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Quantitative real-time polymerase chain reaction (QPCR) assay as a molecular tool to assess rickettsial replications in tick hosts

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QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QPCR) ASSAY AS A
MOLECULAR TOOL TO ASSESS RICKETTSIAL REPLICATION IN TICK HOSTS

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Abstract

During the past century, many species of the Spotted Fever Group *Rickettsia* (SFGR) have been described, especially, through the introduction of a variety of molecular techniques applied to detect rickettsiae inside of their host. In this study we developed a quantitative real-time polymerase chain reaction (qPCR) assay (1) to characterize the growth and the distribution of a SFGR of unrecognized pathogenicity in naturally infected *Amblyomma americanum* ticks during physiological events; and (2) to determinate the influence of the host cell specificity in the replication patterns of recognized and unrecognized SFGR during a reciprocal rickettsiae challenge in both mammalian and tick cell lines. *Rickettsia amblyommii* was identified in the tissue samples of naturally infected *A. americanum* ticks at ratios of ≤ 1 rickettsiae per tick cell. Significant variability in the ratio of rickettsial to tick gene copy numbers between the tissues was identified; however, no single tissue was consistently observed to have the greatest rickettsial burden throughout the feeding event. Furthermore, the ratio of rickettsial to tick gene copy numbers did not significantly differ between eggs, immature ticks, and feeding events. In the *in vitro* study, differences in the ratio of rickettsiae per cell were observed within each cell line. The ratio of rickettsiae per host cell was greatest in *Rickettsia*-infected ISE6 cells, compared to Vero cells. *Rickettsia parkeri* infection load was consistently greater in both cell lines compared to *R. amblyommii* and *Rickettsia montanensis*; and considerable variability between these last two *Rickettsia* species was observed when the ratio of rickettisae per host cell was calculated for each individual cell line. The implications of the use of this technique to understand the pathogenic nature of some SFGR and to investigate the host specificity in the tick-SFGR interactions is further presented and discussed.

Chapter 1: General Introduction

Ticks transmit a greater variety of pathogenic microorganisms compared to any other arthropod vector group; and they are among the most important vector of diseases affecting humans and animals (Jongejan and Uilenberg, 2004). Within this milieu of possible infectious agents, tick-borne rickettsioses have a special importance because zoonoses of the Spotted Fever Group *Rickettsia* (SFGR) are among the oldest known tick-borne diseases (Parola et al., 2005), accounting, therefore, to one of the highest morbidity and mortality rates in humans (Jongejan and Uilenberg, 2004).

Spotted fever group *Rickettsia* (SFGR) are obligate intracellular gram-negative bacteria that belong to the α-subdivision of Proteobacteria. They are members of the genus *Rickettsia* within the family *Rickettsiaceae* in the order *Rickettsiales* (Bechah et al., 2008). The name of this genus honors Howard Taylor Ricketts, who first described the role of ticks in the transmission of SFGR (Ricketts, 1906), and who unfortunately died of typhus fever (another rickettsiosis) in 1910 while studying the causative agent in an outbreak of this disease in Mexico City. Phylogenetically, members of the SFGR are closely related (Stothard et al., 1994); however, pathogenicity ranges considerably among the officially named SFGR, because recognized tick-transmitted spotted fevers share the same group with SFGR of unknown pathogenicity, that, in some instances, have been reported to utilize a truly symbiotic life style with their tick-hosts (Table 1). A good illustration of this phenomenon is the fact that only two of the five officially named SFGR presently identified in the United States are currently associated with human disease (Bechah et al., 2008).

Ticks are the principal vectors and reservoirs of SFGR, effectively sustaining the rickettsial cycle through horizontal transmission to vertebrate hosts during bloodmeal acquisition and vertical (transstadial and transovarial) transmission (Munderloh and Kurtti, 1995).

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Table 1. Officially named Spotted Fever Group *Rickettsia* (SFGR), their tick vectors, life cycle and geographical distribution in the Unites States.

Tick-borne SFGR	Organism	Arthropod vector	Life cycle	Geographical distribution
Tick-transmitted spotted fever	*Rickettsia rickettsii*	*Dermacentor variabilis, Dermacentor andersoni, Rhipicephalus sanguineus, Amblyomma. cajennense and Amblyomma aureolatum*	Transovarian and transtadial	Western hemisphere
	Rickettsia parkeri	*Amblyomma maculatum, Amblyomma triste, Amblyomma dubitatum, A. cajennense, Amblyomma americanum*	Transovarian and transtadial	Western hemisphere
Non-pathogenic Spotted Fever	*Rickettsia montanensis*	*D. variabilis, D. andersoni*	Transovarian and transtadial	Montana, Virginia, Ohio, New York, Massachusetts, Connecticut, North Carolina, South Carolina
	Rickettsia peacockii	*D. andersoni*	Transovarian and transtadial	Montana, Colorado
	Rickettsia rhipicephali	*R. sanguineus, D. andersoni, Dermacentor occidentalis, Haemaphysalis juxtakochi*	Transovarian and transtadial	Mississippi, Connecticut, Montana, California, Brazil
Other rickettsiae	*Rickettsia felis*	*Ctenocephalides felis*	Transovarian and transtadial	Worldwide
	Rickettsia akari	*Liponyssoides sanguinus*	Transovarian and transtadial	Wordwide
	Rickettsia canadensis	*Haemaphysalis leporispalustris*	Transovarian and transtadial	Montana
	Rickettsia bellii	*D. variabilis, D. andersoni Ixodes loricatus, H. juxtakochi, Amblyomma neumanni, A. aureolatum*	Transovarian and transtadial	Western hemisphere

Adapted from Macaluso and Azad (2005)

To date, some consensus exists when considering the tick-SFGR interactions, since only hard ticks (Ixodidae) have been incriminated in maintaining the rickettsial life cycle; however it is now believed that other arthropods could potentially play a role in the epidemiology of these microorganisms, because experimental infections of two SFGR (*R. rickettsia* and *Rickettsia conorii*) have been recently reported in lice (Houhamdi et al., 2003; Houhmadi et al., 2006).

Globally, the complex tick-SFGR relationships have unclear boundaries. Spotted Fever Group *Rickettsia* can only be identified where their specific tick-hosts are naturally found. In the Western hemisphere, *R. rickettsii* and *R. parkeri*, the causative agents of Rocky Mountain Spotted Fever (RMSF) and Maculatum disease (MD), respectively, are only isolated from infected patients or in field-caught ticks where *Dermacentor* (RMSF), *Rhipicephalus sanguineus* (RMSF) and *Amblyomma* (RMSF and MD) ticks are also localized. For the same reason, rickettsioses transmitted by *R. conorii* and *Rickettsia africae*, the causative agents of Boutonneuse fever (BF) and African tick-bite fever (ATBF), respectively, are only reported in Southern Europe (BF), Africa (BF and ATBF), Southern Asia (BF) and West Indies (ATBF), where *Rhipicephalus* (BF) and *Amblyomma* (ATBF) ticks are naturally distributed (Walker and Ismail, 2008). Since the first report in 1896 in the Snake River Valley of Idaho (Ricketts, 1909)*, R. rickettsii* has been assigned as the conclusive tick-borne rickettsiosis associated with human disease in the Western Hemisphere, with the highest incidence rates in regions of the United States, followed by Mexico, Costa Rica, Panama, Colombia, Brazil and Argentina (Parola et al., 2005; Walker, 2007). In fact, for approximately the next 90 years since the first isolation, cases of RMSF in the United States and in Latin America were directly associated with *Dermacentor*, *Amblyomma* and *Rhipicephalus* ticks, particularly *D. variabilis* and *D. andersoni*, in the US; and *A. cajennense*, *A. aureolatum* and *R. sanguineus* in Latin America (Parola et al., 2005). Recent shifts in the rickettsial distribution in the United States identified *R. sanguineus* as an unexpected

vector for RMSF in Arizona (Demma et al., 2005). *R. parkeri*, was first isolated in 1937 from *A. maculatum* ticks found on cattle in the Gulf Coast region of Texas (Parker et al., 1939), but its role as a human pathogen remained unknown for more than 60 years. For many years, investigators speculated that agents other than *R. rickettsii*, including *R. parkeri*, caused mild RMSF-like illnesses in the United States (Walker and Fishbein, 1991; Stothard et al., 1995). However, the role of *R. parkeri* as a pathogen of humans was not confirmed until 2002, when *R. parkeri* was isolated from a patient with a relatively mild febrile illness and multiple eschars (Paddock et al., 2004). Conscious education of physicians about this SFGR in the United States was crucial to add one more differential diagnose to mild-illness followed by tick bite, especially, within susceptible individuals (Whitman et al., 2007). The recognition of the *R. parkeri* in the Western Hemisphere is progressively increasing. Once believed to be naturally maintained in a single tick species (Parker et al., 1939), *A. triste* (Venzal et al., 2008a; Venzal et al., 2008b; Silveira et al., 2007), *A. dubitatum* and *A. cajennense* (Pacheco et al., 2007) are now associated with *R. parkeri* infection in Latin America countries, such as Uruguay and Brazil, even though experimental infection of this microorganism (Goddard, 2003; Sangione et al., 2005) has demonstrated that *A. americanum* and *A. cajennense* ticks could potentially maintain *R. parkeri*.

In Europe, the first case of SFGR was reported in Tunis (Conor and Bruch, 1910) but the role of the *R. sanguineus* ticks in the rickettsial epidemiology was not described until the 1930s (Brumpt, 1932). For many years, *R. conorii* was considered to be the sole tick transmitted spotted fever in the old continent. Recent advances in diagnostic methods offered better differentiation of the *R. conorii* strains as soon as new human cases were diagnosed in Europe, Africa and Southern Asia countries. *R. conorii* is now subdivided by genetic isotypes and location where new phenotypes are isolated (Bechah et al., 2008). Even though *R. sanguineus* is

still the major vector for BF in those countries, in 1994, *Rhipicephalus pumilio* ticks were also found to be infected with the *R. conorii* subsp *caspia* (Eremeeva et al., 1994). ATBF was first described in 1911 (McNaught, 1911), but the agent related with the cases reported in Mozambique and South Africa during that time remained uncharacterized for 80 years. In 1990, *R. africae* was isolated from *Amblyomma hebraum* ticks in Zimbabwe (Kelly and Mason, 1991) and isolation of the bacterium from a patient suffering from a tick bite fever was confirmed 2 years later in the same country (Kelly et al., 1992). The rickettsial strain isolated from *A. hebraum* ticks in 1990 could not be compared with the first strain identified (Pijper, 1934) because such culture was lost and further studies at that time were unable to confirm the previous findings (Pijper, 1936); however, the strain isolated by Kelly and Mason (1991) was identical to the microorganism isolated from naturally infected *Amblyomma variegatum* ticks 20 years earlier in Ethiopia (Burgdorfer et al., 1973). To date, ATBF cases are restricted to African and West Indies countries where these *Amblyomma* ticks are found (Walker and Ismail, 2008).

During the past century, many others SFGR rickettsiae were isolated from ticks, especially in the United States. Due to the lack of apparent pathogenicity, the role of these microorganisms in the tick-SFGR interactions was basically overlooked. Contributions regarding the characterization of most of these SFGR of unknown pathogenicity remain contradictory as novel techniques are applied with the aim of taxonomically including them as new species (Walker, 2007). In the Western hemisphere, *R. montanensis*, *R. peacockii*, *R. rhipicephali*, are officially named tick-borne SFGR of unrecognized pathogenicity (Parola et al., 2005). In the United States, the distribution of these SFGR is well established (Macaluso and Azad, 2005). *R. montanensis* (formerly *R. montana*) was first isolated from *D. variabilis* and *D. andersoni* ticks in eastern Montana (Bell et al., 1963). In the past 45 years, the presence of *R. montanensis* has been also reported in naturally infected *Dermacentor* ticks collected in the states of Maryland

(Ammerman et al., 2004), Ohio (Pretzman et al., 1990), Massachusetts (Feng et al., 1980), Connecticut (Anderson et al., 1986), North Carolina (Breitschwerdt et al., 1988), South Carolina, Virginia and New York (Ammerman et al., 2004). *R. peacockii* (Niebylski et al., 1997), also known as the East Side agent, was first isolated from *D. andersoni* ticks found in the east side of Bitterroot Valley in western Montana (Burgdorfer and Brinton, 1975). Additional reports of the presence of this endosymbiont SFGR in others states is only available from wood ticks collected in Colorado (Baldridge et al., 2004; Simser et al., 2001). *R. rhipicephali* (Burgdorfer et al., 1978) was first isolated from *R. sanguineus* ticks in Mississippi (Burgdorfer et al., 1975). Further detection of *R. rhipicephali* in the United States was reported in *R. sanguineus* collected in Connecticut (Magnarelli et al., 1982), *D. andersoni* in Montana (Philip and Casper, 1981) and *D. occidentalis* in California (Philip et al., 1981; Wikswo et al., 2008). Phylogenetic analyses of *Rickettsia*-like organisms isolated from *Haemaphysalis juxtakochi* ticks also confirmed the presence of *R. rhipicephali* in Brazil (Labruna et al., 2005; 2007a).

Human migration patterns toward the South and Western United States during the past 300 years have been recently correlated with the geographic distribution and increased incidence of some tick-transmitted pathogens. Contributing factors include the gradual, but continuous human environmental interventions, which provided favorable microclimatic conditions for tick survival and an optimum habitat for their vertebrate hosts (Childs and Paddock, 2003). In 1754, *A. americanum* became the first North American tick species to be formally described by European naturalists due to its relative abundance (Figure 1). The population of the wood ticks, as they were known during that time, rapidly decreased due to the profound changes in regional microclimate, hydrology, and soil mechanics provoked, mainly, by the expansion of the deforestation of the virgin longleaf pine (*Pinus palustris*) forests of the Southeast to produce charcoal and land for crops and pastures.
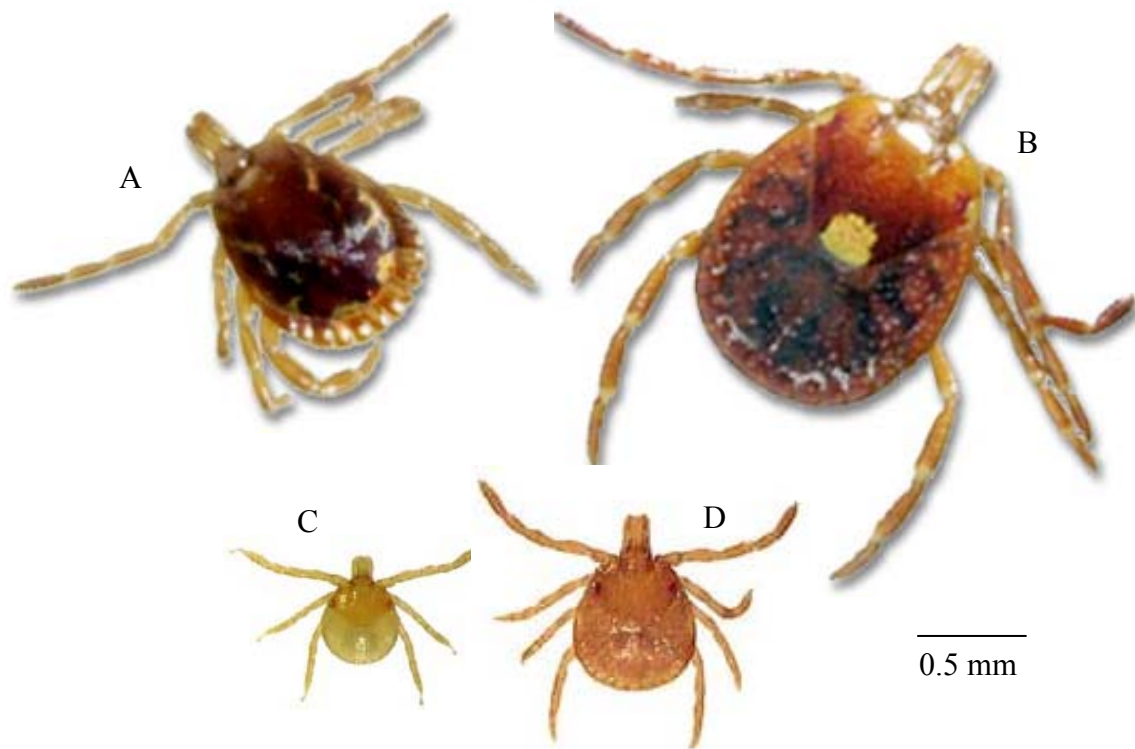
Figure 1. The lone star tick. Photography illustrating the different life stages of *A. americanum* ticks. (A) male, (B) female, (C) larva and (D) nymph. Bar 0.5 mm Source: http://www.ticktexas. org/ticks/afaa_lone_star_tick.

The vast cleared lands rapidly became inhospitable to the survival of these moisture-sensitive ticks. By 1870, *A. americanum* ticks once described as abundant were considered extinct in many North American regions (Paddock and Yabsley, 2007). During the first half of the twentieth century, reforestation of the extensively longleaf harvested regions took place by replacing *P. palustris* partly or entirely by mixed pines and hardwoods, particularly scrub oak (*Quercus sp*), which was observed to be accompanied by vigorous growth of formerly suppressed understory flora, creating, once again, ideal microclimatic conditions for the lone star tick survival through the establishment of ecotones comprised of smaller trees and more abundant surface vegetation (Wahlenberg, 1946). This profuse source of accessible vegetal protein coupled with the lack of natural predators and the increased hunting regulations contributed to a resurgence of the white-tailed deer population in the mid-1900s. White-tailed deer are the major wildlife host of *A. americanum* in the United States (Kollars et al., 2000). They play a dual role in the survival and proliferation of *A. americanum* ticks by serving as a preferred food source for all tick life stages and as a vehicle for transport and localization within the preferred habitat (Paddock and Yabsley, 2007). Due to the increased number of deer in the United States, estimated to be around 18 million animals in 1992 (Mcdonald and Miller, 1993), and therefore, the increased number of *A. americanum* ticks in the vegetations where the vertebrate host circulates, the number of tick-biting incidents among humans dramatically increased during the past few decades (Childs and Paddock, 2003). As humans can be accidentally bitten by all three tick life stages (larvae, nymphs and adults), and microclimate conditions favor optimal proliferation of these ticks, we might be facing one of the most serious problems with ticks in the entire American history.

