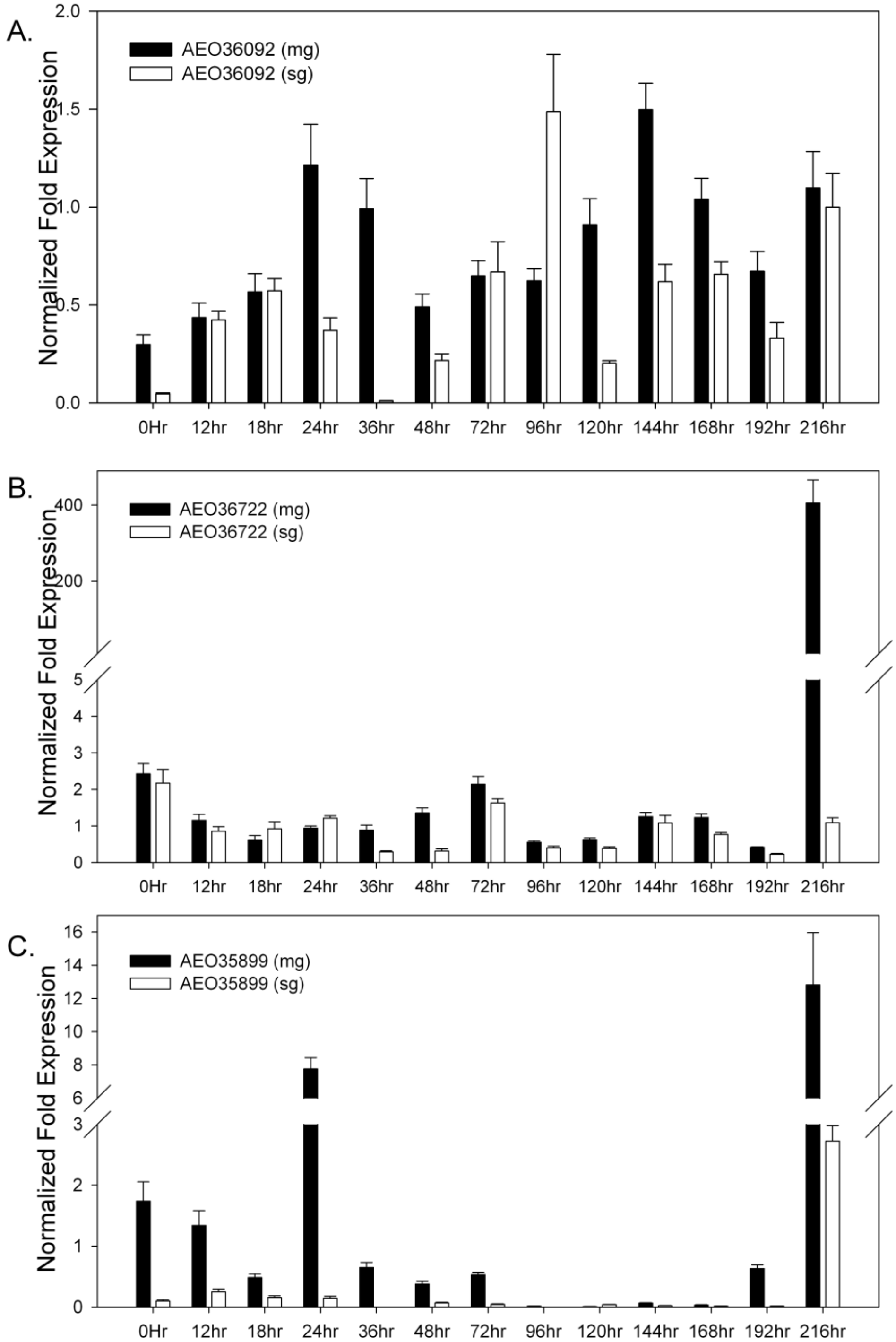


Figure 11. The temporal transcriptional expressions of cystatins in *A. maculatum*. The *A. maculatum* cystatins *cystatin-AEO36092*, *cystatin-AEO36722* and *cystatin-AEO35899* transcript level were determined in (A) midgut tissues and (B) salivary gland tissues across the blood meal cycle of nine days.

The intracellular *cystatin-AEO35899* (Fig 11C) gene expressed during early feeding stage and remained at lower during entire cycle except abruptly seen increased at repletion in midgut tissues. Similarly, the expression of this cystatin remained very low during entire feeding stage while abrupt increase was observed at repletion.

Rickettsia parkeri Induced Differential Expression of *A. maculatum* Cystatins

qRT-PCR

The transcriptional expression of selected cystatins were performed in field infected, field uninfected, and lab colony uninfected *A. maculatum* midgut tissues, salivary glands tissues of two different time points (two-day and six-day) on host. We further expanded the transcriptional expression studies in field infected, uninfected and lab colony *A. maculatum* egg batches. The *R. parkeri* infected tick tissues used for the differential expression were shown in Table 7 and transcriptional expression in different tick tissues with different infection were given in Figures 12 and 13.

The *cystatin-AEO35364* down regulated at six-day fed infected midgut tissues (-4.18 fold) and salivary gland tissues (-18.4 fold) compared to lab colony ticks of same day fed tissues (Figures 12 A, B).

We did not observe the any change in two-day fed tick tissues. While this cystatin was upregulated in infected egg batch 2 (+25.9 fold) compared to lab colony (Figure 12C).

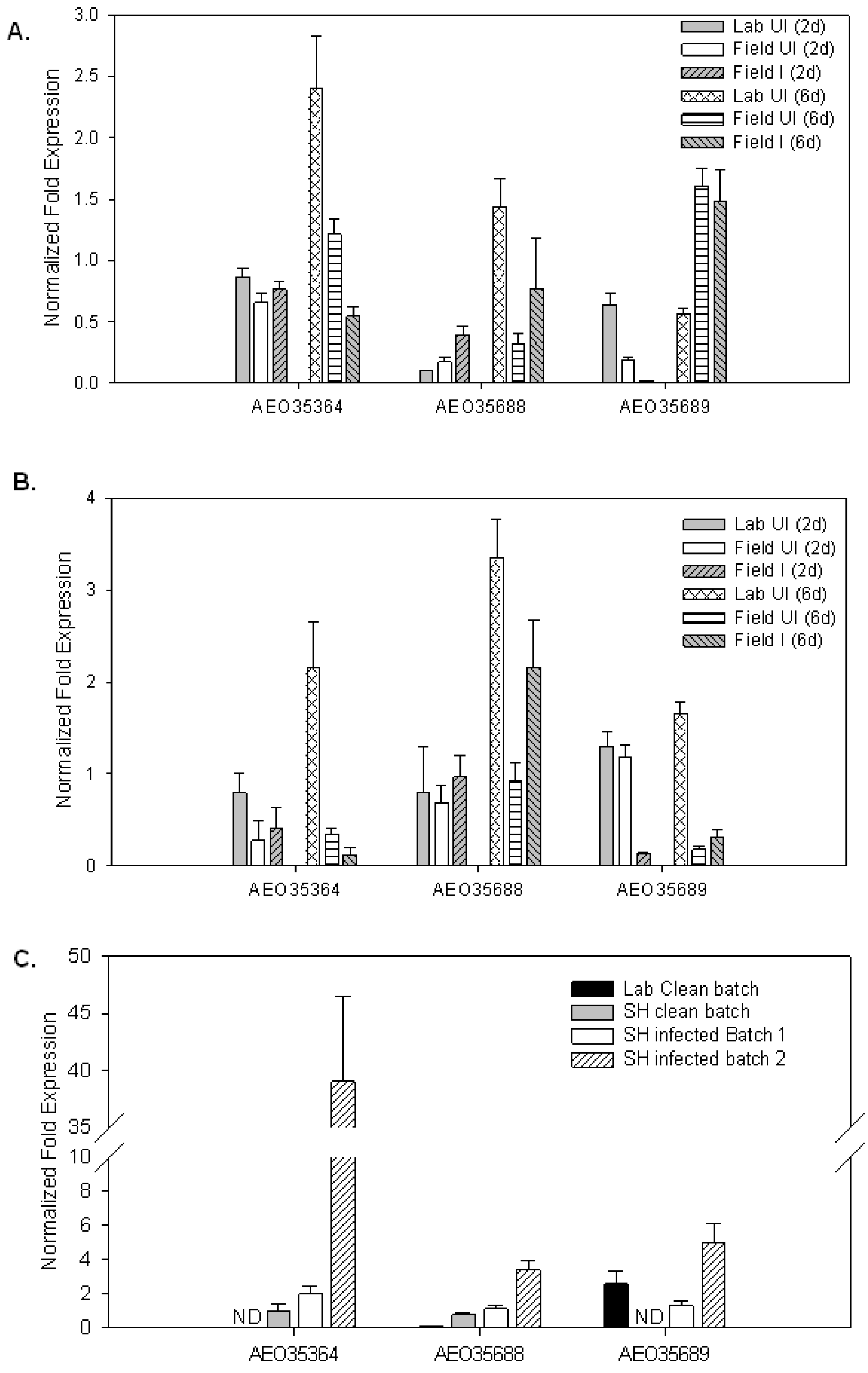


Figure 12. The *R. parkeri* induced differential gene expressions of *A. maculatum cystatins.* The differential expression of the selected secretary cystatins *cystatin-AEO35364*, *cystatin-AEO35688* and *cystatin-AEO35689* studied in *A. maculatum* tissues (A) midgut tissues (B) salivary gland tissues and (C) egg batches.

The *cystatin-AEO35688* showed up-regulation at two-day fed (+4.07 fold) midgut tissues and infected tick egg batches (+16.1 and +49.1 fold) while there were not significant differences in other tissues (Figures 12 A, B, C). The *cystatin-AEO35689* was down regulated in two-day fed midgut tissues (-38.2 fold) while slightly increased expression at six-day fed (+2.63 fold). Similarly, it was down regulated in salivary glands in two-day fed (-9.7 fold) and six-day fed (-5.3 fold) (Figures 12 A, B). But, it remained unchanged in infected egg batches (Figure 12C).

The *cystatin-AEO36092* was down regulated in midgut tissues with *R. parkeri* infection in midgut tissues at six-day fed (-9.9 fold) but remained unchanged at two-day fed (Figures 13 A, C). It was down regulated in both time points in infected salivary glands: two-day (-2.17 Fold) and 6-day (-4.62 Fold) (Figure 13C). Similarly, two different infected eggs batches were simultaneously up or down regulated in infected egg batches (Figure 13B).

The *cystatin-AEO36722* showed sharp down regulation at two-day (-112.36 fold) but observed upregulated at 6-day (+9.87 fold) midgut tissues. Similarly, in salivary gland tissues, it was down regulated in two-day (-46.14 fold) but slightly upregulated at 6-day (+2.77) (Figures 13 A, C). In egg batches, it was up-regulated in both batches (7.87 and 14.6 fold, respectively) with *R. parkeri* infection (Figure 13B).

The *cystatin-AEO35899,* intracellular cystatin, was observed up regulation at two-day fed (2.55 fold) and six-day fed (+1450.9 fold) midgut tissues but it remained fairly down regulated at two-day fed (-2.07 fold) and six-day fed

(+72.93 fold) with *R. parkeri* infection (Fig 13 A, C) in salivary gland tissues. The same cystatin was upregulated in infected egg batches 2 (+8.07 fold).

We observed field uninfected tick tissues expressing differently compared to lab colony tissues. We have seen differential regulation of selected cystatins in those tissues (Figures 12 and 13).