As a consequence, the number of tick-borne diseases transmitted by *A. americanum* ticks also increased. Once believed to be a tick of minor public health importance, the lone star ticks

are now known to be the vector of important zoonotic pathogens (Table 2). It is accepted that the human ehrlichioses are the most important diseases transmitted by *A. americanum* ticks, since a total of 1,050 human monocytic ehrlichiosis (HME) cases have being reported in a 6 year study of several United States locations (Paddock and Yabsley, 2007). However, natural infections of lone star ticks with other recognized pathogens and with agents of undetermined pathogenicity have been extensively identified throughout the range of *A. americanum.* More recently, laboratory-reared and field-collected lone star ticks were described to be highly infected with *Coxiella* and *Rickettsia* spp (Jasinkas et al., 2007; Zhong et al., 2007). *Coxiella burnetii,* the causative agent of Q fever, had already been identified (Parker and Kohls 1943; Philip and White, 1955), but it is thought that the transmission of this bacterium to humans is not significantly important in these ticks, even though ticks can be naturally infected (Childs and Paddock, 2003). The ascendency of *A. americanum* ticks as vectors of SFGR in the United States is well substantiated. *R. rickettsii* is believed to be the cause of infection in two fatal cases reported in a region heavily infested with *A. americanum* ticks back almost 70 years ago (Anigstein and Bader, 1943), even though attempts to isolate this SFGR in large field collections of *A. americanum* were unsuccessful (Burgdorfer et al., 1981b; Goddard and Norment, 1986).

R. parkeri has only been isolated from *A. maculatum* ticks in the United States (Parker et al., 1939; Whitman et al., 2007), but artificial infection of this microorganism has already been demonstrated in *A. americanum* ticks (Goddard, 2003). According to the current literature, '*Rickettsia amblyommii'* is the most prevalent SFGR in *A. americanum* ticks (Mixson et al., 2006; Apperson et al., 2008, Stromdahl et al., 2008). The WB-8-2 agent, as official classifications still designate this SFGR (Rault et al., 2005), was first isolated, at high levels, in 1974 during an unsuccessful attempt to associate *A. americanum* ticks with *R. rickettsii*

Table 2. Bacteria isolated or identified from *Amblyomma americanum.*

Bacterial agent	Disease in humans	Comments
Ehrlichia chaffeensis	Human monocytic ehrlichiosis (HME)	The most severe of the three ehrlichioses of humans in the United States. Underreported and probably as common as Rocky Mountain spotted fever.
Ehrlichia ewingii	*E. ewingii* ehrlichiosis	Most commonly diagnosed in immunosuppressed persons. Less than 20 cases documented.
Rickettsia rickettsii	Rocky Mountain spotted fever	Role of lone star ticks in transmission is uncertain, as recent surveys have not identified *R. rickettsii* in ticks.
Coxiella burnetii	Q fever	Tick transmission is not thought to play a significant role in human disease, although many species of ticks are naturally infected
Francisella tularensis	Tularemia	Tick transmission remains important in endemic occurrence. Other routes of transmission, such as direct contact with wild rabbits, are also significant.
"*Borrelia lonestari*"	Probable cause of southern tick-associated rash numbers	Likely to become recognized as a common disease where lone star ticks exist in high. Agent as yet uncultivable.
85-1034 ("*Rickettsia amblyommii*")	Possible mild spotted fever rickettsiosis	Association with human disease based on serologic reactivity only.
Rickettsia parkeri	Maculatum disease	Originally isolated from *Amblyomma maculatum* in Texas.
WB-8-2	None described	Nonpathogenic or mildly pathogenic in guinea pigs and meadow voles. Most closely related to MOAa.
MOAa	None described	Most closely related to WB-8-2.

Source: Childs and Paddock (2003) modified.

infections in field-collected ticks from Arkansas, South Carolina and Tennessee (Burgdorfer et al., 1981b). Subsequently, molecular analysis of a SFGR isolated from field-collected *A. americanum* ticks (MOAa) in Missouri (Weller et al., 1998) was found to be closed related with WB-8-2. In fact, both WB-8-2 and MOAa strains are believed to represent the same SFGR (Paddock and Yabsley, 2007). The most comprehensive survey examining infection of *A. americanum* with '*R. amblyommii*' was performed between 1998 and 2005 in nine states (Mixson et al., 2006). From the 2,038 adult ticks collected, the outer membrane protein A gene (*OmpA*), encoding the '*R. amblyommii*' DNA sequence was detected in 42% of those ticks, with infection prevalence ranging as high as 97% in all 29 sites surveyed. '*R. amblyommii*' has also being identified in other countries, particularly in Brazil, Argentina and French Guyana, where *Amblyomma coelebs* (Labruna et al., 2004a; Parola et al., 2007), *A. cajennese* (Labruna et al., 2004b) and *A. neumanni* (Labruna et al., 2007b) are the major vectors. Considering the pathogenic nature of this microorganism, a lot of speculation based on serological evidences (Dasch et al., 1993; Sanchez et al.,1992; Labruna et al., 2007b; Apperson et al., 2008; Saito et al., 2008), epidemiological studies (Mixson et al., 2006) and molecular analyses (Billeter et al., 2007) suggest that this SFGR might be the cause of mild-illness in humans, however the lack of classical RMSF clinical signs (Sanchez et al.,1992), and the impossibility to replicate the infection in animal models (Burgdorfer et al., 1981b) coupled with the failure to isolate the bacterium from human patients previously exposed to infected *A. americanum* ticks (Billeter et al., 2007), make the inclusion of this *Rickettsia* in the group of tick-transmitted spotted fever difficult (Walker and Ismail, 2008). As noted by Childs and Paddock (2003), some of these obstacles will sooner or later be overcome and '*R. amblyommii*' transmitted by *A. americanum* will eventually be isolated from human patients, as has been reported in the past, but for the best of our knowledge '*R. amblyommii*' is still considered as a SFGR of unrecognized pathogenicity.

Ticks have a complex and very old association with the rickettsiae species that they harbor. It is fascinating to note that *R. rickettsii* (Parola et al., 2005; Demma et al., 2005), *R. rhipicephali* (Burgdorfer et al., 1975; Magnarelli et al., 1982; Philip and Casper, 1981; Philip et al., 1981; Labruna et al., 2005; 2007a; Wikswo et al., 2008) and *R. bellii* (Gordon et al., 1984; Labruna e t al., 2004b; Horta et al., 2006; Pinter et al., 2006; Labruna et al., 2007a; 2007b) throughout their evolution became highly capable to associate with tick vectors from several different genera, while *R. peacockii*, seems to be restricted to *D. andersoni* ticks only (Burgdorfer and Brinton, 1975; Niebylski et al., 1997). Discrepancy between these ranges seems to be also true, because *R. parkeri* (Parker et al., 1939; Goddard et al 2003; Pacheco et al 2007; Silveira et al 2007; Venzal et al 2008a; Venzal et al 2008b), *R. conorii* (Brumpt, 1932; Eremeeva et al., 1994), *R. africae* (Burgdorfer et al., 1973; Kelly and Mason, 1991), *R. montanensis* (Bell et al., 1963) and '*R. amblyommii*' (Burgdorfer et al., 1981b; Labruna et al., 2004a; 2004b; Parola et al., 2007; Labruna et al., 2007b), apparently hold an intrinsic relationship with several different species within the same tick genus. One possibility for why these phenomena might happen seems to be related with the way that the genome of each one of these bacteria split from the common α-proteobacteria-like ancestor, the same eubacterial ancestor that mitochondria are believed to originate from, early in the rickettsial evolution (Gray 1998; Gray et al., 2001). According to this hypothesis, rickettsial species that retained homology with some eukaryotic mitochondria proteins after the process of reductive evolution that both rickettsiae and mitochondria, independently, originate from, are more likely prone to better associate with each other (Ogata et al., 2006; Fitzpatrick et al., 2006); therefore, rickettsiae species that share some similarities with the tick host cell proteome, could potentially develop more malleable interactions, sustaining infection in tick vectors of more than one genus. In this process, some rickettsiae could take advantage of the favorable conditions and proliferate more than the load

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supported by the tick host, crossing a line that could ultimately result in the end of their interactions (Niebylski et al., 1999). This argument, even though plausible, seems to be novel among rickettsiologists, since no investigations have being conducted so far to address the question at this level.

In order to understand the tick-*Rickettsia* interactions, a variety of methods have been used by scientists throughout the years. Burgdorfer and coworkers (1981b) were some of the first scientists to describe rickettsial interspecies competition inside the tick host. According with these authors, *R. rickettsii* transovarial transmission was observed to be interfered by the presence of *R. peacockii* in natural infected *D. andersoni* ticks (Burgdorfer et al., 1981a). Similar responses have been demonstrated under laboratory conditions (Burgdorfer, 1988; Macaluso et al., 2002). *Dermacentor* ticks infected either with *R. montanensis* or *R. riphicephali* were unable to transmit transovarially acquired *R. rickettsii* infection (Burgdorfer, 1988). In a similar fashion, detection of *R. montanensis* or *R. riphicephali* in the progenies (F1 and F2) of *R. montanensis* or *R. riphicephali*-infected cohorts of *D. variabilis* ticks (Macaluso et al., 2001) was not observed after reciprocal rickettsial challenges (Macaluso et al., 2002). Under these circumstances, blockage of transovarial transmission was suggested to be related with shifts in the expression of some molecules in the ovaries of *D. variabilis* ticks (Macaluso et al., 2002; 2003). The ability that some rickettsiae have to propagate their life cycle without negatively impacting fecundity of infected female ticks and the number of viable post-embryonic tick stages is consistent with endosymbiosis (Azad and Beard, 1998). Strictly vertically transmitted symbionts can manipulate the host reproductive fitness to their own benefit (Burgdorfer et al., 1981a; Lawson et al., 2001; Hagimori et al., 2006; Zhong et al., 2007). *R. peacockii*, for example, is only found in the oocytes and interstitial cells of the ovarian tissues of *D. andersoni* ticks (Munderloh et al., 2005; Niebylski et al., 1997); while *R. rickettsii* is responsible for high mortality rates in post-

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embryonic stages of *D. andersoni* ticks (Niebylski et al., 1999). When *R. peacockii*-infected *D. andersoni* ticks are exposed to *R. rickettsii*, interspecies competition in the reproductive system of female ticks may be one of the reasons for the blockage of *R. rickettsii* transovarial transmission (Burgdorfer et al., 1981a). *R. bellii*-like bacteria were demonstrated to be related with male-killing and arthropod host parthenogenesis (Lawson et al., 2001; Hagimori et al., 2006). In order to enhance their own transmission, these microorganisms alter the embryonic maturation in infected insects to increase the number of females in the next generation. Antibiotic-treated insects were related with increases in the production of male offspring (Lawson et al., 2001; Hagimori et al., 2006). Similarly, *Coxiella* sp-infected *A. americanum* ticks suffered substantial reduction in reproductive fitness after antibiotic administration. Reduction of *Coxiella* sp loads in antibiotic-treated ticks was correlated with decreased weight, prolonged time to oviposition and lower progeny viability (Zhong et al., 2007). Understanding the underlying mechanisms in some of these processes could lead to the development of alternative approaches to control ticks and tick-borne diseases. Throughout the years, though, studies have being focused mainly on the vector competence of ticks, with considerably less attention to the relationships of SFGR and various tick cells, tissues, organs and with specific physiologic processes of acarines (Parola et al., 2005).

In a tick host, SFGR utilize both horizontal and vertical transmission pathways. As a general rule, pathogenic SFGR are horizontally transmitted to vertebrate hosts during blood feeding of infected ticks, while nonpathogenic or SFGR of unrecognized pathogenicity are those microorganisms that lack such ability, maintaining the rickettsial life cycle in nature principally by transovarial transmission and transstadial survival in ticks. The tick's role in the pathogenic SFGR life cycle is believed to take place after ticks are exposed to infected blood during feeding on rickettsemic vertebrates (Munderloh and Kurtti, 1995). Cofeeding infection has also been

suggested as a method of rickettsial acquisition by ticks, since *R. rickettsii* infection was observed in previously uninfected-*D. andersoni* ticks when *R. rickettsii*-infected *D. andersoni* ticks were allowed to feed in the same host in closely situated bite sites (Philip, 1959). Once inside the tick host, most SFGR can infect all tick tissues, including the salivary glands, midgut, and ovaries (Munderloh and Kurtti, 1995); however, the contributing arthropod and *Rickettsia*-derived factors that facilitate host infection are not clearly defined. Transovarial and transtadial transmissions serve as the primary mechanism for maintenance of rickettsiae of low or no pathogenicity, and may serve a lesser role in the maintenance of pathogenic rickettsiae, as evidenced by the lethal effects of *R. rickettsii* on ticks (Burgdorfer and Brinton, 1975, Niebylski et al., 1999). *R. peacockii*, seems to be restricted to the oocytes and interstitial cells of the ovarian tissues of *D. andersoni* ticks (Munderloh et al., 2005; Niebylski et al., 1997), a fact attributed to the lack of functional *OmpA*, a putative adhesion protein (Li and Walker, 1998). *Rickettsia monacensis* transformed to express green fluorescent protein (GFP) can be easily visualized in *Ixodes scapularis*, *A. americanum* and *D. variabilis* tick tissues after infection by capillary feeding (Baldridge et al., 2007). This SFGR migrate to the midgut first, before disseminate to other organs, particularly salivary glands and ovaries in *I. scapularis* ticks, but not in *A. americanum* and *D. variabilis*. In those ticks, dissemination of GFP-transformed rickettsiae was restricted to the midgut. GFP-*R. monacensis* was not transmitted to vertebrate hosts during feeding and the life cycle was maintained exclusively through vertical transmission in *I. scapularis*, a feature related to the nonpathogenic nature of this SFGR (Baldridge et al., 2007).

Serological analyses had been the golden standard for the detection of SFGR in both vertebrate and tick hosts (Parola et al., 2005). However, the development of molecular methods to diagnose rickettsial infection in the past 20 years facilitated the recognition of several distinct tick-borne SFGR. Polymerase chain reaction (PCR) rapidly became a reliable tool to diagnose

rickettsioses in the blood, tissues or within the tick vectors (Macaluso et al., 2001; Paddock et al., 2004; Mixson et al. 2006; Walker, 2007). Several different genes are targeted in the diagnosis of SFGR. For phylogenetic analysis purposes, the outer membrane protein A (*ompA*), citrate synthase and *Rickettsia* genus-specific 17-kDa-antigen are the most common genes (Roux et al., 1996; 1997; Ellison et al. 2008), and SFGR differentiation through restriction fragment length polymorphism (RFLP) is suitable only in *ompA* amplicons (Roux et al., 1996). Quantitative real-time polymerase chain reaction (qPCR) was recently introduced as a tool to enumerate rickettsiae infection in the host (Rolain et al., 2002; Eremeeva et al., 2003). To date, qPCR has been used to quantify SFGR in cell culture and whole ticks and for diagnostics of SFGR in field-caught ticks (Rolain et al., 2002; Eremeeva et al., 2003; Labruna et al., 2004a, 2004b; Stenos et al., 2005; Zhong et al., 2007). However, the application of qPCR to quantify SFGR in specific tick tissues during tick physiologic events has not been described.

Deciphering the tick-SFGR interactions is complicated; to examine the roles that pathogenic and nonpathogenic SFGR play within the tick host in relation to the epidemiology of tick-borne rickettsioses, the mechanisms of rickettsial infection within the tick must first be characterized. In this study, a qPCR assay was used as a tool to investigate the tick-SFGR interactions by accessing the ratio of *Rickettsia* per tick cell during tick feeding events and in atypical host cells. The importance of *A. americanum* tick as a vector of SFGR in the United States was stressed by using this tick as a model in the tick feeding experiment. To explore the pathogenic nature of SFGR, the ratio of *Rickettsia* per tick cells in selected tissues of *A. americanum* ticks and post-embryonic life stages was accessed in unrecognized SFGR. "*R. amblyommii*"-naturally infected *A. americanum* ticks were allowed to feed and mate on vertebrate hosts before salivary glands, midgut and ovaries tissues were dissected out for DNA extraction and qPCR analysis of rickettsial infection. A portion of the mated infected female

ticks were allowed to complete the life cycle and the F1 generation (eggs, larvae and nymphs) were also accessed for rickettsial infection. To investigate if host specificity plays a role in the tick-SFGR interactions, equal amounts of *R. montanensis*, "*R. amblyommii*" and *R. parkeri* were inoculated in an atypical tick cell line (ISE6) and followed infectivity rates and rickettsial burden inside the cell throughout the experiment. The working hypotheses in this study are that (1) The ratio of *Rickettsia* per tick cell during tick feeding and transovarial/transtadial transmissions in *A. americanum* ticks infected with unrecognized rickettsiae is directly correlated to the significance of the horizontal versus vertical route of transmission in SFGR; (2) The specificity of associations among rickettsiae and a particular tick species is correlated with replication under laboratory conditions. The conclusions presented in this study offer insightful thoughts about the tick-SFGR interactions. Due to the apparent low prevalence of *R. parkeri*-naturally infected *A. americanum* (Goddard and Norment, 1986; Chils and Paddock, 2003), further investigations should be conducted to establish *R. parkeri*-artificially infected cohorts of *A. americanum* to examine the potential roles of this SFGR in selected tissues and post-embryonic life stages during physiologic tick events. At the same time, the *in vitro* model established in this study should be expanded to other tick cell lines for a better understanding of the role of host specificity among SFGR.

Chapter 2. Characterization of Rickettsial Infection in *Amblyomma americanum* Ticks (Acari: Ixodidae) by Quantitative Real-Time Polymerase Chain Reaction

2.1. Introduction

Spotted fever group *Rickettsia* (SFGR) are obligate intracellular gram-negative bacteria that belong to the α-subdivision of Proteobacteria. Phylogenetically, members of the SFGR are closely related (Stothard et al., 1994); however, pathogenicity ranges considerably among the officially named SFGR, because only four of the nine SFGR presently identified in the United States are currently associated with human disease (Macaluso and Azad, 2005). Ticks and mites serve as the principal vectors and reservoirs of SFGR, effectively sustaining the rickettsial cycle through horizontal transmission to vertebrate hosts during bloodmeal acquisition and vertical (transstadial and transovarial) transmission (Munderloh and Kurtti, 1995). Infection with some SFGR, e.g., *R. rickettsii,* is detrimental to both the vertebrate and tick hosts (Burgdorfer and Brinton, 1975, Niebylski et al., 1999), whereas other SFGR, not associated with vertebrate infection, employ a symbiotic lifestyle with unapparent effects on their invertebrate hosts (Noda et al., 1997; Macaluso et al., 2001, 2002). Interestingly, there are several distinct tick-associated rickettsiae that have yet to be formally classified. Within the genus *Amblyomma,* Burgdorfer et al., (1981b) provided microscopic analysis of infection by SFGR designated WB-8-2 in *A. americanum* (L.). The *A. americanum*-associated *Rickettsia* was referred to as "*R. amblyommii"* (Stothard and Fuerst, 1995), and molecular analysis of a SFGR isolated from *A. americanum* (MOAa) found it to be closely related to WB-8-2 (Weller et al., 1998); subsequent surveys and molecular analysis of SFGR in *Amblyomma* ticks (Labruna et al., 2004b; 2007b) identified additional genotypes of the SFGR, referred to here as *R. amblyommii.* The influence of nonpathogenic SFGR on the ecology of tick-borne rickettsioses (Burgdorfer et al., 1981a) has facilitated the discovery of numerous tick-associated SFGR, which serve as models to explore

the complex *Rickettsia*-arthropod relationship. Most SFGR can infect all tick tissues, including the salivary glands, gut, and ovaries (Munderloh and Kurtti, 1995); however, the contributing arthropod and *Rickettsia*-derived factors that facilitate host infection are not clearly defined. SFGR respond to cues coupled with tick bloodmeal acquisition; ultrastructural changes in the SFGR, associated with tick feeding, are correlated with infectivity for the vertebrate host (Hayes and Burgdorfer, 1982), and replication of SFGR during tick feeding has been demonstrated (Santos et al., 2002, Baldridge et al., 2007). Quantitative real-time polymerase chain reaction (qPCR) has been used to enumerate SFGR in cell culture and whole ticks and for diagnostics of SFGR in field-caught ticks (Rolain et al., 2002; Eremeeva et al., 2003; Labruna et al., 2004a, 2004b; Stenos et al., 2005; Zhong et al., 2007). However, the application of qPCR to quantify SFGR in specific tick tissues during tick feeding events has not been described. Deciphering the tick-SFGR interactions is complicated; to examine the roles that pathogenic and nonpathogenic SFGR play within the tick host in relation to the epidemiology of tick-borne rickettsioses, the mechanisms of rickettsial infection within the tick must first be characterized. Therefore, in the current study, a qPCR assay was applied to describe the growth and tissue distribution of *R. amblyommii* in selected tissues of constitutively infected *A. americanum,* collected on regular intervals during tick bloodmeal acquisition events. Comparable bacterial load was observed between adult tick tissues during tick feeding and among life cycle stages; these results suggest a physiological balance in bacterial load exists during vertical transmission.

2.2. Materials and Methods

2.2.1. Ticks

Unfed *R. amblyommii*-infected adult *A. americanum* ticks were obtained from a colony maintained by the Tick Research Lab, Department of Entomology, Texas A&M University

(College Station, TX). Ticks used in this study were originally collected in Sutton Co., TX. This colony of ticks is maintained without regular introduction of wild-caught ticks. At the Louisiana State University School of Veterinary Medicine (LSU-SVM), adult male and female ticks were fed on a male New Zealand White rabbit as described previously (Sonenshine, 1993). Engorged females were maintained in a mini incubator (Labnet, Woodbridge, NJ) at 28ºC, 91% RH, and a photoperiod of 14:10 (L:D) hours through oviposition. The subsequent postembryonic stages, larvae and nymphs, were fed on BALB/c mice by using an encapsulation method as described previously (Macaluso and Wikel, 2001) (Figure 2). All use of animals in this research was done in accordance with protocols approved by the Louisiana State University Institutional Animal Care and Use Committee. The approved protocols are on file in the office of the Division of Laboratory Animal Medicine at Louisiana State University.

2.2.2. Tick Feeding and Sample Collection

In total, 64 female ticks were fed on a rabbit host for up to 19 days (d). In the first 4d, groups of three partially fed virgin female ticks were forcibly detached from the host at 0, 12, 24, 36, 48, 60, 72, 84, and 96 hours (h) postattachment. The capsule was partitioned and a portion of female ticks were exposed to male ticks at a ratio of 2:1 (male: female) on day 5. During the next 5d, groups of three ticks, mated and unmated females, were collected at 120 (mated only), 144, 168, 192, and 216h postattachment. Ten female ticks were allowed to feed to repletion naturally detach, and undergo oviposition.

All egg clutches were collected between 5 and 11d postdetachment. The postembryonic stages, larvae and nymphs, were fed for 3 and 5d, respectively. An aliquot of each egg clutch, ≈ 300 eggs from each engorged female, and F1 immature ticks ($n = 40$ larvae and $n = 8$ nymphs) were used for the vertical transmission study. At each collection time point and for each life stage, tick weights were determined using an analytical balance (Denver Instrument, Arvada, CO).
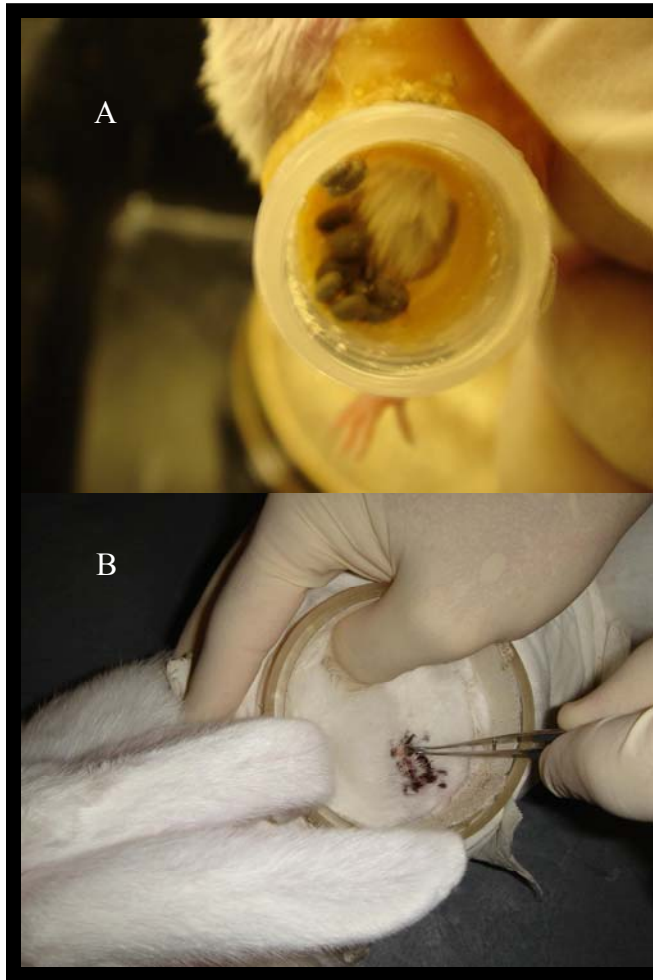
Figure 2. Lab-reared *A. americanum* ticks. At SVM/LSU *A. americanum* ticks are allowed to complete their life cycle in laboratory animals. Larvae and nymphs (A) are usually fed on Balb/c mice, while adult ticks (B) are fed on New Zealand White rabbits.

2.2.3. Tissue Recovery and DNA Isolation

Ticks were surface sterilized by immersion in 70% ethanol for 10 min followed by three washes in sterile water. Selected tick tissues (salivary glands, guts, and ovaries) were dissected out from adult females and rinsed in phosphate-buffered saline by using standard microdissection technique (Macaluso et al., 2003) (Figure 3). Individual adult tick tissues, egg clutches, whole larvae, and nymphal ticks were homogenized in ATL buffer (DNeasy tissue kit,QIAGEN,Valencia,CA) with plastic pestles in 1.5-ml microcentrifuge tubes and immediately stored at -80ºC until used for genomic DNA (gDNA) extraction. All sample preparation followed the manufacturer's protocol for purification of total gDNA from animal tissues with the DNeasy tissue kit (QIAGEN). The Final elution of gDNA was in 100 µl of elution buffer, and the sample was stored at -20ºC until used as PCR template.

2.2.4. Rickettsial Detection and Identification by PCR

Rickettsia infection in the adult tick gut samples and the subsequent vertical transmission of SFGR to the eggs, larvae, and nymphs was detected by PCR amplification of portions of the genes encoding the *Rickettsia* genus-specific 17-kDa-antigen and the SFGR specific outer membrane protein A (*ompA*). The mitochondrial 16S rDNA (mt16SrDNA) primer set of *A. americanum* was used as a control to check the integrity of the template DNA. All primers used for standard PCR and qPCR were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and they are listed in Table 3. PCR products were amplified using PCRMaster Mix (Promega, Madison, WI) together with the gene-specific primers. *Rickettsia montanensis* (M5/6) gDNA and water served as the positive and negative controls, respectively, for the PCR. The conditions were as follows: initial denaturation at 94ºCfor 3 min, followed by 35 cycles of denaturation at 94ºC for 30s, annealing at 55º (*ompA*) or 60ºC (17-kDa antigen gene and mt16SrDNA) for 45s, extension at 72ºC for 1 min, and a final extension at
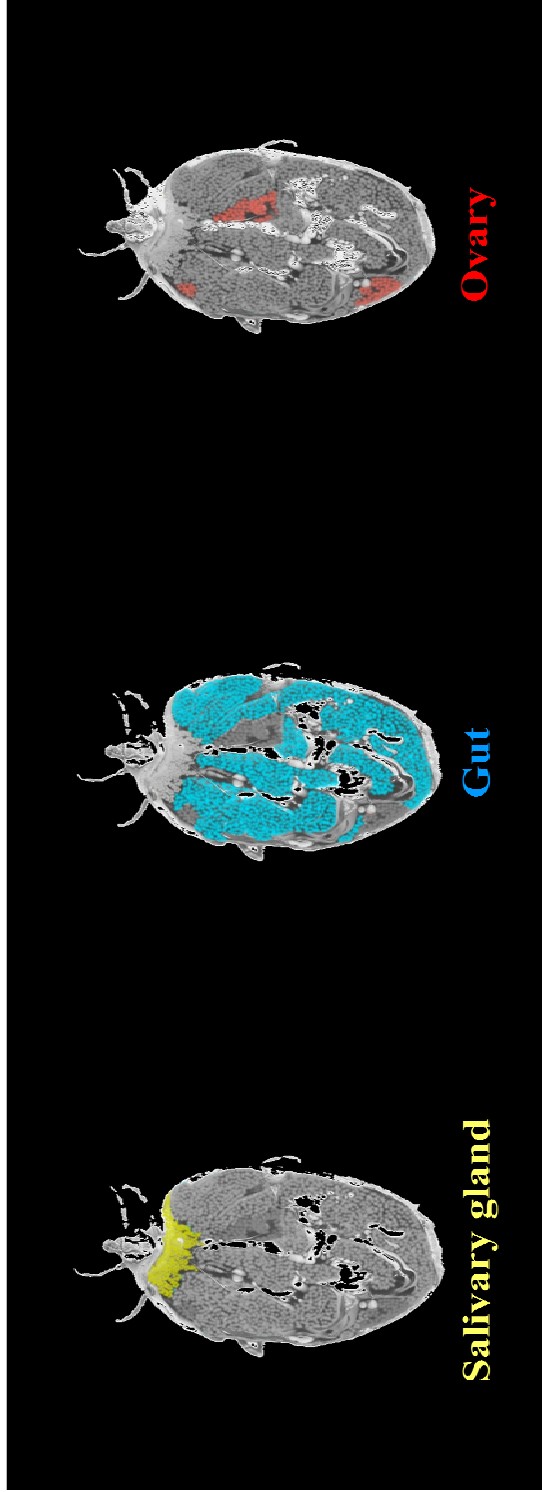
Figure 3. Schematic representation of the tissues selected for genomic DNA extraction.

72ºC for 7 min. The target PCR products were visualized by electrophoresis on an ethidium bromide stained 1.5% agarose gel. Three clones of a single PCR amplicon for *ompA* were sequenced as described previously (Pornwiroon et al., 2006).

2.2.5. Construction of an Internal-Control Plasmid for Quantitative Real-Time PCR

To quantify the copy numbers of rickettsial and tick genes in samples of tick tissues during tick feeding, egg clutches, and immature stages, serial dilutions of a plasmid harboring a single copy of both rickettsial and host genes were used to generate a standard curve. The 128-base pair (bp) fragment of the 17-kDa antigen gene of *R. amblyommii* was PCR amplified with *Ra*17kDaF and *Ra*17kDaR primers and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The insert fragment (*Ra*17kDa) was sequenced to confirm its identification as the rickettsial 17-kDa antigen gene. Likewise, the 188-bp portion of the gene encoding *A. americanum* macrophage migration inhibitory factor (*Aa*MIF) (Jaworski et al., 2001) was PCR amplified using the primers *Aa*MIFF and *Aa*MIFR, cloned, and sequenced. Both amplicons (*Ra*17kDa and *Aa*MIF) were amplified using a gene-specific primer and either M13forward or M13reverse primer.

The obtained PCR products, the template for the primer pair *Ra*17kDaF and *Aa*MIFF were digested with *EcoRI* and ligated together.

The PCR product, containing both amplicons, was cloned and sequenced; the resulting plasmid, pCR4-TOPO-*Aa*MIF_*Ra*17kDa, served as the standard template (Figure 4).

2.2.6. Quantitative Real-Time PCR

For each gene, a qPCR reaction mixture was created in a Final volume of 35μl with 2X iTaq SYBRGreen Supermix (Bio-Rad, Hercules, CA), 100nM of each primer, DNase/RNasefree water; and either gDNA template, water, or serial 10-fold dilutions (1×10^7 to 10 copies) of pCR4- TOPO-*Aa*MIF_*Ra*17kDa (Figure 5).

Table 3. Primers used for standard PCR amplification and quantitative PCR.

Primers	Gene	Nucleotide sequence (5'- 3')	Reference
*Ral/kDa*F	17-kDa genus-specific antigen	GCATTACTTGGAGCAGTTCTTG	This study
*Ral/kDa*R		CGCCATTCTACGTTACTACCAC	
*Rr*190.70p	190-kDa antigen (*ompA*)	ATGGCGAATATTTCTCCAAAAA	(Regnery et al., 1991)
*Rr*190.701n		GTTCCGTTAATGGCAGCAT	(Roux et al., 1996)
*AaMIF*F	Amblyomma americanum macrophage migration	TGTTTGCTCTGATGGAGTGG	This study
*AaMIF*R	inhibitory factor	CTGTACGGAAATCGCACTCA	
*Aa*16SF	Mitochondrial 16S mRNA	GCGACCTCGATGTTGGATTAGGA	This study
*Aa*16SR		CCTGATTCTCATCGGTCTAAACTCA	
*ISCRT*F	*Ixodes scapularis* calreticulin protein	AGCAGGGAACTTTCAAGCTG	This study
*ISCRT*R		AGAAAGGCTCGAACTTGGTG	
*β-actin*F	Rhesus macaque	AACCTTCCTTCCTTGG_GCAT	(Ohno et al., 2003)
*β-actin*R		CAGGAGGAGCAATGAT_CTTGAT	

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Because the amount of tissue, and therefore gDNA recovered, from individual ticks varied, we used 100-600 ng of gDNA in 5µl added to the qPCR master mix for each reaction. Copy numbers per 5µl were calculated, and the total copy numbers per sample was based on the 100µl elution volume. For each assay, reaction components and template were premixed in 96-well plates for both genes of interest; a qPCR of selected tick tissues, egg clutches, and immature samples were conducted in individual 384-well plates. For each sample, three wells were filled with 10µl of the reaction mixture. qPCR was performed with an ABI 7900HT unit (Applied Biosystems, Foster City, CA) at the LSU-SVM under the following cycling condition; denaturation step at 95ºC for 10 min, 40 cycles of denaturation at 95ºC for 15s, and annealing at 60ºCfor 1 min. The dissociation stage (melting curve) was performed with one cycle at 95ºC for 15s, 60ºC for 15s, and 95ºC for 15s. Analysis of the amplification was carried out with ABI 7900HT sequence detection system (SDS version 2.3) software. To determine the specificity of the PCR assay, the dissociation curve was assessed for each sample; the expected single peak was verified present in the pCR4-TOPO-*Ra*17kDa/*Aa*MIF and gDNA wells and absent in the water (negative control) samples. The baseline and threshold were optimized for each gene to maximize efficiency. Additionally, representative PCR products were verified by gel analysis to confirm the specificity of the reaction (data not shown), and they were sequenced to confirm specific amplification. Both of the genes encoding for *Ra*17kDa and *Aa*MIF are single copy genes; therefore, infection was quantified in terms of rickettsiae 17-kDa copy numbers per tick MIF copy numbers (*Ra*17kDa/*Aa*MIF).