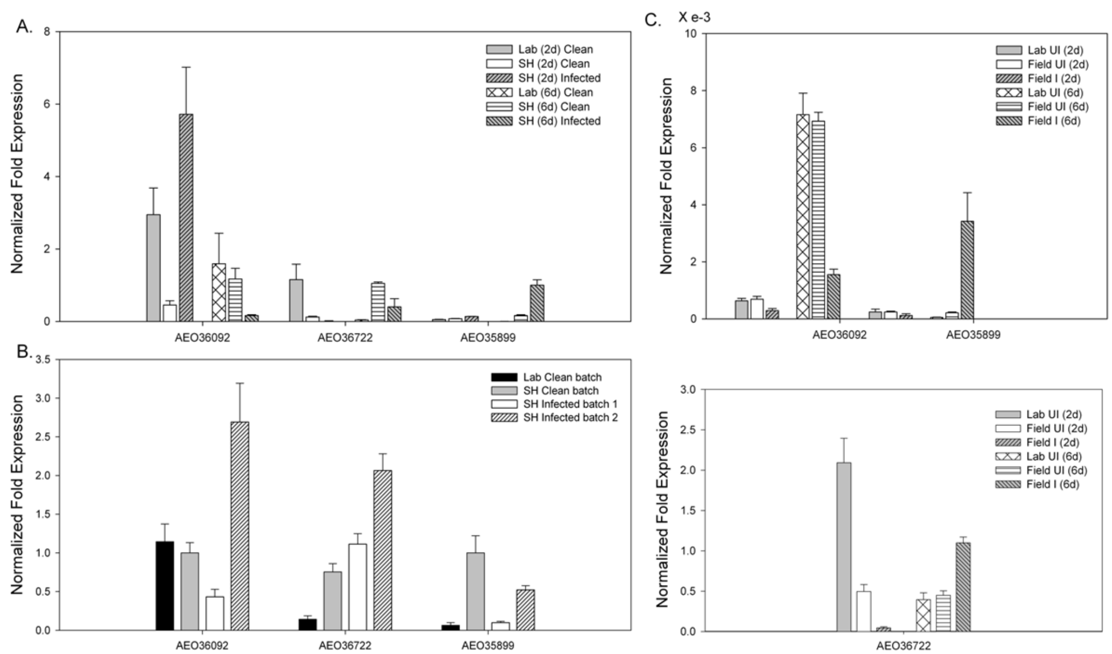


Figure 13. The *R. parkeri* induced differential gene expressions of *A. maculatum* cystatins. The differential expression of the selected secretary cystatins *cystatin-AEO36092* and *cystatin-AEO36722* and an intracellular *cystatin-AEO35899* studied in *A. maculatum* tissues (A) midgut tissues (B) egg batches and (C) salivary gland tissues.

RNAseq- Differential Gene Expression of the Selected Cystatins

The RNAseq method performed the differential gene expression between *A. maculatum* salivary glands tissues with and without *R. parkeri* infections (Table 7) in three-day and five-day fed *A. maculatum*. The RNAseq method provided the differential expressions of total 15,886 mRNA transcripts of *A.*

maculatum. We were interested in six different results for those from entire pool. The differential expressions of selected cystatins with *R. parkeri* infection in salivary glands tissues were represented in logarithmic of fold change (Figure 14). We observed the down regulation of cystatins: *cystatin-AEO35364*, *cystatin-AEO36092*, *cystatin-AEO36722* and up-regulation of *cystatin-AEO35688*. The intracellular cystatin, *cystatin-AEO35899,* remained unchanged with the pathogen infection in tick salivary glands. The *cystatin-AEO35689* down regulated at three-day while upregulated at five-day fed infected salivary glands tissues.

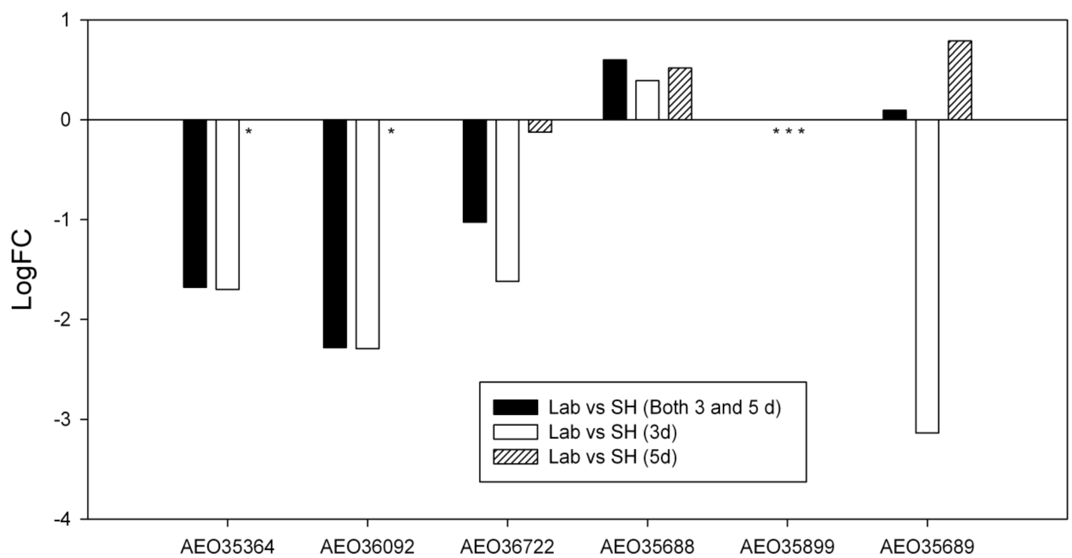


Figure 14. The *R. parkeri* induced differential gene expressions of *A. maculatum* cystatins in salivary glands by RNAseq. The asterisk (*) sign means the no differential expression observed.