2.2.7. Statistical Analysis

The SAS statistical package (version 9.1.3) GLM procedure in an analysis of variance (ANOVA) was used to examine potential differences between the weights of mated and unmated female ticks, larvae, and nymphs during feeding.
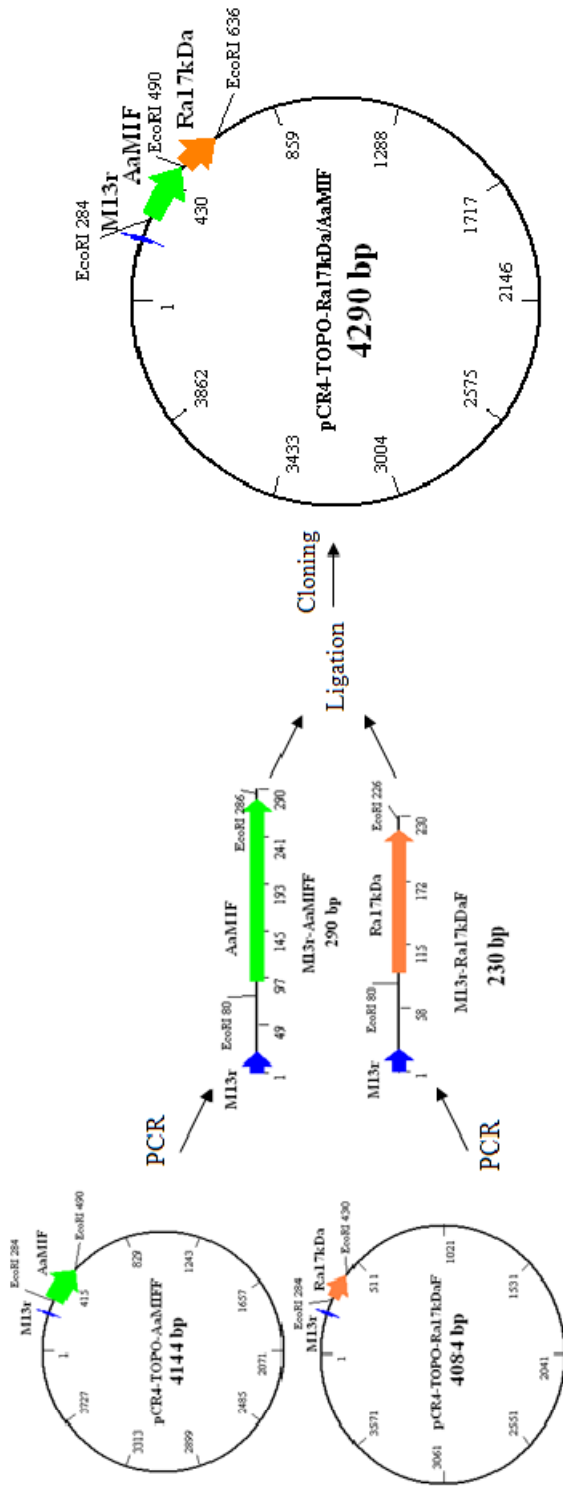
Figure 4. Schematic representation of the construction of the internal-control plasmid pCR4-TOPO-*Ra*17kDa/*Aa*MIF.
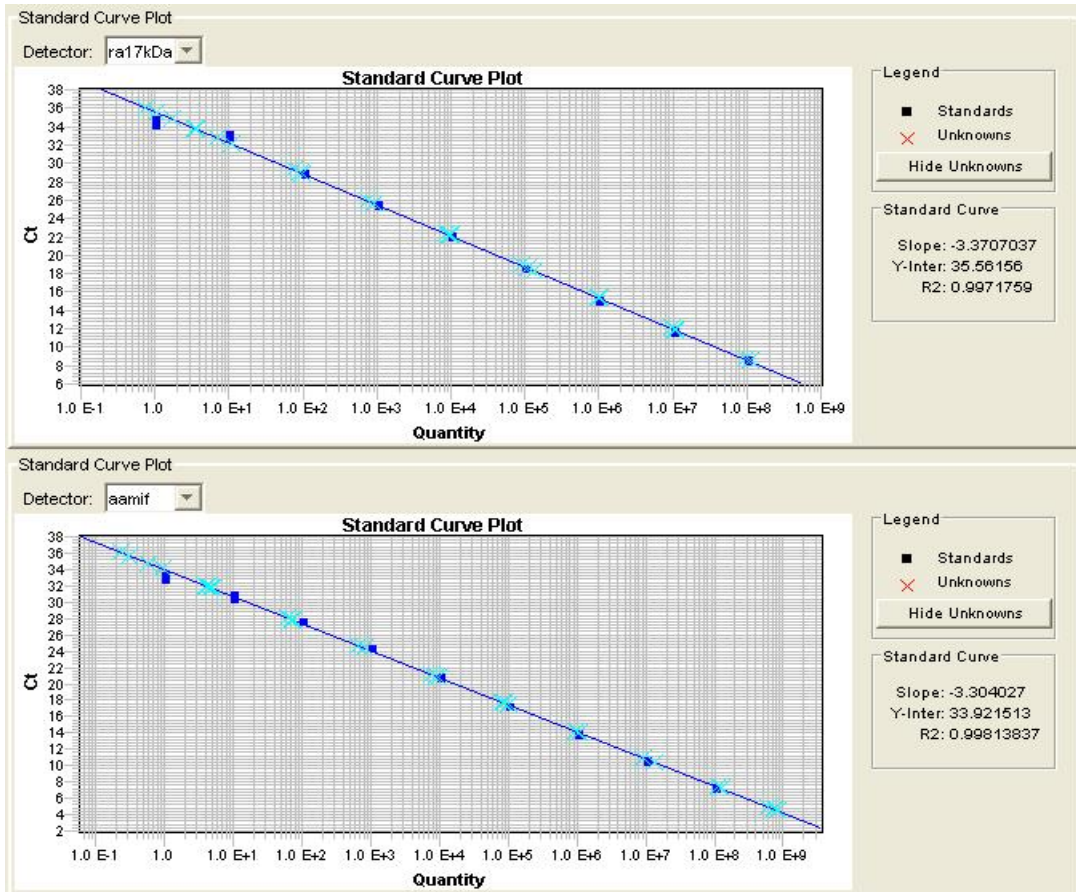
Figure 5. 10-fold serial dilutions of both genes used to quantify rickettsiae in *A. americanum* ticks.

Enumeration data presented are from one of two qPCR assays with similar results. The ratios of rickettsial to tick copy numbers were calculated after the logarithmic transformation of the quantity of both genes (Ra17kDa and AaMIF) was analyzed. When the overall significance was found, Tukey's honestly significant difference (HSD) post hoc test was used to examine pairwise differences of means of main effects. Pairwise t-tests of least square means were performed for interaction effects to identify significant differences in the ratio of Ra17kDa/AaMIF and weights, between tissues and time points, in addition to comparing the rickettsial load among egg clutches, fed, and unfed immature ticks. An F-test was used for a general comparison of grouped means. For all comparisons, a P value < 0.05 was considered significantly different.

2.3. Results

2.3.1 Tick Biology

Four-day-fed unmated female ticks ($n = 27$) and 5-9d-fed groups of unmated ($n = 12$) and mated ($n = 15$) female ticks were forcibly detached at 12- and 24h intervals, respectively. Of the remaining mated female ticks ($n = 10$) that were left feeding, only seven ticks underwent oviposition, beginning 4d postdetachment. Eggs began to hatch at 21d postoviposition. Larvae were immediately fed on BALB/c mice, and molting was observed in fed larvae at 19d posthatch. Nymphs were subsequently fed on BALB/c mice for 7-10d. Although not quantified, a portion of both immature feeding stages failed to molt after feeding. Changes in the mean weight of adult ticks were associated with feeding events (Figure 6). Significant differences were observed between the average weights of the virgin females during early stages of feeding (0-96h; 6.7 ± 3.8 mg) and the average of the weights of both mated (14.4 ± 3.7 mg; $P < 0.002$; t-test) and unmated (10.0 ± 0.70 mg; $P < 0.01$; t-test) female ticks in the late stage of feeding (120-216h). Mating also influenced tick weight as mated ticks weighed more than unmated ticks on each day 6 through 9 ($P < 0.02$; t-test). A subsample of each egg clutch (300 eggs) weighed on

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average 26.0 ± 6.4 mg. The average weights of unfed larvae ($n = 20$) and nymphs ($n = 4$) were 0.05 and 0.25 mg, respectively. Upon feeding, significant increases in the average weights of larvae (0.69 mg) and nymphs (10.4 mg) were observed ($P < 0.001$; t-test).

2.3.2. *Rickettsia* Prevalence and Identification in Tick Samples

The presence of rickettsiae in the homogenized guts of mated and unmated female ticks, a subsample of each egg clutch, and immature ticks was confirmed by PCR amplification and sequencing a portion of the SFGR-specific *ompA*.

For all tick samples, 100% of the tested samples generated a 128-bp band for the 17-kDa antigen gene. For every gut from the adult ticks, a 628-bp portion of *ompA* was amplified by PCR. The partial sequence of *ompA* from a representative sample was determined and deposited in the GenBank database under accession no. EF194096. Comparative analysis of our sequence to other sequences deposited in GenBank demonstrated a 99% identity (529/531) to *R. amblyommii* (accession no. AY062007).

2.3.3. Analysis of Rickettsial Infection by qPCR

A standard curve generated using serial dilutions of pCR4- TOPO-*Aa*MIF_*Ra*17kDa allowed for the determination of both tick and rickettsial gene copy numbers. For both genes, there was no patterned variation in copy numbers between individual time points during adult tick feeding (data not shown); the mean quantities (copy number) in each life cycle stage and individual tissue are presented in Table 4. The minimum detection limit for pCR4-TOPO-*Aa*MIF_*Ra*17kDa was 10 copies. The number of DNA copies of *Aa*MIF ranged from 1.9×10^4 to 7.4×10^5, and the average copy number was greatest in egg samples, followed by salivary gland, gut, unfed larvae, unfed and fed nymphs, ovary, and fed larvae. During adult tick feeding, the average *Aa*MIF copy number in the ovary was significantly lower compared with the salivary gland (4.2-fold) and gut (3.3-fold; $P < 0.001$; f-test), and no statistical differences were observed
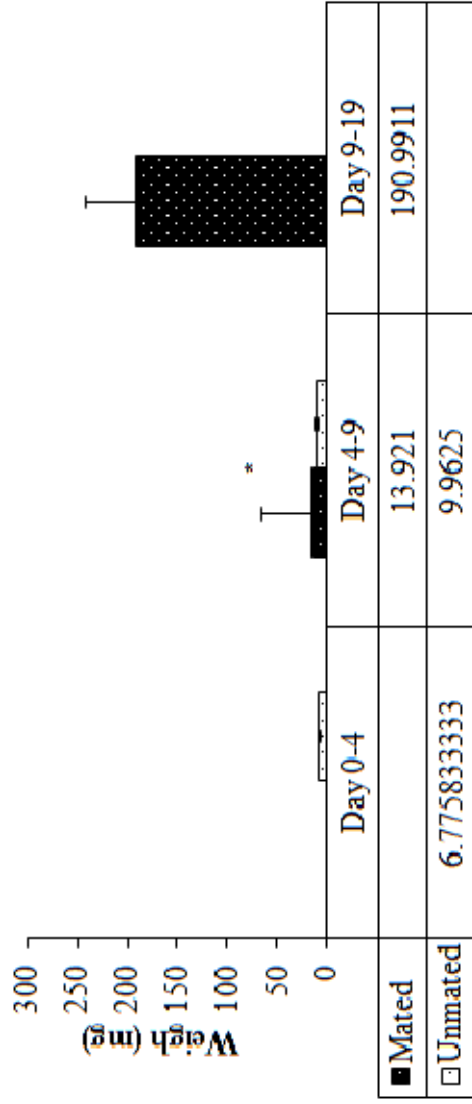
Figure 6. Average of weights (mg) of mated and unmated *Amblyomma americanum* ticks throughout bloodmeal experiment. Asterisk indicate significant differences between mated and unmated ticks. ($P < 0.05$; *t*-test).

	Day 0-4	Day 4-9	Day 9-19
Mated		13.921	190.9911
Unmated	6.775833333	9.9625	

between the average *Aa*MIF copy number in the salivary gland and gut ($P < 0.05$; *f*-test). *Aa*MIF copy number also significantly differed between samples of fed larvae compared with the other immature life stages ($P < 0.05$; *f*-test).

The average number of *R. amblyommii* ranged from 1.3 X 10^2 to 2.5 X 10^5, with the greatest rickettsial numbers in the salivary glands, followed by the egg samples, gut, ovary, unfed nymphs and larvae, and fed nymphs and larvae. Similar to *Aa*MIF copy numbers in the adult, the average rickettsial load in ovary samples was significantly less than rickettsial numbers in the salivary glands (10.3-fold; $P < 0.001$; *f*-test) and in the gut (5.2-fold; $P < 0.001$; *f*-test). The number of rickettsiae in the salivary glands was significantly greater than that observed in the gut (two-fold; $P < 0.05$; *f*-test). Significant variation in rickettsial numbers in immature ticks was observed for fed larvae compared with other postembryonic stages ($P < 0.05$; *f*-test) (Table 4).

2.3.4. Rickettsial Distribution and Growth in Tick Samples

To compare the rickettsial load in individual tick samples, a ratio of *R. amblyommii* 17-kDa antigen gene copy numbers per *A. americanum* MIF copy numbers was generated for each sample by logarithmic transformation of the copy numbers for rickettsial *Ra*17kDa and tick *Aa*MIF then calculating the ratio *Ra*17kDa/*Aa*MIF) for each sample or for individual ticks, when combined ratios from each sample were assessed. In adult ticks, significant decreases in ratios of *Ra*17kDa/*Aa*MIF were observed during early and late feeding time points (Figure 7). Compared with unfed ticks, the mean ratio of *Ra*17kDa/*Aa*MIF, when tissue sample ratios were combined, was significantly lower up to 48h of feeding, and in some unmated (144 and 192h postattachment) and mated (168 and 216h postattachment) ticks ($P < 0.05$; HSD). When examining the 54 samples (three ticks per 18 time points) for each tissue, significant variability was also observed in the ratio of *Ra*17kDa/*Aa*MIF for each tissue, between time points, with an

Table 4. Mean copy number ± S. E. M. of both rickettsial and tick genes in eggs, larval, nymphal (pre- and post-feeding), and adult (0-9-d-fed) A. americanum.

Life cycle stage[a,b]	Mean copy no. ± SEM			
	Ra17kDa		AaMIF	
Egg	$4.5 \times 10^4 \pm 7.0 \times 10^3$		$6.9 \times 10^5 \pm 1.0 \times 10^5$	
	Unfed	Fed	Unfed	Fed
Larva	$6.7 \times 10^2 A \pm 2.0 \times 10^0$	$1.3 \times 10^2 B \pm 2.0 \times 10^0$	$2.0 \times 10^5 a \pm 1.5 \times 10^5$	$5.0 \times 10^4 b \pm 3.7 \times 10^4$
Nymph	$7.0 \times 10^2 A \pm 4.8 \times 10^2$	$4.5 \times 10^2 A \pm 1.0 \times 10^0$	$1.2 \times 10^5 a \pm 3.3 \times 10^4$	$1.2 \times 10^5 a \pm 8.9 \times 10^4$

	Ra17kDa Tissue			AaMIF Tissue		
	Salivary gland	Gut	Ovary	Salivary gland	Gut	Ovary
Adults	$5.8 \times 10^4 A \pm 1.4 \times 10^4$	$2.9 \times 10^4 B \pm 5.4 \times 10^3$	$5.6 \times 10^3 C \pm 1.5 \times 10^3$	$2.9 \times 10^5 a \pm 5.9 \times 10^4$	$2.3 \times 10^5 a \pm 3.9 \times 10^4$	$6.8 \times 10^4 b \pm 1.3 \times 10^4$

Eggs (≈ 300 per tick) from seven ticks, whole immature ticks from the same life cycle stage were pooled (20 larvae, 4 nymphs), and selected tissues from adult female ticks ($n = 54$) were assessed by qPCR.

[a] Within each life cycle stage average of rickettsial copy numbers (Ra17kDa) with the same uppercase letter were not significantly different.

[b] Within each life cycle stage average of tick copy numbers (AaMIF) with the same lowercase letter were not significantly different ($P < 0.05$; f-test).

average of 0.82 ± 0.02 *Ra*17kDa/*Aa*MIF in the salivary glands during the entire tick feeding

event, followed by 0.79 ± 0.02 *Ra*17kDa/*Aa*MIF in the gut and 0.74 ± 0.02 *Ra*17kDa/*Aa*MIF in

the ovaries ($P < 0.05$; HSD). Within each time point assessed, except after 12 and 168h

postattachment ($P < 0.143$; *t*-test), there was significant variability in the ratio of

*Ra*17kDa/*Aa*MIF between individual tissues (Figure 8). Although salivary glands and guts were

generally observed to have the greatest ratio of *Ra*17kDa/*Aa*MIF, no single tissue was

consistently observed to maintain the highest rickettsial burden throughout the feeding event.

Likewise, analyses of individual tissue samples identified unique patterns of rickettsial load

during the course of adult bloodmeal acquisition (Figure 8). In comparison with unfed female

ticks, a decrease in the ratio of *Ra*17kDa/*Aa*MIF was followed by both salivary gland and gut

ratios at, or above ($\leq$ 36h postattachment) those observed in unfed ticks for both salivary glands

and gut samples ($\leq$ 168h postattachment). During the tick-feeding event, the ratio of

*Ra*17kDa/*Aa*MIF in the ovaries was similar to that observed after the first 12h postattachment.

Tick mating had little effect on the ratio of *Ra*17kDa/*Aa*MIF in the gut samples, while in the

salivary glands, unmated ticks typically had higher ratios compared with mated ticks. Similar to

the salivary glands, unmated female ticks had increased ratios of *Ra*17kDa/*Aa*MIF in ovary

samples compared with mated counterparts. Finally, the ratio of *Ra*17kDa/ *Aa*MIF in the egg

clutches and immature ticks identified no significant differences between life cycle stages or in

association with larval or nymphal tick feeding events ($P < 0.05$; HSD) (Figure 9).

2.4. Discussion

    In the present report, a qPCR assay was used to analyze the growth and distribution of *R.*

amblyommii in individual *A. americanum* tissues during tick feeding and in whole ticks through

transovarial and transstadial transmission events. The infection of *A. americanum* by SFGR has

been examined with regards to the prevalence of different species of SFGR, transmission
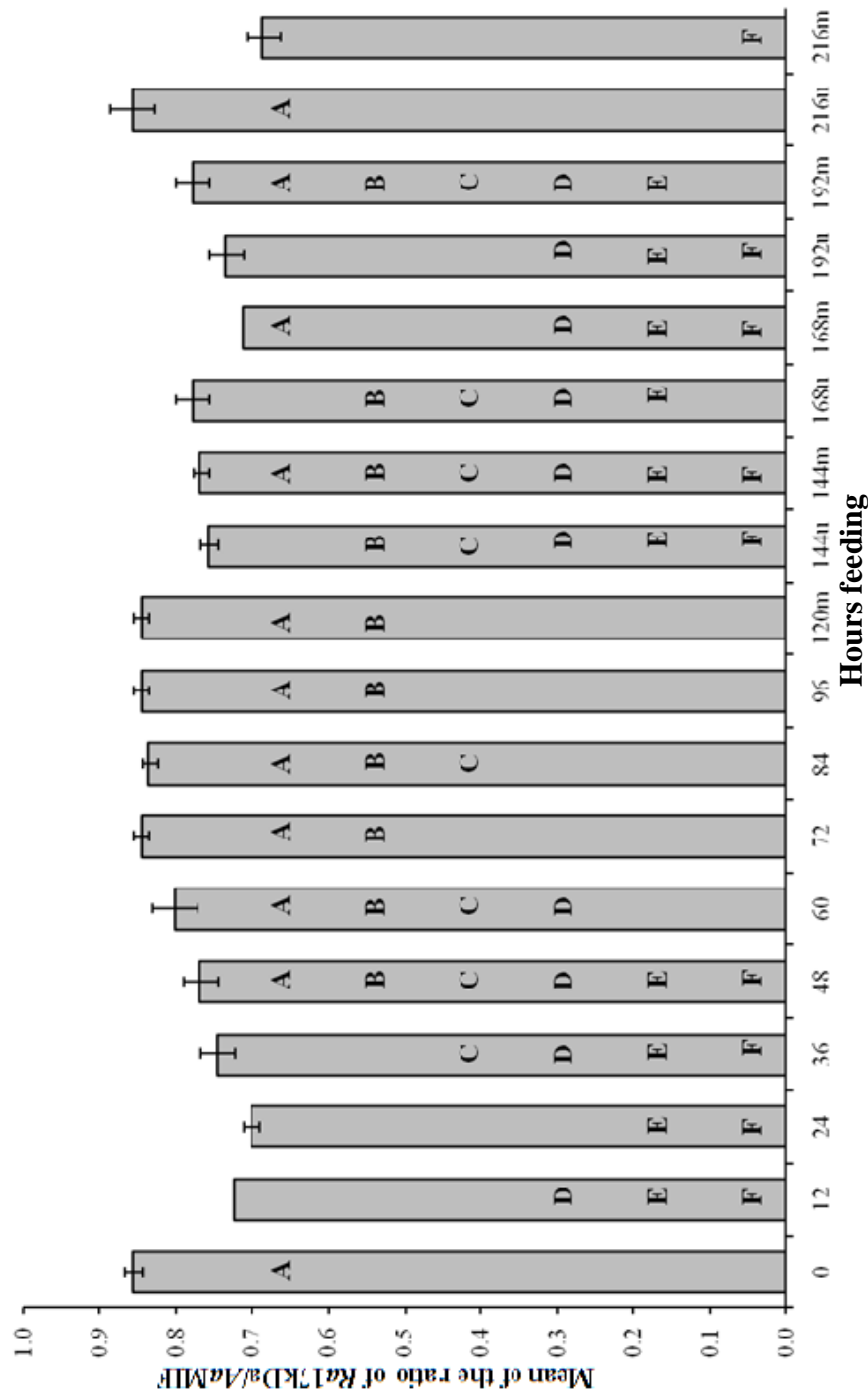
Figure 7. Growth and distribution of *R. amblyommii* in ticks. Cumulative mean ± SEM $\log_{10}$ ratios of rickettsial (17-kDa antigen gene) to tick (*A. americanum* MIF) genes quantified in salivary gland, gut, and ovaries during tick bloodmeal acquisition. Across the time points, means with the same letter on the bar are not significantly different. Male ticks were introduced to a group of females (mated females – m) at a ratio of 2:1 (male:female) after 96h of experiment (120m). Mated and unmated (u) females were collected during intervals of 24h during the remaining time points ($P > 0.05$; HSD).

potential, and influence of SFGR on tick fitness (Goddard and Norment, 1986; Goddard, 2003; Mixson et al., 2006; Jasinskas et al., 2007; Zhong et al., 2007). This study now provides the kinetics of SFGR growth in *A. americanum,* an emerging vector in the United States (Childs and Paddock, 2003).

Molecular detection of SFGR has been accomplished via real-time PCR in arthropod hosts (Labruna et al., 2004a, Henry et al., 2007), whereas the analysis of growth and quantification of SFGR have been assessed via qPCR in cell culture and arthropod models in terms of rickettsial whole numbers and ratio to host cell (Rolain et al., 2002; Jasinskas et al., 2007; Zhong et al., 2007). Eremeeva et al., (2003) described a qPCR technique for SFGR in cell culture, clinical samples, and tick hosts. The SYBR Green-based assay used primers designed on a conserved region of *ompA* to quantify *R. rickettsii* in *A. americanum;* however, tick sample details (i.e., life cycle stage, sex, whole tick or specific tissue, and feeding status) were not provided and the quantification of SFGR in ticks (10^6-10^7 rickettsiae per tick) cannot be directly compared with the number of rickettsiae ($\approx 10^5$ per tick) for combined tissues (salivary gland, gut, and ovary) from adult ticks in our study. The tick model described here facilitates the understanding of infection and rickettsial growth by representing the natural variability SFGR infection in ticks. The amount of tissue, and therefore DNA, from individual ticks varies; to account for this variation, the copy numbers for rickettsial 17-kDa antigen gene and *A. americanum* MIF were determined and then the rickettsial burden was calculated as a ratio of single copy genes *Ra*17kDa/*Aa*MIF. Although *R. amblyommii* is present in all samples assessed, variability was observed in both the copy number of tick MIF and rickettsial 17-kDa antigen genes counted among the individual tissues and between the life cycle stages. Both rickettsial species and source of ticks (field-caught versus laboratory reared) may influence rickettsial burden as *A. americanum* collected in state parks had greater ratio of a *Rickettsia bellii*-like
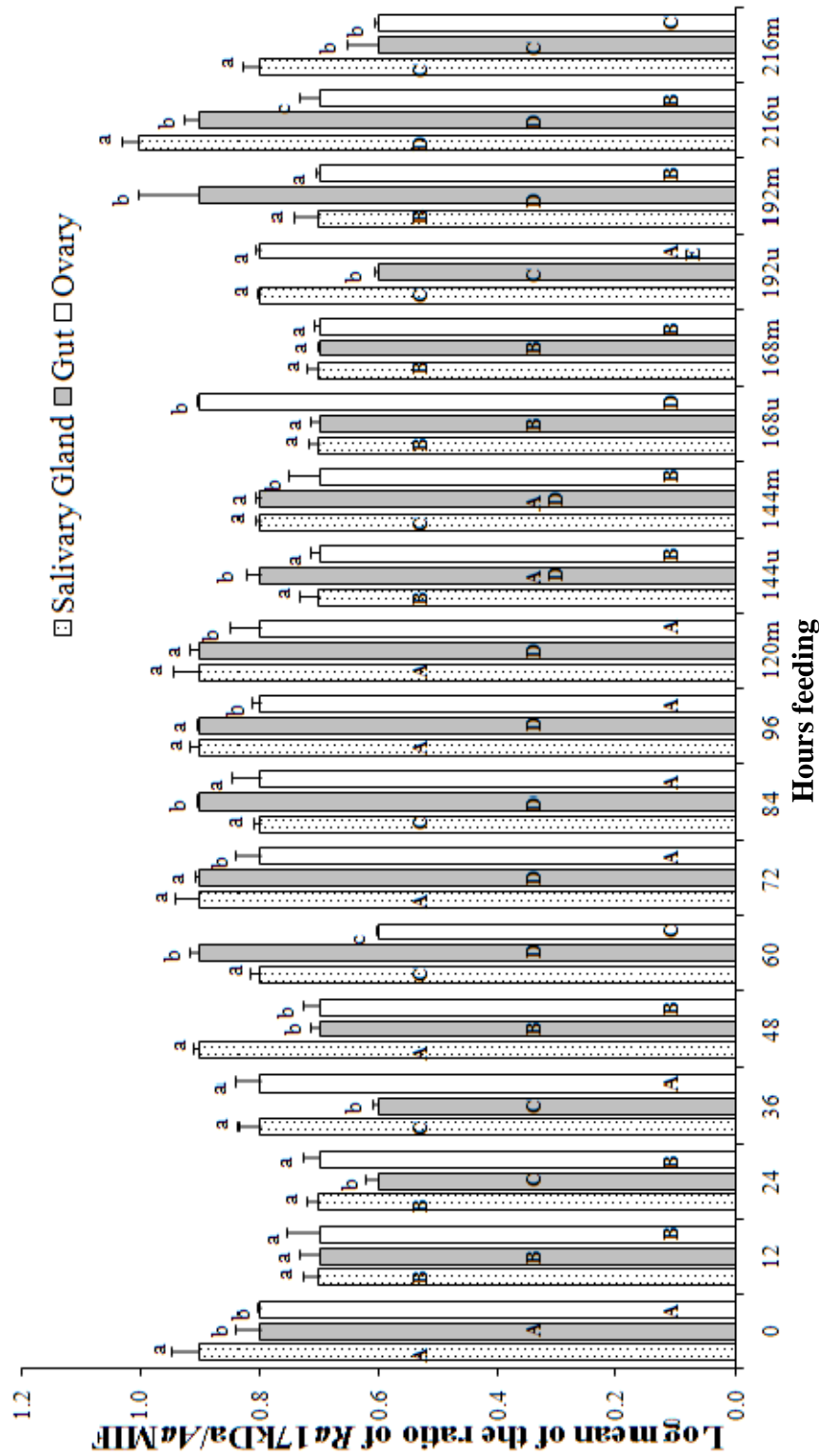
Figure 8. *R. amblyommii* burden in female *A. americanum* salivary gland, gut, and ovaries during tick bloodmeal acquisition. Mean ± SEM $\log_{10}$ ratios of rickettsial (17-kDa antigen gene) to tick (*A. americanum* MIF) genes were calculated for individual tick tissues salivary gland, gut, and ovaries during tick (n = 3) bloodmeal acquisition on 12- and 24-h intervals. Different lowercase letters above bars indicate significant differences between tissues with individual time points (*P* < 0.05; t-test). Different uppercase letters on the bars indicate significant difference between time points for each individual tissue (*P* < 0.05; t-test). Male ticks were introduced to a group of females (mated females – m) at a ratio of 2:1 (male:female) after 96h of experiment (120m). Mated and unmated (u) females
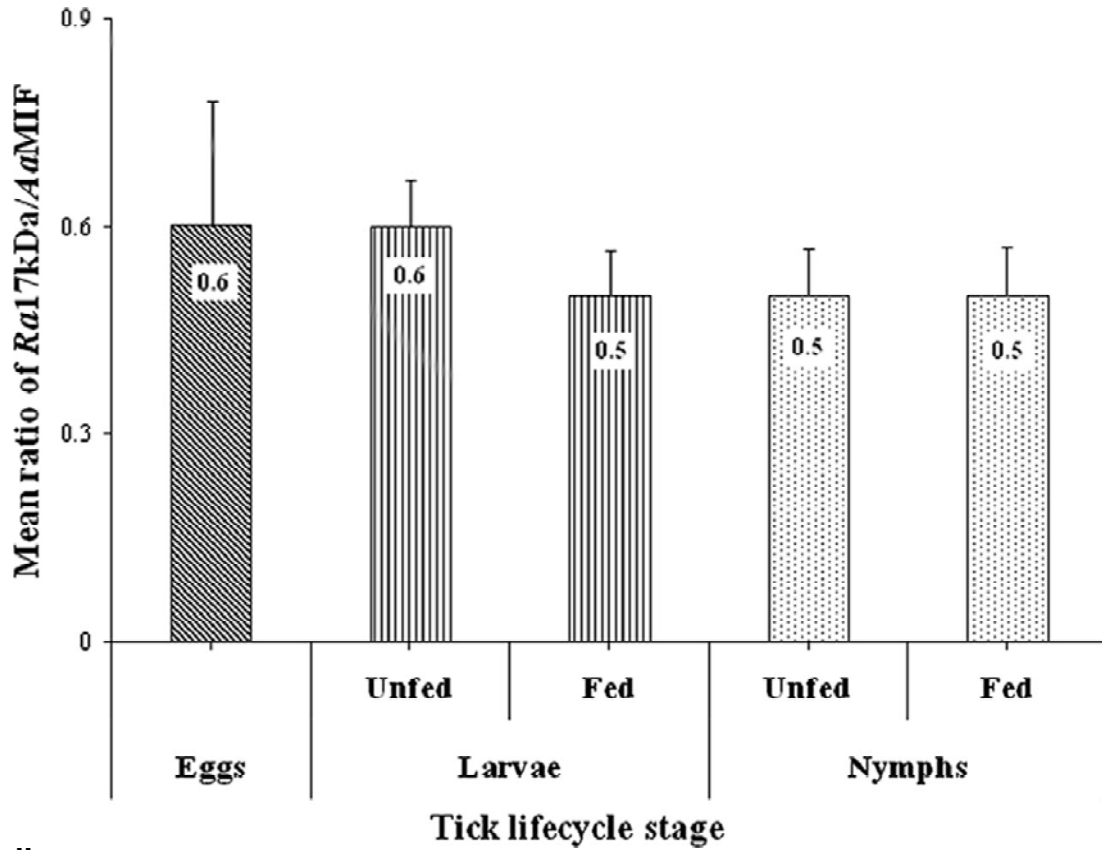
Figure 9. *R. amblyommii* burden in immature *A. americanum*. Mean ± SEM $\log_{10}$ ratios of rickettsial (17-kDa antigen gene) to tick (*A. americanum* MIF) genes were calculated for pooled *A. americanum* eggs, larvae, and nymphs. Single copy tick and rickettsial genes quantified by qPCR were subjected to logarithmic transformation and the ratio of *R. amblyommii* 17 kDa per *A. americanum* MIF gene copy numbers was calculated for each sample.

species to tick cell (4.3) compared with laboratory-reared *R. amblyommii*-infected *A. americanum* (Jasinskas et al., 2007). For example, analyses of rickettsial burden in a *R. amblyommii-A. americanum* model by using genes encoding rickettsial citrate synthase (*gltA*) and *A. americanum* MIF, identified the ratio of *gltA*/MIF in postmolt larvae (0.002) and adult ticks (0.002-0.71) consistent with the whole tick ratios in this study. As the ratio of less than one *Rickettsia* per host cell suggests, not every cell was infected. An infection of *Ixodes scapularis* (Say) with *R. monacensis* expressing green fluorescent protein demonstrated the organisms growing in intracellular clusters, in a stage-dependent manner (Baldridge et al., 2007). Based on the different cell types within the assessed tissues and the preferential infection of distinct cell types by some SFGR (Santos et al., 2002), the authors suspect certain cell types within each tissue become infected with more than one organism per cell. Analysis of *R. amblyommii* in field-caught *A. americanum* also identified "light" infection predominately in ovary and Malpighian tubules, with only 10% of the tick hemocytes infected with small numbers (< 10 per cell) of rickettsiae (Burgdorfer et al., 1981b, Goddard and Norment, 1986); although consistent with the results of this study, every tissue was infected. Most SFGR are maintained in nature via vertical and horizontal transmission by ticks; the data presented here compliment our understanding of rickettsial infection in ticks by enumerating SFGR in individual tick tissues during bloodmeal acquisition and vertical transmission events. A direct correlation between specific tick tissue infection and SFGR pathogenicity has been proposed (Santos et al., 2002; Matsumoto et al., 2005) and a potential role for *R. amblyommii* in human disease has been suggested (Marshall et al., 2003; Parola et al., 2005). However, the generalized infection of ticks by most SFGR, combined with the observed infection of salivary glands by both pathogenic and nonpathogenic SFGR, indicates that presence alone in the salivary glands is not sufficient criteria for the pathogenic classification of SFGR for vertebrate hosts. In the current study, no adverse

effects to animal hosts and no distinct shift in growth of SFGR in any individual tissue during feeding were observed, consistent with studies in which *R. amblyommii* (WB-8-2) was observed to be nonpathogenic to meadow voles (*Microtus pennsylvanicus*) and guinea pig (*Cavia porcellus*) hosts via both needle and tick inoculation (Burgdorfer et al., 1981b). Some SFGR may have characteristics typically associated with endosymbiotic and pathogenic SFGR (Baldridge et al., 2007); therefore, vertebrate infectivity should be further assessed for *R. amblyommii.* Vertical transmission of the human pathogens *R. rickettsii* and *R. conorii* is associated with decreased tick fitness (Niebylski et al., 1999; Burgdorfer and Brinton, 1975; Santos et al., 2002); whereas the toll on tick fitness in the transmission of other pathogenic and nonpathogenic SFGR is less clear (Macaluso et al., 2001; 2002; Goddard, 2003; Matsumoto et al., 2005; Zhong et al., 2007). Consistent with the previous analysis of vertical transmission of *R. amblyommii* by ticks, transovarial transmission was observed in 100% of the adult ticks that laid eggs. The filial infection rate for *R. amblyommii* by *A. americanum* is between 30 and 100% (Goddard and Norment, 1986). In this study, filial infection rates were not assessed, therefore, infection cannot be presumed in all eggs. It is possible that *R. amblyommii* infection did affect the ticks, as seen in *A. americanum* experimentally infected with *R. parkeri* (Goddard, 2003), because in this study not all ticks underwent oviposition, and mortality in larvae and nymphs was not observed. A SFGR-free line of *A. americanum* was not available; therefore, even though a 70% survivorship is typical for these ticks under laboratory conditions for this study, correlation between SFGR infection and tick fitness cannot be made. Several factors (e.g., temperature and humidity) are associated with tick survivorship when maintained in a laboratory, particularly if the ticks are *Rickettsia* infected (Niebylski et al., 1999). Additionally, the association between alternate bacterial symbionts and tick fitness must be furthered examined, because recent analyses demonstrated that *Coxiella* symbionts, not rickettsiae, were correlated to tick fitness (Zhong et

al., 2007). In the current study, a relatively constant level of infection during transmission and feeding events was observed. The rickettsial burden was slightly lower in immature ticks compared with adult ticks. Groves and Kelly (1989) reported that among different strains of *Orientia* (*Rickettsia*) *tsutsugamushi,* the rate of bacterial replication was the contributing factor to virulence. Likewise, rickettsial replication (*R. rickettsii*) during tick feeding is considered to be a component of reactivation and contributes to rickettsial infectivity for vertebrate hosts (Hayes and Burgdorfer, 1982). A similar *Orientia*-like scenario may exist for SFGR, and defining the kinetics of growth for highly pathogenic SFGR is part of ongoing studies. Although assessing rickettsial growth in vitro can lend insight into the pathogenic nature of rickettsiae, to fully understand the mechanisms of SFGR transmission, the whole-tick model of analyses is required and will provide accurate representation of the dynamics of rickettsial growth in nature and facilitate our understanding of rickettsial pathogenicity.