CHAPTER V

DISCUSSION

The study of bacterial diversity in *A. maculatum* ticks and within that specific characterization of *R. parkeri* and its effect on tick genes was the aim of this study. We collected the field ticks couple of times during fall of 2011 and 2012 from the Sand Hill Crane National wild life refuge, Gautier, MS. The study of bacterial diversity and specific characterization of *R. parkeri* in field collected ticks was performed in collected ticks (2011). Based on these findings, we collected (2012) ticks again for further study of *R. parkeri* transmission in tick progenies and global gene regulation in infected tick tissues by RNAseq.

The Rickettsial diversity assay was performed with specific amplification of the rickettsial outer membrane protein A (*ompA*) gene (Blair et al. 2004). The amplicon sequencing revealed the presence of three important rickettsial species after aligning at BLAST program of Gene Bank. The male and female *both A. maculatum* were harboring *R. parkeri*, *R. amblyommii* and *R. endosymbiont of A. maculatum.* Within the female *A. maculatum*, only the *R. parkeri* was detected in tick salivary glands compared to all three different rickettsiae in tick midgut. It may be due to its unique ability to traffick to salivary gland from midgut tissues via haemocoel. The pathogen acquired in tick midgut tissues traffick to salivary glands and transmitted to host via salivation (Ribeiro et al. 1987). But we were not able to amplify *ompA* gene in tick saliva (results not shown). This may suggest that either a lack of SFGR secretion altogether or that the bacteria were secreted at a different time point though we observed few rickettsial reads from pyrosequencing approach. The *ompA* gene screening of rickettsial agent was followed by *ompB* gene based specific qPCR assay for *R. parkeri* in same samples. The specific detection of *R. parkeri* by qPCR assay showed only three out of 11 *R. parkeri* infection in female tick midgut tissues and one out of four *R. parkeri* infection in salivary gland tissues. While there was no detection in male ticks. The very small differences between the *ompA* gene fragments of 540 base pairs (Figure 5) among the rickettsial agents could be the reason behind lower level of detection of *R. parkeri* with *ompB* assay.

The qPCR assay for detection of *R. parkeri* became important assay for further screening of tick tissues for infection rate and selection of tissues for differential gene expressions (Tables 5, 6, 7, and 8). We showed the *R. parkeri* infection rate as 12-40% in total samples tested in field collected *A. maculatum*. In various literatures, we have seen that the *R. parkeri* infection rates among field collected *A. maculatum* ticks ranged from 28-43.1% (Cohen et al. 2009, Paddock et al. 2010, Trout et al. 2010, Varela-Stokes et al. 2011, Wright et al. 2011). Unlike previous studies, our study deals with the specific tissues of *A. maculatum,* which were unfed to partially blood fed on vertebrate host. The quantification of *R. parkeri* in tick midgut tissues, a site of pathogen acquisition and development and in salivary gland tissues, a important tissues of pathogen development before transmission via salivation while infesting in vertebrate host were the important findings with respect to biology of *R. parkeri* in vector.

The *R. parkeri* trafficking from midgut to salivary gland and secretion via salvation is important with respect to pathogen and disease spread. The transovarial and transstadial characteristic of *R. parkeri* would provide the *A. maculatum* itself could be a reservoir of *R. parkeri*. We proved the *R. parkeri* is transovarial and transstadially (Tot) transmitted to *A. maculatum* progenies in natural conditions. Though, Tot of *R. parkeri* was shown in lab condition in *Amblyomma americanum* (Goddard 2003). We are the first to show the *R. parkeri* transovarial and transstadial transmitted in its natural host *A. maculatum.*

Further, we proved *R. parkeri* infection in field collected tick midgut and salivary gland tissues with the polyclonal *R. parkeri* antibody. The cross reactivity with the *R. parkeri* antibody in vero cells and tick tissues were not at same band (Figure 7) which may be due to the polyclonal nature of our antibody may cross reacted with differently expressed rickettsial protein in tick tissues and mammalian tissues. Though, we observed the negative control (tick tissues from Texas A & M) working perfectly but the lab colony tick tissues showed cross reactivity with antibody used for detection of *R. parkeri*. The *R. endosymbiont* present in our lab colony ticks may have cross reacted with *R. parkeri* polyclonal antibody and it was reported earlier that antibody generated against one rickettsial pathogen showed frequent cross reaction to other rickettsial species (Anderson and Tzianabos 1989).