Chapter 3: Dynamics of Growth and Infectivity of *Rickettsia amblyommii* (WB-8-2), *Rickettsia parkeri* and *Rickettsia montanensis* in Vertebrate and Tick Cells: A Preliminary Study

3.1. Introduction

During the past century, many Spotted Fever Group *Rickettsia* (SFGR) were isolated from ticks, especially in the United States. They account for a mixed cluster of microorganisms where recognized tick-transmitted spotted fevers are combined with SFGR of unknown pathogenicity, that, in some instances, are reported to engage in a truly symbiotic life style with their tick hosts (Niebylski et al., 1997, Simser et al., 2002). Of the five officially named tick-borne SFGR in the United States, specificity ranges among the different tick vectors available. *R. rickettsii* (Parola et al., 2005; Demma et al., 2005) and *R. rhipicephali* (Burgdorfer et al., 1975; Magnarelli et al., 1982; Philip and Casper, 1981; Philip et al., 1981; Labruna et al., 2005; 2007a; Wikswo et al., 2008) have evolved to associate with tick vectors from several different genera (Parola et al., 2005), while *R. peacockii* seems to be restricted to *D. andersoni* (Burgdorfer and Brinton, 1975; Niebylski et al., 1997) ticks only. Discrepancy between these ranges seems also true, since *R. parkeri* (Parker et al., 1939; Goddard et al., 2003; Pacheco et al., 2007; Silveira et al., 2007; Venzal et al., 2008a; 2008b) and *R. montanensis* (Bell et al., 1963), apparently hold an intrinsic relationship with several different species within the same tick genus. Recently, *R. amblyommii* loads in naturally infected *A. americanum* ticks was reported to remain relatively constant during tick physiologic events (Zanetti et al., 2008). *R. amblyommii* is a SFGR of unknown pathogenicity isolated exclusively from *Amblyomma* ticks (Burgdorfer et al., 1981b; Labruna et al., 2004a; 2004b; Parola et al., 2007; Labruna et al., 2007b). The replication pattern observed in this study is apparently correlated with the pathogenic nature of this SFGR (Hayes and Burgdorfer, 1982; Groves and Kelly, 1989). Due to the lack of information regarding the specificity of associations among SFGR and their tick hosts (Parola et al., 2005), we

hypothesized that replication is also correlated with the specificity of the host. Therefore, a quantitative real time PCR (qPCR) assay was performed to access the rickettsial burden inside the cell after SFGR challenge in atypical host cells. Recognized tick-transmitted spotted fever (*R. parkeri*) and SFGR of unrecognized pathogenicity (*R. amblyommii* and *R. montanensis*) were inoculated in mammalian (Vero) and tick (ISE6) cell lines at equal amounts to investigate the replication patterns of these rickettsiae within different intervals. *R. parkeri* and *R. amblyommii* are SFGR naturally associated with *Amblyomma* ticks (Parker et al., 1939; Silveira et al 2007; Venzal et al 2008a; 2008b; Burgdorfer et al., 1981b; Labruna et al., 2004a; 2004b; Parola et al., 2007; Labruna et al., 2007b) while *R. montanensis* infection is reported only in naturally infected *Dermacentor* ticks (Bell et al., 1963). The contributing factors associated with replication changes in host cells not naturally associated with these SFGR could offer supporting evidence of an intrinsic relationship between some SFGR and ticks of a determinate genus. In the present study, differences in the replication of the SFGR examined were observed within each cell line. In general, the ratio of rickettsiae per cell was greater in ISE6-infected cells with the *R. parkeri*-load being the most prominent. The additional information regarding the replication changes observed between different rickettsiae species, cell lines and time points are further presented and discussed.

3.2. Materials and Methods

3.2.1. Cells Lines and *Rickettsia* Species

Vero cells (passage 43) were originally provided from the Tissue and Organ Culture Laboratory of the School of Veterinary Medicine/ Louisiana State University (LSU-SVM). Cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and were maintained in a humidified 5% CO_2 incubator at 34°C. ISE6 cells (passage 117) were originally provided by T. Kurtti (University of Minnesota). Cells were cultured in

L15B supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 10% tryptose phosphate broth (Sigma) at pH 6.8 in a humidified 5% CO_2 incubator at 32°C according to published protocol (Pornwiroon et al., 2006). Cell density of 1 X 10^6 cells/200μL was seeded per well of 24-well plates (6 plates in total) one day prior to the beginning of the experiment. *R. parkeri* (Portsmouth strain) was originally provided by C. Paddock (CDC, Atlanta)*; R. amblyommii* (Wb-8-2) was a courtesy of T. Kurtti (University of Minnesota); and *R. montanensis* (m5-6) was originally obtained from Abdu Azad (University of Maryland). *Rickettsia* species were first inoculated in Vero cells, after two passages, a portion of the infected cells were treated in blocking buffer (5 % skim milk in PBS – 0.1 % Tween 20) at room temperature, and then incubated with rabbit-anti-spotted fever group-specific antibody (polyclonal NIH/RML-I7198; diluted 1:1000 in blocking buffer) for 1.5 hour at room temperature (Figure 10) as previously described (Sunyakumthorn et al., 2008). Infected cells were then partially purified from Vero cells before inoculation in ISE6 cells according to Kurtti et al. (2005). Briefly, cells (Vero and ISE6) infected with *R. montanensis*, '*R. amblyommii*' and *R. parkeri* were harvested from 25-cm^3 tissue culture flasks (Griener), lysed by mechanical disruption using a 27-gauge needle attached to a 1mL syringe and suspended microorganisms were pelleted by high-speed centrifugation (Pornwiroon et al., 2006). Lysed cells were then washed twice with 0.85% sterile sodium chloride solution between two centrifugations steps at 13,000 x g for 10 min. Viability of partially purified rickettsiae was estimated using LIVE/DEAD$^®$ BacLight^{TM} stainning (Invitrogen, Carlsbad, CA). Rickettsiae were counted using a Petroff-Hausser chamber under a Leica microscope.

3.2.2. Cell-Rickettsiae Challenge

Partially purified rickettsiae were resuspended in growth medium (DMEM or L15B) to yield a final concentration of 7.8 X 10^5 rickettsiae/well, the equivalent to a ratio of 0.78
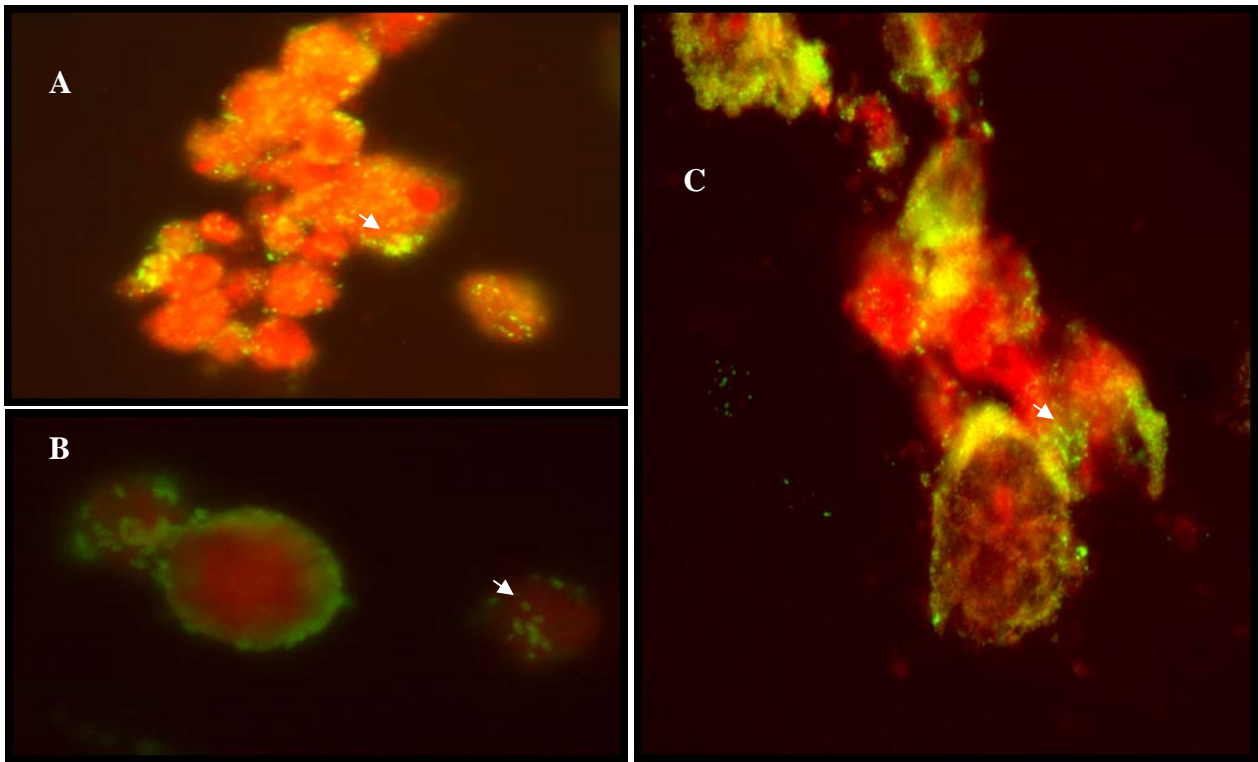
Figure 10. Photomicrographs illustrating (A) *R. parkeri*; (B) *R. amblyommii*; and (C) *R. montanensis* coated with rabbit-anti-SFGR polyclonal antibody NIH/RML-I7198. — arrow heads indicate microorganism; 1:1000 dilution, UV/100X.

rickettsiae/cell. Rickettsiae were inoculated in triplicate for each *Rickettsia* strain and time points. Rickettsial infection of both tick and mammalian cell lines was assisted by quick centrifugation at 1,000 x g for 5 min at 4°C, before infected plates were stored in a humidified 5% CO_2 incubator at 34°C (Vero) or 32°C (ISE6). Data collection was performed three hours post-inoculation (0 hours), followed by subsequent time points (24; 48; 72; 144 and 216 hours), as schematically illustrated in Figure 11.

3.2.3. Infectivity Determination by Diff Quick[TM] and Transmission Electron Microscopy

For each time point, samples were collected for infectivity analysis. Cytospin centrifuge (Wescor) was performed using 200 µL of suspended cells. Cells were then stained according to the manufacture's protocol Diff-Quik[TM] (Dade Behring). Infected cells collected in the last time point (216 hours) were used for transmission electron microscopy. Infected cells were fixed and prepared according published protocols (Pornwiroon et al., 2006; Ito and Rikihisa, 1981).

3.2.4. High Through-Put DNA Extraction

Cells were harvested from each well by pipetting media up and down. Suspended cells were transferred into 1.5 mL tubes and immediately centrifuged at 1,000 X g for 2 min before storage in – 80 °C. DNA extraction was performed using BloodPrep® DNA Chemistry for Cultured Cells and Blood according manufacture protocol (Applied Biosystems[TM]). Stock DNA was final eluted in 200 µL of elution buffer.

3.2.5. Construction of Internal-Control Plasmids for Host-Microorganism Quantification

To quantify the copy numbers of rickettsial and host cells genes, serial dilutions of a plasmid harboring a single copy of both rickettsial and host genes Vero (Ohno et al., 2003) and ISE6 (Xu et al., 2005) were used to generate a standard curve.

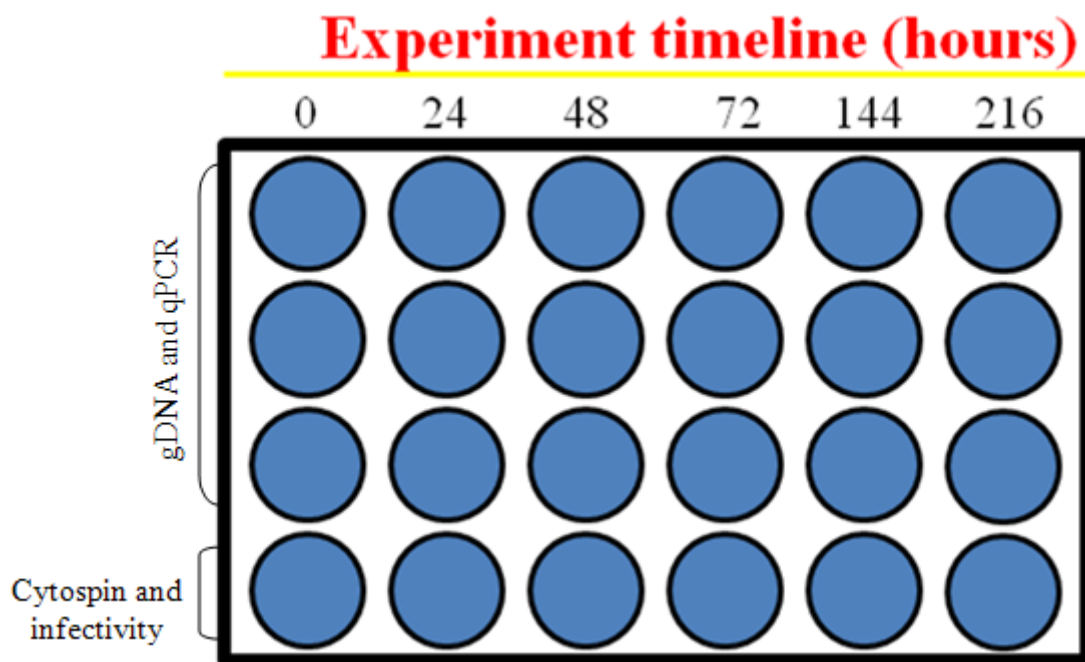**Figure 11.** Layout of in vitro study. *R. amblyommii*, *R. montanensis* and *R. parkeri* were inoculated in each individual 24-well plate containing Vero or ISE6 cells. For each time point (0; 24; 48; 72; 144 and 216 hours post-inoculation) three wells were harvested for gDNA isolation and qPCR analysis while only one well was harvested to assess infectivity in each culture.

The 128-base pair (bp) fragment of the 17-kDa antigen gene of *R. amblyommii* was PCR amplified with *Ra*17kDaF and *Ra*17kDaR primers and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA). The insert fragment (*Ra*17kDa) was sequenced to confirm its identification as the rickettsial 17-kDa antigen gene. Likewise, the 122-bp portion of the gene encoding *Ixodes scapularis* calreticulin protein and the 198-bp portion of the gene *ß*actin (Ohno et al., 2003) were PCR amplified using the primers *IS*E6CrtF, *IS*E6CrtR, *ß*actinF and *ß*actinR, cloned, and sequenced. Both amplicons (*Ra*17kDa and *ß*actin*/IS*E6Crt) were amplified using a gene-specific primer (Table 3) and either M13forward or M13reverse primer (Invitrogen, Carlsbad, CA). The obtained PCR products, the templates for the primer pair *Ra*17kDaF and both *ß*actinF*/IS*E6CrtR, were digested with *EcoRI* and ligated together. The PCR product, containing both amplicons, was cloned and sequenced; the resulting plasmid, pCR4-TOPO-*Ra*17kDa/*ß*actin or pCR4-TOPO-*Ra*17kDa/*IS*E6Crt, served as the standard template (Figures 12 and 13).

3.2.6. Quantitative Real-Time PCR

For each gene, a qPCR reaction mixture was created in a final volume of 35µl with 2X iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA), 100nM of each primer, DNase/RNasefree water; and either gDNA template, water, or serial 10-fold dilutions (1×10^7 to 10 copies) of pCR4-TOPO-*Ra*17kDa/*ß*actin or pCR4-TOPO-*Ra*17kDa/*IS*E6Crt (Figure 14). We used 100 ng of gDNA in 5µl added to the qPCR master mix for each reaction. Copy numbers per 5µl were calculated, and the total copy numbers per sample was based on the 200µl elution volume. For each assay, reaction components and template were premixed in 96-well plates for both genes of interest; a qPCR of each gene was conducted in individual 384-well plates. For each sample, three wells were filled with 10µl of the reaction mixture.
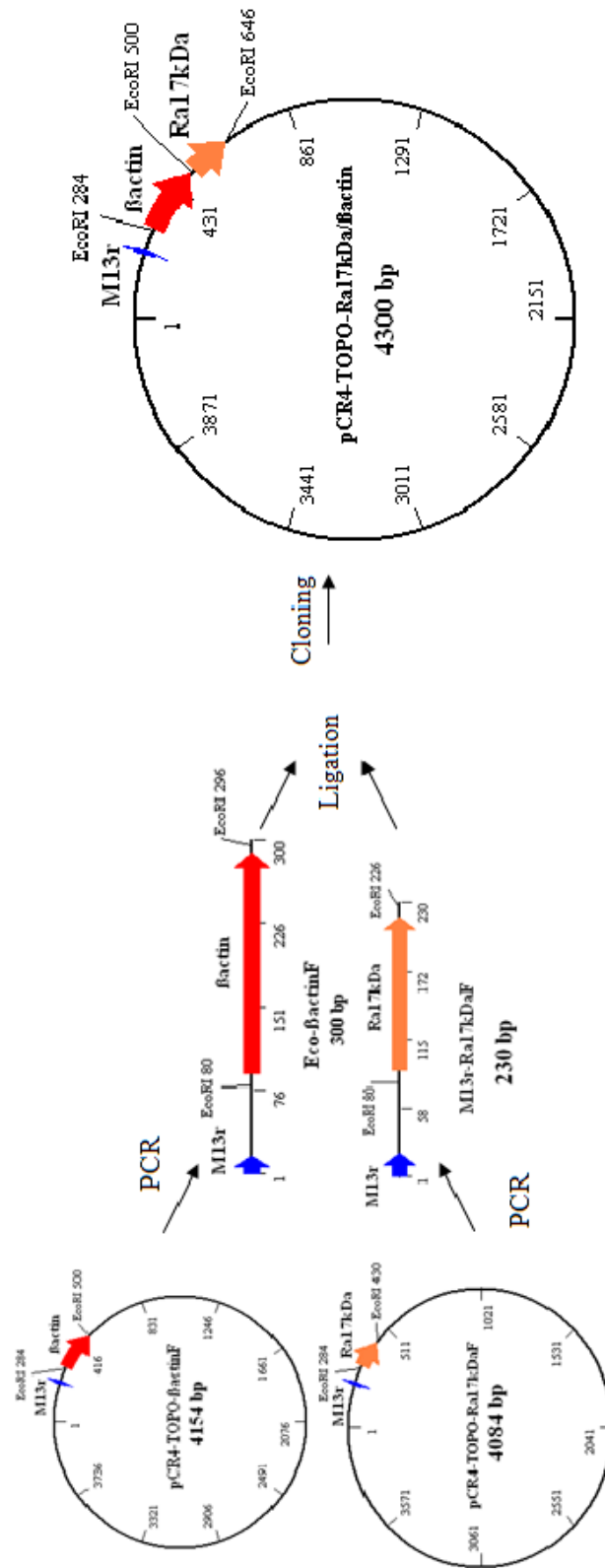
Figure 12. Schematic representation of the construction of the internal-control plasmid pCR4-TOPO-*Ra*17kDa/*β*actin.