The association of different bacteria in tick midgut, salivary glands and saliva provided the profiling of bacteria in tick tissues. For the assessment of the entire bacterial community we used recently developed culture independent metagenomic approach, a revolutionizing tool in microbiology, in 454-pyrosequencing platform. The pyrosequencing approach of identification of bacterial diversity across the midgut, salivary glands and saliva revealed the dynamic presence of *Rickettsia* species along with the other microbial species which may have role in interaction with other bacterial communities in tick tissues. The detection of microbial genera across the tick tissues and saliva (Figure 8) with predominantly enterobacteria in midgut tissues and endosymbionts in salivary glands with dynamic presence of rickettsia is the important finding in this study. The *Enterobacteria* genera: *Raoultella, Ewingella, Escherichia* and *Klebsiella* were present about 30% of total species diversity in gut tissues. The dominance enterobacterial species in tick gut tissues may have role in digestion or stress tolerance (Wang et al. 2011). Further the *Stenotrophomonas, Pseudomonas, Rhodococcus* and *Propriobacterium,* which were detected *in A. maculatum* midgut and salivary glands, were detected also in *I. ricinus* and proposed to be a part of core microbiome of Ixodid tick (Carpi et al. 2011). The *Propionibacterium* were found in both midgut and salivary gland which has been related with stress response ability (Wang et al. 2011). The detection of *Mycobacterium, Bacillus, Streptococcus, Clostridium, Streptomyces, Pseudomonas, Streptococcus, Corynebacterium, Staphylococcus, Papilibacter, Coprococcus, Eubacterium, Roseburia, Pantoea, Ruminococcus,* and many other environmental and soil bacterial species had been reported earlier from tick but the biological significance of these huge bacterial diversity has not been known yet (Andreotti et al. 2011, Carpi et al. 2011). The study on biological significance of bacteria or bacterial community is required to provide further significance of metagenomic studies.

A rich diversity of bacterial genera in tick midgut tissues could be the direct environmental contact to tick midgut and less than half in salivary gland tissues could be due to the differential level of pathogen trafficking across the midgut to salivary gland or could be the described with tissue specificity of microbes. We saw even less bacterial diversity in saliva which could be due to the less level of secretion or the environmental contamination during saliva sampling. Though few reads of *Rickettsia* from saliva but it had great significance provided evidence of rickettsial secretion in saliva.

The salivary glands revealed the dominance of *Francisella endosymbionts* and *Devosia endosymbionts* (Figure 9). Though, *Devosia endosymbiont* had not been reported from tick earlier but the *Francisella* were identified from other tick species as well viz. *D. variabilis*, *D. andersoni*, *D. hunteri*, *D. nitens*, *D. occidentalis*, and *D. albipictus* (Niebylski et al. 1997, Sun et al. 2000, Scoles 2004). The huge presence of *Francisella endosymbionts* in tick salivary glands but <1% in midgut tissues was interesting. We hypothesized that *Francisella endosymbionts* may facilitate the rickettsia in salivary glands tissues. The detection of *Wolbachia* in both midgut and salivary glands in similar level and recent studies on role of *Wolbachia* in pathogen transmission (Hughes et al. 2011), pest control by cytoplasmic incompatibility (Zabalou et al. 2004) seek the further characterization and functional significance of *Wobachia* in ticks. The *Wolbachia* and *Spiroplasma endosymbionts* has shown role building insect immunity and helping the tick immune system during the pathogen infection and they proposed endosymbiont could be the guardian of insect immune system (Eleftherianos et al. 2013).

The microbes identified in saliva *Shigella, Bacillus, Escherichia, Micrococcus and Micrococcus* (Figure 8) may be due to the contamination of host skin or importantly these detected species are common species in soil and environmental samples (Carpi et al. 2011).

The manipulation of the microbial communities with the altering or inhibiting particular bacteria could alter the pathogen transmission ability of vector (Hughes et al. 2011) which could be further direction in the *A. maculatum* and rickettsial interactions. Though, the factors behind tick susceptibility to rickettsial infection are unknown yet. However, exactly how the tick midgut microbiome influences pathogenic rickettsial development and transmission would be of great interest.

The identification of role of *R. parkeri* with the endosymbionts is the important further direction of bacterial diversity in *A. maculatum*. In the other hand, we wanted to see the how tick genes were affected with presence of *R. parkeri*? We focused on the cysteine protease inhibitors (cystatins) as they are important during tick blood meal acquisition and facilitation of pathogen transmission (Karim et al. 2005, Kotsyfakis et al. 2010). The presence of the pathogen in tick tissues changes the expression pattern of different tick molecules and which the pathogen utilizes for the survival, replication, and development of virulence and transmission to host via tick. In this study, we observed the expression patterns of the tick cysteine protease inhibitors during normal blood meal cycle as well as with the presence of *R. parkeri* to understand the molecular mechanism important for the tick and for pathogen in tick.

The transcriptional expression provides the mRNA activity of particular gene at particular time points in cell. The *cystatin-AEO35688* (Figure 10C) activity increased in midgut and salivary glands as the feeding progresses and became peak at the repletion stage. Similarly, we observed the peak expression at repletion stage for *cystatin-AEO36722* (Figure 11B) and *cystatin-AEO35899* (Figure 11C) in both midgut and salivary glands tissues. The temporal expression study of sialostatin L2 had also shown to be increased as the feeding progressed (Kotsyfakis et al. 2007). They have further showed the functional role in tick blood feeding success of sialostatin L2, and immunomodulation role in host (rabbit). The sialostatin L2 further characterized with the inhibition of cathepsins L, V, S, and C. Recently, it has been shown that tick blood digestion takes place utilizing the cysteine proteases and aspartic endopeptidases, and blood digestion (haemoglobinolysis) starts with the ingestion of the blood meal and it increases towards the fast feeding stage and become maximum at fully fed ticks (Horn et al. 2009). The cystatins regulate the cysteine protease activity or proteolysis reversibly; we assume that role of cystatins *cystatin-AEO35688, cystatin-AEO36722, and cystatin-AEO35899* during the blood meal digestion though further reverse genetic approach (RNAi) needs to be performed. The higher mRNA expression of these cystatins at the repletion stage may have role in blood digestion after the tick drop off the host (Sojka et al. 2013). The transcriptional expression of the most of the cystatins in salivary gland follows the same pattern as in midgut tissues. The higher expression could be related with stress for osmoregulation (removing excess amount of water via salivation) and secretion of the huge amount of the biomolecules helping in the disguising the host advanced blood coagulation, immunity, and pain reactions (Francischetti et al. 2009).

The transcriptional expression of *cystatin-AEO35364* (Figure 10A) higher at the early stage (salivary glands), similarly, *cystatin-AEO35689* (Figure 10C) had peak expression during tick attachment stage. The peak expression of the cystatins during initial attachment on host is important for the attachment success of ticks and countering initial response of host immune responses. We have seen the cyclical gene expression pattern of *cystatin-AEO36092* (Figure 11A) in both tissues across the blood meal which may have role across the tick blood feeding. The early cystatin expression and decreased in the expression with the feeding had also been seen in sialostatin L (Kotsyfakis et al. 2007). Our results showed the importance of cystatins during blood feeding of *A. maculatum*. The cystatins role in ticks' blood feeding success had observed in *A. americanum* (Karim et al. 2005) and *Ixodes scapularis* (Kotsyfakis et al. 2007). The further study of cystatins expressing peak during early stage may provide important information on tick initial attachment success which could be important vaccine target for tick and tick borne disease control.

The cystatins could be the molecular target for the *R. parkeri* development and transmission in *A. maculatum* as well. We observed the selected cystatins were differentially regulated with presence of *R. parkeri* in all the tick tissues tested including the egg batches (Figure 12 and 13).The selected cystatins expression study in *R. parkeri* infected tick midgut, salivary glands and egg batches provided the importance of cystatin in selected tissues.

We observed gene expression of an intracellular cystatin *cystatin-AEO35899* (Figure 13) up regulated in all tissues tested with intracellular bacteria, *R. parkeri*, infection though we did not see any regulation in RNAseq assay (Figure 14). *R. parkeri* induced transcriptional expression of intracellular cystatin provided the important information to consider this while identification of molecular target controlling tick borne diseases. The cystatin differential expression study provided important results in infected tick eggs. All the selected cystatins were observed to be up regulated with *R. parkeri* infection in tick egg batches (Figures 12C, 13B). The possible explanation could be the cystatins' role in inhibition of various classes of the proteases, protection of eggs from the microbial or parasite attack and regulates egg protein degradation (Golab et al. 2001). It has been found that vitellin-degrading cysteine endopeptidases (VTDCE) was associated with tick egg-yolk processing cascades (Seixas et al. 2003) in *Boophilus microplus,* a hard tick. The upregulated tick cystatins which may potentially mediates the degradation of VTDCE-like activity during establishment of *R. parkeri* in tick eggs.