49
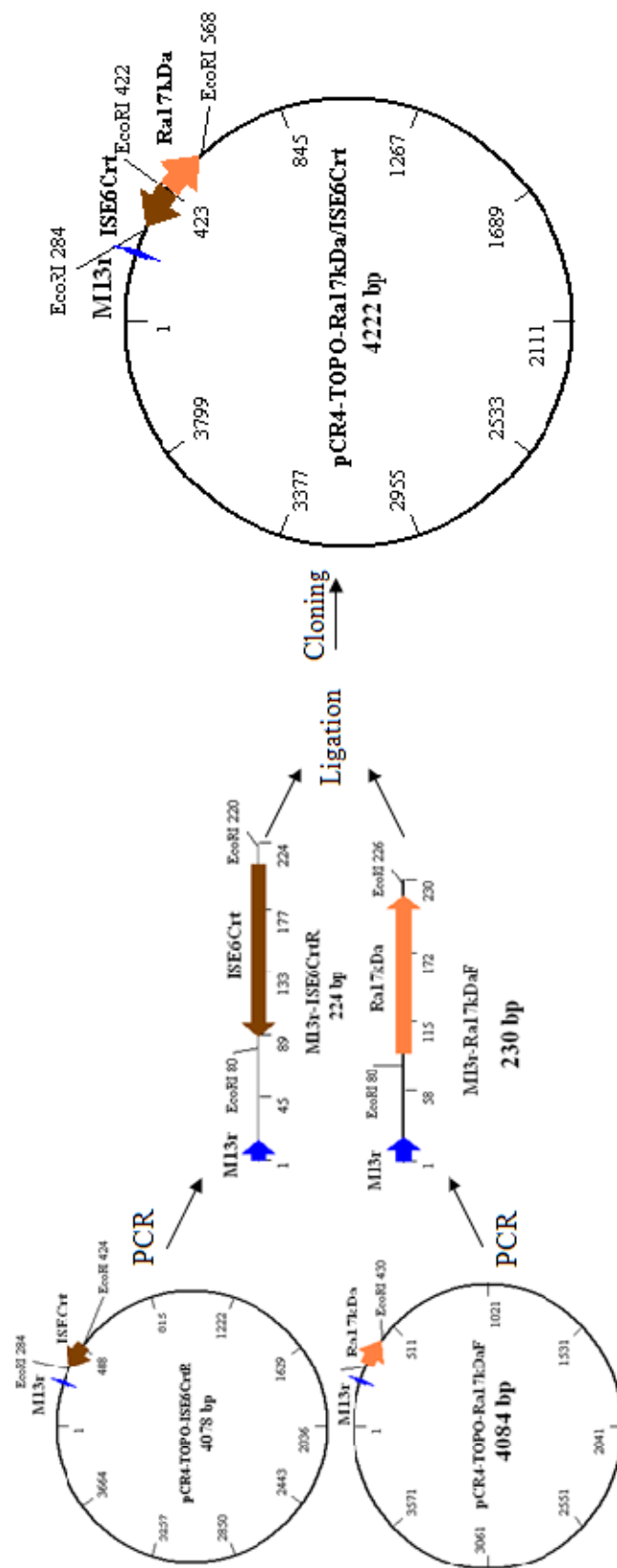
Figure 13. Schematic representation of the construction of the internal-control plasmid pCR4-TOPO-*Ra*17kDa/*ISE*6Crt.

qPCR was performed with an ABI 7900HT unit (Applied Biosystems, Foster City, CA) at the

LSU-SVM under the following cycling condition; denaturation step at 95ºC for 10 min, 40

cycles of denaturation at 95ºC for 15s, and annealing at 60ºC for 1 min. The dissociation stage

(melting curve) was performed with one cycle at 95ºC for 15s, 60ºC for 15s, and 95ºC for 15s.

Analysis of the amplification was carried out with ABI 7900HT sequence detection system (SDS

version 2.3) software. To determine the specificity of the PCR assay, the dissociation curve was

assessed for each sample; the expected single peak was verified present in both pCR4-TOPO-

*Ra*17kDa*/ß*actin and pCR4-TOPO-*Ra*17kDa*/IS*E6Crt and gDNA wells and absent in the water

(negative control) samples. The baseline and threshold were optimized for each gene to

maximize efficiency. The rickettsial burden inside the cell was expressed as 17-kDa DNA copies

per 10^6 host cells (*ß*actin or *IS*E6Crt) as previously described (Xu et al., 2006).

3.2.7. Statistical Analysis

The normalized average of the quantity of rickettsiae of the triplicates of each treatment

were entered in the SAS statistical package (version 9.1.3) to be logarithmic transformed and

finally used to determine the ratio of rickettisae per host cells. The influences of the ratio,

treatments, and time points were accessed using GLM procedure in an analysis of variance

(ANOVA). Enumeration data presented are from one of two qPCR assays with similar results.

Tukey's honestly significant difference (HSD) post hoc test was used to examine pairwise

differences of means of main effects while differences within each main effect was assessed

using least squares means (LSM). *F*-test was used to compare infectivity among cell lines. $P <$

0.05 (one-tailed distribution) was considered significant different.

3.3. Results

3.3.1. Rickettsial Infectivity in Host Cells

Three hours post-inoculation (0 hours) medium in both infected cells lines (Vero and
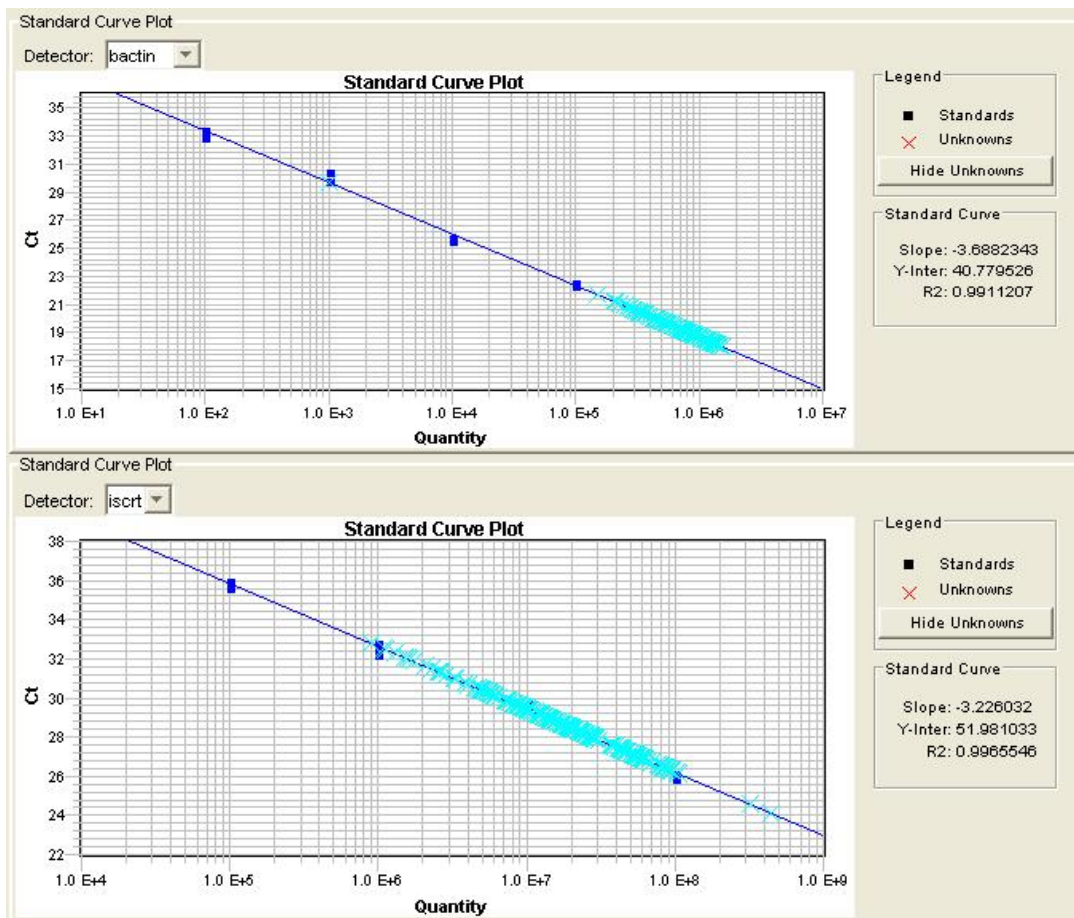
Figure 14. Serial dilutions of both host genes used to quantify rickettsiae in mammalian and tick cell lines.

ISE6) was replaced by the respective fresh medium before the collection of the first time point. For each Diff-Quik® stained slide, infectivity was assessed by counting one hundred cells in two separate measurements and both procedures were performed by the same individual (A.S.Z.). The averages as well as the standard deviation (S.D.) of both percentages are depicted in Figure 15.

Considerable variability in the number of infected cells was observed 3 hours post-inoculation. In this time point, rickettsial infection was greater in Vero compared with ISE6 cells. Infectivity in *R. amblyommii*- and *R. parkeri*-infected Vero cells was slightly comparable (50% and 55%, respectively, $P > 0.05$, *f*-test), but significantly greater than the observed in Vero cells infected with *R. montanensis* (42%, $P < 0.05$, *f*-test). Likewise, the number of infected ISE6 cells was comparable in both *R. amblyommii*- and *R. montanensis*-infected cells (6% and 10%, respectively, $P > 0.05$, *f*-test), but statistically lower than the observed in cells infected with *R. parkeri* (22%, $P < 0.05$, *f*-test) (Figure 15). Twenty-four hours post-inoculation, variability in the number of infected cells changed in both cell lines. Infectivity increased 1.82-fold in ISE6-infected cells compared with 1.67-fold in Vero cells, but the absolute percentage of rickettsiae infection remained greater in Vero cells (3.44-fold greater than the observed in ISE6 for the same time point). The number of *R. amblyommii*- and *R. parkeri*-infected ISE6 cells increased together 1.89-fold (25% and 29%, respectively) when compared with the previous time point (0 hours). When rickettsiae were compared individually, a greater increase in the number of infected ISE6 cells 24 hours post-inoculation was observed in *R. amblyommii* (3.84-fold), followed by *R. montanensis* (1.71-fold; 18%) and *R. parkeri* (1.28-fold), but the accounted increase remained statistically comparable within each *Rickettsia* species ($P > 0.05$, *f*-test).

Figure 15. Comparison of infectivity of *R. amblyommii, R. montanensis* and *R. parkeri* in Vero and ISE6 cells throughout *in vitro* experiment within each time point (hours). Significant difference within each time point for each *Rickettsia* species and cell line is respectively expressed as different uppercase letters, *F-test* ($P \leq 0.05$).
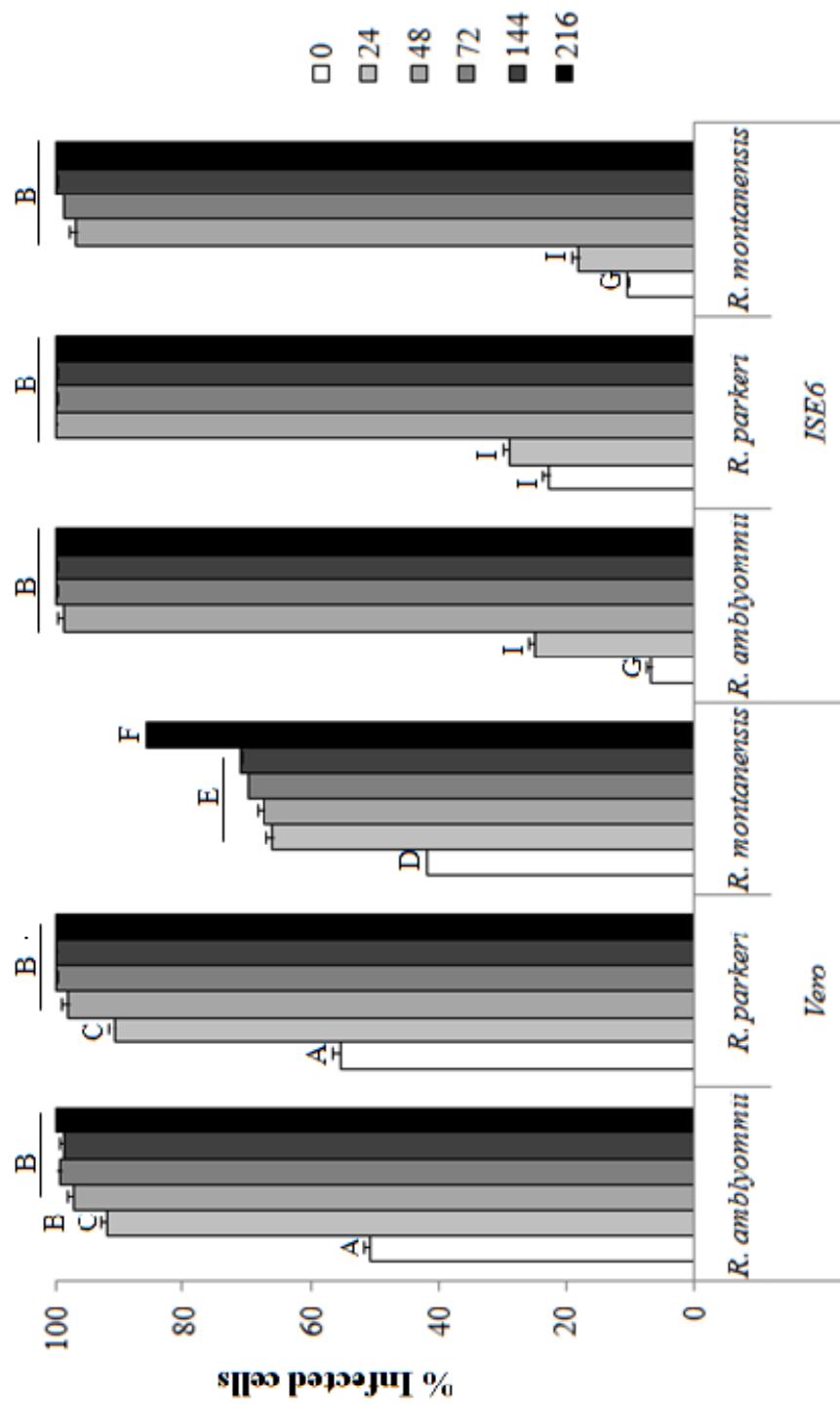
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Likewise, infectivity in *R. amblyommii*- and *R. parkeri*-infected Vero cells increased together 1.73-fold (92% and 90%, respectively) after 24 hours of experiment, and, individually, a greater increase in the number of infected Vero cells was observed in *R. amblyommii* (1.81-fold), followed by *R. parkeri* (1.63-fold) and *R. montanensis* (1.57-fold; 66%). The percentage of infected ISE6 cells didn't differ between *R. amblyommi* and *R. parkeri*, but they were statistically greater than the observed in ISE6 cells infected with *R. montanensis* ($P < 0.05$, *f*-test) (Figure 15). After 48 hours of inoculation, infectivity reached almost the highest level in both cell lines, with the exception of *R. montanensis*-infected Vero cells. At this time point, the number of *R. montanensis* in Vero cells increased only 1-fold (67%), remaining relatively comparable for the next 96 hours of experiment, before significantly increase 1.28-fold (86%) 216 hours post-inoculation ($P < 0.05$, *f*-test). Infectivity increased 4.11-fold in ISE6-infected cells compared with 1.05-fold in Vero cells during the next 24 hours, but no differences in the percentage of infected cells were observed among the others rickettsiae species throughout the rest of the experiment. Individual comparisons of infectivity in both cell lines 48 hours post-inoculation revealed that in infected ISE6 cells a greater increase during these 24 hours of interval was observed in *R. montanensis* (5.38-fold; 97%), followed by *R. amblyommii* (3.96-fold; 99%) and *R. parkeri* (3.44-fold; 100%). Similarly, when assessing the number of infected Vero cells 48 hours post-inoculation compared with the past 24 hours of experiment, a greater increase was observed in *R. parkeri* (1.08-fold; 98%), followed by *R. amblyommii* (1.05-fold; 97%) and *R. montanensis* (1.01-fold; 67%).

3.3.2. Rickettsial Growth in Atypical Host cells

Rickettsial quantity didn't significantly differ within the variables assessed throughout the experiment. Averages of large samples sizes coupled with the fluctuation of the 17-kDa DNA quantity in each *Rickettsia* species or time points revealed not significantly different ($P > 0.05$,

HSD). Exception for this rule, was observed only in ISE6 cells 144 hours post-inoculation ($P =$ 0.0149, LSM). An overall average of 3.0 X $10^{10} \pm$ 8.7 X 10^9 copies of rickettsiae was enumerated in this time point. Such greater quantity turned to be a reflex of the increased *R. parkeri* copy number (9.0 X $10^{10} \pm$ 2.7 X 10^{11}) after 144 hours of experiment ($P = 0.0029$, LSM).

A summary of the other quantities for each *Rickettsia* species per hours post-inoculation and cell lines is illustrated in Tables 5. In addition, we also performed the logarithmic transformation of the mean copy number of SFGR in normalized host cells and this data is depicted in Table 6.

In general, the average quantity of rickettsiae in ISE6-infected cells (5.1 X $10^9 \pm$ 3.5 X 10^9) was 3,791-fold greater than the overall quantity in Vero cells infected with SFGR (1.3 X 10^6 $\pm$ 3.5 X 10^9), however the variation within all 162 samples computed for each cell line revealed that the differences within these numbers were not statistically relevant ($P > 0.05$, HSD). Likewise, in general, *R. parkeri* copy number (7.5 X $10^9 \pm$ 4.3 X 10^9) was 495.4-fold greater than *R. montanensis* (1.5 X $10^7 \pm$ 4.3 X 10^9) and 91.8-fold greater than *R. amblyommii* (8.2 X $10^7 \pm$ 4.3 X 10^9) as well as *R. amblyommii* copy number was 5.39-fold greater than *R. montanensis*, but fluctuation in the quantity of the 17-kDa gene throughout the experiment in all 108 samples related with each *Rickettsia* species revealed that the differences within these copy numbers of rickettsial gene were not significant ($P > 0.05$, HSD). Finally, no statistical differences in the copy number of rickettsiae were either observed per time points throughout the *in vitro* experiment. The overall quantity mean was the greatest at 144 hours (1.5 X $10^{10} \pm$ 6.1 X 10^9), followed by 72 (1.8 X $10^8 \pm$ 6.1 X 10^9), 3 hours post-inoculation (1.5 X $10^7 \pm$ 6.1 X 10^9, 0 hours), 24 (7.7 X $10^6 \pm$ 6.1 X 10^9), 48 (7.4 X $10^6 \pm$ 6.1 X 10^9) and 216 hours post-inoculation (3.2 X 10^6 $\pm$ 6.1 X 10^9).

Table 5. Mean copy number ± S.E.M. of *R. parkeri*, *R. montanensis* and *R. amblyommii* in ISE6 and Vero infected cells from 3 hours (0 hours) until 216 hours post-incubation.