The differential expression of the cystatins with RNAseq and qRT-PCR in salivary glands revealed similar pattern of down or up regulation of cystatins expect intracellular showing no regulation with RNAseq results. The benefit of

RNAseq assay is that it provided differential expressions for all the tick genes in sialotranscriptome project discussed earlier.

The selected cystatins were differentially regulated in *R. parkeri* infected midgut tissues. Except in a few cases, up or down regulation of selected cystatins had similar pattern with salivary glands tissues with *R. parkeri* infection. The *cystatin-AEO35689* upregulated in midgut tissues (six-day fed), whereas down regulation in salivary glands (Figures 12A, B). It was observed from both RNAseq and qRT-PCR assay that *cystatin-AEO35364*, *cystatin-AEO36092*, *cystatin-AEO36722* were down regulated with the presence of *R. parkeri* in tick midgut and salivary glands. While *cystatin-AEO35688* remained up regulated and *cystatin-AEO35689* up down regulated in 2 and 3 day midgut/salivary glands while up regulated in five-day by RNAseq (Figure 14) while down regulated in six-day by qRT-PCR (Figure 12B) in salivary glands tissues with *R. parkeri* infection. The qRT-PCR served as the validation of the RNAseq therefore the qRT-PCR data may have higher confidence for the results or incongruity may be due to different samples viz. three and five-day for RNAseq while two and six-day salivary glands tissues for qRT-PCR. Though there lacks differential expression with pathogen infections, cystatins had been reported facilitating pathogen transmission success in case of *Borrelia burgdorferi* transmission from *I. scapularis* (Kotsyfakis et al. 2010).

The differential expressions were observed in field uninfected tick tissues along with the infected to make comparison studies. In many cases, the selected cystatins differentially expressed in field uninfected (*R. parkeri* negative by qPCR) tissues. The differential expressions observed might be due to the effect of pathogenic microbial species other than *R. parkeri*. The differential expressions in tick midgut and salivary glands tissues were performed at two different time points with two different levels of *R. parkeri* infections. The different levels of *R. parkeri* infection had been used using two egg batches. The infection dependent differential level of cystatins expression had not been assessed due to non-uniformity in expressions in tissues. The further separate study in tick cell lines with different level of *R. parkeri* infection could be performed to answer the pathogen load dependent differential expressions.

CHAPTER VI

CONCLUSIONS

The *A. maculatum* rickettsial diversity is comprised of *R. parkeri, R. amblyommii* and *R. endosymbiont of A. maculatum*. Only *R. parkeri* detected in female salivary glands provided its unique ability to traffic from midgut tissues. We found the *R. parkeri* infection rate of 12-40% in field collected *A. maculatum* ticks. The *R. parkeri* was detected in field *A. maculatum* tick eggs, and larvae showing for the first time transovarial and transstadial transmission of *R. parkeri* in wild *A. maculatum*. The *A. maculatum* microbial diversity as assessed by pyrosequencing comprised of *Rickettsia* along with abundance of Enterobacteria in midgut, *Francisella endosymbionts* in salivary glands tissues and other environmental bacteria in saliva. *A. maculatum* cystatins were transcriptionally active during early or fast blood feeding suggesting their importance in tick feeding. *A. maculatum* responds the *R. parkeri* presence by differentially regulating cystatins in tick tissues and eggs.

APPENDIX

IACUC PROTOCOL

THE UNIVERSITY OF SOUTHERN MISSISSIPPI

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Phone: 601.266.4063
Fax: 601.266.4377

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**10042001**
PROJECT TITLE:	**Tick Sialome**
PROPOSED PROJECT DATES:	**February 2013 – September 2015**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Shahid Karim**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**NIH NIAID, DOS NAS, AHA**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	September 30, 2015

Jodie M. Jawor, Ph.D.
IACUC Chair

19 February 2013
Date

Inclusions – see 'Procedures'

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