Time points	Mean copy no. ± SEM					
	ISE6			Vero		
	R. parkeri	*R. montanensis*	*R. amblyommii*	*R. parkeri*	*R. montanensis*	*R. amblyommii*
0	$4.9 \times 10^7 \pm 1.2 \times 10^8$	$1.1 \times 10^5 \pm 1.7 \times 10^5$	$3.9 \times 10^7 \pm 1.1 \times 10^8$	$1.3 \times 10^6 \pm 1.6 \times 10^6$	$1.6 \times 10^1 \pm 1.4 \times 10^1$	$1.2 \times 10^4 \pm 3.7 \times 10^4$
24	$2.6 \times 10^7 \pm 4.5 \times 10^7$	$2.3 \times 10^5 \pm 3.3 \times 10^5$	$1.8 \times 10^7 \pm 5.6 \times 10^7$	$1.3 \times 10^6 \pm 1.6 \times 10^6$	$2.6 \times 10^1 \pm 2.9 \times 10^1$	$1.2 \times 10^4 \pm 3.7 \times 10^4$
48	$4.3 \times 10^7 \pm 9.3 \times 10^7$	$1.4 \times 10^5 \pm 1.9 \times 10^5$	$8.8 \times 10^3 \pm 9.9 \times 10^3$	$1.4 \times 10^6 \pm 1.6 \times 10^6$	$2.2 \times 10^1 \pm 2.9 \times 10^1$	$1.5 \times 10^2 \pm 7.4 \times 10^1$
72	$1.6 \times 10^7 \pm 2.4 \times 10^7$	$1.8 \times 10^8 \pm 5.4 \times 10^8$	$9.2 \times 10^8 \pm 2.8 \times 10^9$	$6.2 \times 10^6 \pm 1.0 \times 10^7$	$2.1 \times 10^1 \pm 1.8 \times 10^1$	$9.6 \times 10^1 \pm 8.9 \times 10^1$
144	$9.0 \times 10^{10}\dagger \pm 2.7 \times 10^{11}$	$5.1 \times 10^4 \pm 1.0 \times 10^5$	$2.3 \times 10^5 \pm 3.5 \times 10^5$	$6.5 \times 10^6 \pm 1.0 \times 10^7$	$2.8 \times 10^1 \pm 1.7 \times 10^1$	$8.4 \times 10^1 \pm 6.8 \times 10^1$
216	$1.2 \times 10^7 \pm 1.7 \times 10^7$	$5.6 \times 10^4 \pm 1.2 \times 10^5$	$2.3 \times 10^5 \pm 3.6 \times 10^5$	$7.4 \times 10^6 \pm 1.1 \times 10^7$	$2.5 \times 10^1 \pm 1.9 \times 10^1$	$9.6 \times 10^1 \pm 8.9 \times 10^1$

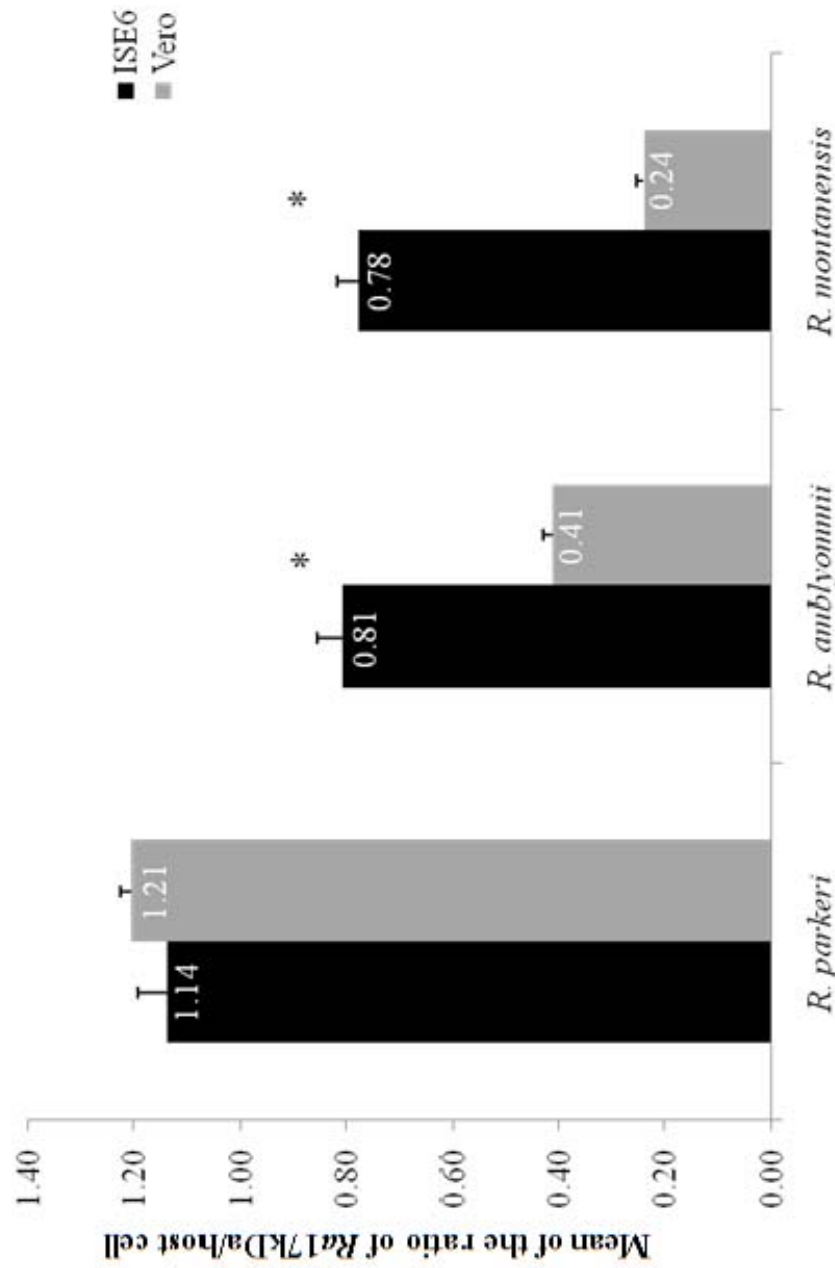† Significant different (*P* < 0.05; LSM).

Significant differences in the kinetics of rickettsiae in both Vero and ISE6 cells were observed only after the calculation of the ratio of rickettsiae per host cell in the normalized data. Generally, the ratio of rickettsiae per ISE6-infected cells (0.91 ± 0.021) was 1.4-fold significantly greater than in infected Vero cells (0.62 ± 0.021) ($P < 0.05$, HSD). Throughout the experiment, the ratio of *R. parkeri* per host cell (1.17 ± 0.026) was 2.34-fold greater than the ratio in *R. montanensis* (0.50 ± 0.026) and 1.95-fold greater than the ratio in *R. amblyommii* (0.61 ± 0.026) while the ratio of *R. amblyommii* per host cell was only 1.22-fold significantly greater than in *R. montanensis* ($P < 0.05$, HSD). Considering the ratio of rickettisae per host cell in individual cell lines (Figure 16), the ratio of *R. amblyommii* and *R. montanensis* per ISE6 cell was, respectively, 1.91- and 3.34-fold greater than in Vero cell ($P < 0.0001$, LSM) and no significant difference was observed in the ratio of *R. parkeri* per host cell ($P = 0.199$, LSM). Under multiple comparisons, the mean of the ratio of *R. parkeri* in both cell lines (1.17) was significantly greater than this ratio in all other rickettsiae ($P < 0.0001$, LSM). Interestingly, the ratio of *R. amblyommii* was 1.71-fold greater than the ratio of *R. montanensis* per Vero cell ($P = 0.001$, LSM), but no statistical differences ($P = 0.5604$, LSM) were observed in the ratio of these two *Rickettsia* species per ISE6-infected cell (Figure 16). Differences in the ratio of SFGR per host cell, but not within individual cell lines, were also observed throughout the *in vitro* study (Figure 17). For each time point the mean of the ratio of rickettsiae per ISE6 cell (0.90 ± 0.03) was in average 1.4-fold greater ($P < 0.0001$, LSM) than the mean of the ratio of rickettsiae per Vero-infected cells (0.61 ± 0.01).

The ratio of *R. parkeri* per ISE6 and Vero cells didn't differ within the 216 hours post-inoculation, but was significantly greater than the ratio observed in both *R. amblyommii* and *R. montanensis* ($P < 0.0001$, LSM). Considering the ratio of those last two *Rickettsia* species per ISE6 and Vero cells their ratio fluctuated within comparable levels throughout the *in vitro* study

Table 6. Logarithmic transformation of the mean copy number ± S.E.M. of *R. parkeri*, *R. montanensis* and *R. amblyommii* in ISE6 and Vero infected cells from 3 hours (0 hours) until 216 hours post-inoculation.

Log Mean Copy no. ± SEM

Time points	ISE6			Vero		
	R. parkeri	*R. montanensis*	*R. amblyommii*	*R. parkeri*	*R. montanensis*	*R. amblyommii*
0	7.69 ± 8.08	5.04 ± 5.23	7.59 ± 8.04	6.11 ± 6.20	1.20 ± 1.15	4.08 ± 4.57
24	7.42 ± 7.65	5.36 ± 5.52	7.26 ± 7.75	6.11 ± 6.20	1.42 ± 1.46	4.08 ± 4.57
48	7.63 ± 7.97	5.15 ± 5.28	3.94 ± 4.00	6.15 ± 6.20	1.34 ± 1.46	2.18 ± 1.87
72	7.20 ± 7.38	8.26 ± 8.73	8.96 ± 9.45	6.79 ± 7.00	1.32 ± 1.26	1.98 ± 1.95
144	10.95† ± 11.43	4.71 ± 5.00	5.36 ± 5.54	6.81 ± 7.00	1.45 ± 1.23	1.92 ± 1.83
216	7.08 ± 7.23	4.75 ± 5.08	5.36 ± 5.56	6.87 ± 7.04	1.40 ± 1.28	1.98 ± 1.95

†Significant different ($P < 0.05$; LSM)

Figure 16. Overall mean of the ratio of rickettsiae per host cell (ISE6 or Vero) throughout the *in vitro* study. *Significant difference between cell lines infected with the same *Rickettsia* ($P < 0.01$, LSM).
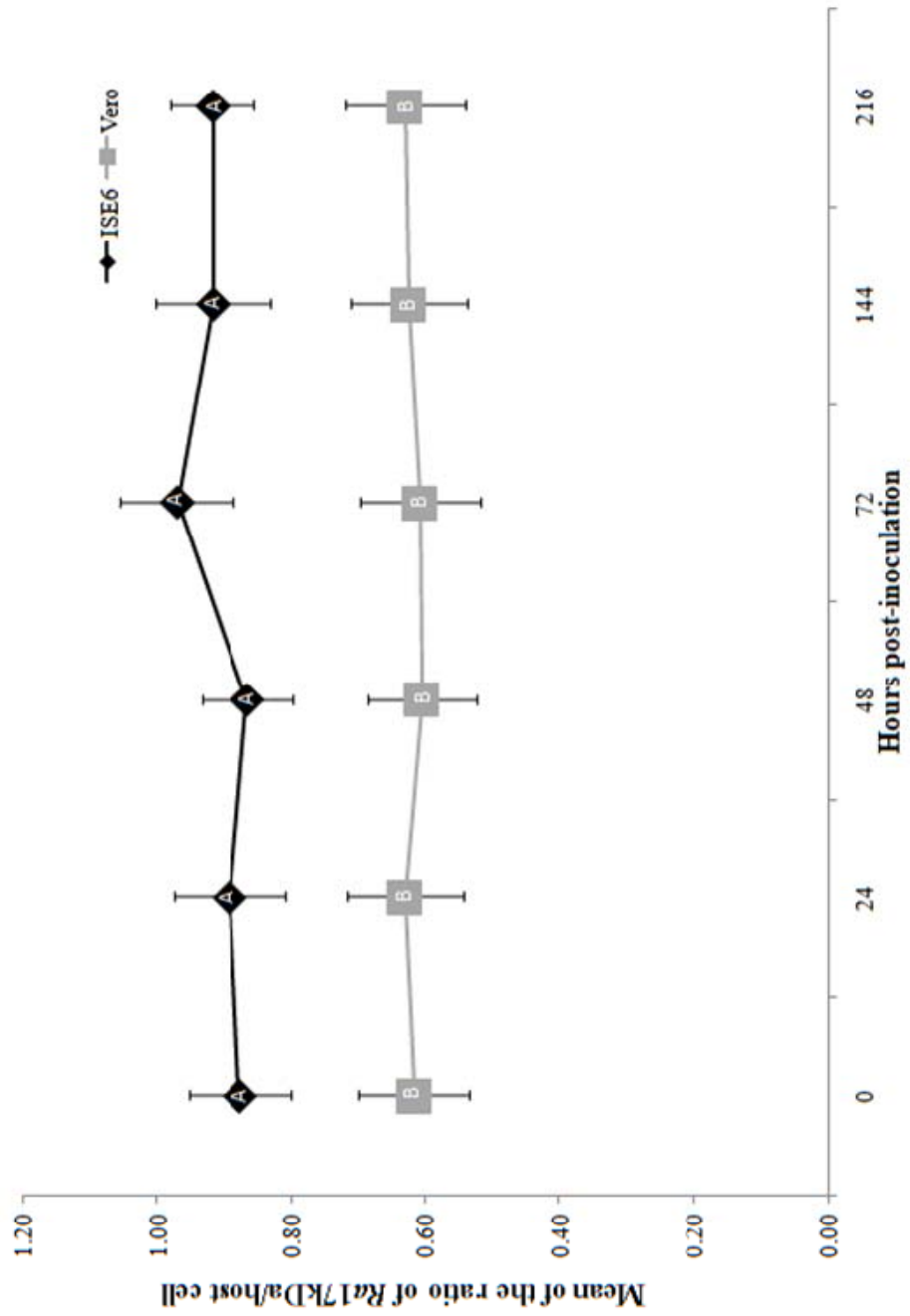
Figure 17. Growth of rickettsiae in Vero and ISE6 cells within each time point (hours post-inoculation). Means with the same letter inside the marker are not significantly different ($P > 0.05$; LSM).

in ISE6 cells ($P = 0.1$, LSM), but slightly differ within the first 48 hours in Vero cells ($P < 0.05$, LSM) (Figures 18 and 19). Throughout each time point no differences in the ratio of *R. parkeri* per host cell were observed when replication in individual cell lines was compared ($P \geq 0.311$, LSM). Variation in the replication when both cell lines were compared within each time point was observed only in cells infected with *R. amblyommii* and *R. montanensis* ($P \leq 0.0498$, LSM). For each of those last two *Rickettsia* species the ratio of *Rickettsia* per host cell ranged from 0.67 – 0.94, 0.36 – 0.48 in *R. amblyommii*- and 0.70 – 0.81, 0.21 – 0.27 in *R. montanensis*-infected ISE6 and Vero cells, respectively. The ratio of *R. amblyommii* in ISE6 cells was, on average, 2-fold (0.39 ± 0.12) greater than in Vero cells. Likewise, in *R. montanensis*-infected ISE6 cells, on average, the ratio of *Rickettsia* per cell was 3.38-fold (0.54 ± 0.06) greater than in Vero cells. Additional, comparisons of the rickettsiae replication in logarithmic transformed samples revealed that in normalized cells the mean copy number of rickettsiae remained the same in most of the samples throughout the *in vitro* study ($P > 0.05$, LSM – Table 7). Compared to time point 0 (3 hours post-inoculation) increases in the replication of rickettsiae was observed only after 72 hours of experiment in ISE6 cells infected with *R. montanensis* (63.7 % of increase in rickettsiae replication; $P < 0.05$, LSM), while decreases in the replication of rickettsiae could be observed only in *R. amblyommii*-infected cells, particularly, in ISE6 cells 48 hours post-inoculation (5 % of decrease in rickettsiae replication) and then at time point 144 throughout the remaining hours of experiment (70.6 % of decrease in rickettsiae replication; $P < 0.05$, LSM). Regarding the replication of *R. amblyommii* in Vero cells, replication decreased after 48 hours (53.3 % of decrease in rickettsiae replication; $P < 0.05$, LSM) and the copy number of *Rickettsia* gene remained statistically lower than the copy number observed 3 hours post-inoculation (0 hours) throughout the rest of the time points (Table 7).

3.3.3. Ultrastructural Comparison of Infected Cell Lines

Analyses of the TEM 216 hours post-inoculation revealed differences in the ultrastructural morphology of the cells infected with *R. amblyommii* (Figure 20 A and B) and *R. montanensis (*Figure 20 E and F) only. For both cell lines, *R. parkeri* infection resulted in cell death because only cell debris can be seen in both host cells infected with this SFGR 216 hours post-inoculation (Figure 20 C and D).

The ultrastructural architecture of Vero cells infected with *R. amblyommii* and *R. montanensis* retained its integrity after 216 hours of experiment (Figure 20 A and E), but when both *Rickettsia* species were inoculated in ISE6 cells, vacuolization of the cytoplasm can be seen in cells infected with *R. amblyommii* (Figure 20 B) and only cell debris resultant from cell death can be appreciated in *R. montanensis*-infected cells (Figure 20 F).

3. Discussion

Among SFGR, it is fascinating to note the wide range of possible tick and rickettsiae associations. Some SFGR can be detected in several different tick genera (Burgdorfer et al., 1975; Philip and Casper, 1981; Philip et al., 1981; Magnarelli et al., 1982; Gordon et al., 1984; Labruna e t al., 2004b; Parola et al., 2005; Demma et al., 2005; Labruna et al., 2005; Horta et al., 2006; Pinter et al., 2006; Labruna et al., 2007a; 2007b; Wikswo et al., 2008), while *R. peacockii*, has been only isolated from the ovaries of *D. andersoni* (Burgdorfer and Brinton, 1975; Niebylski et al., 1997). Within these extremities, other SFGR have also been observed to infect different tick species within the same tick genus (Brumpt, 1932; Parker et al., 1939; Bell et al., 1963; Burgdorfer et al., 1973; Burgdorfer et al., 1981b; Kelly and Mason, 1991; Eremeeva et al., 1994; Goddard et al 2003; Labruna et al., 2004a; 2004b; Parola et al., 2007; Labruna et al., 2007b;Pac heco et al 2007; Silveira et al 2007; Venzal et al 2008a; 2008b). Because limited research has been conducted to understand the mechanism behind these specific types of

Table 7. Rickettsiae replication (percentage of change) in normalized host cells within each time point after comparison of the log mean copy number of SFGR 3 hours (0 hours) post-inoculation.

Cell lines	Rickettsia spp	Time points (h) Replication (% of change)					
		0	24	48	72	144	216
ISE6	R. parkeri	1.00 (100)	0.96 (96.4)	0.99 (99.3)	0.94 (93.7)	1.42 (142.4)	0.92 (92.1)
	R. montanensis	1.00 (100)	1.06(106.4)	1.02 (102.1)	1.64 (163.7)†	0.93 (93.4)	0.94 (94.2)
	R. amblyommii	1.00 (100)	0.96 (95.6)	0.52 (52)†	1.18 (118.1)	0.71 (70.6)†	0.71 (70.6)†
Vero	R. parkeri	1.00 (100)	1.00 (100)	1.01 (100.5)	1.11 (111.1)	1.11 (111.4)	1.12 (112.4)
	R. montanensis	1.00 (100)	1.18 (117.5)	1.14 (114.2)	1.10 (109.8)	1.20 (120.2)	1.16 (116.1)
	R. amblyommii	1.00 (100)	1.00 (100)	0.53 (53.3)†	0.49 (48.6)†	0.47 (47.2)†	0.49 (48.6)†

†Significant different ($P < 0.05$; LSM)
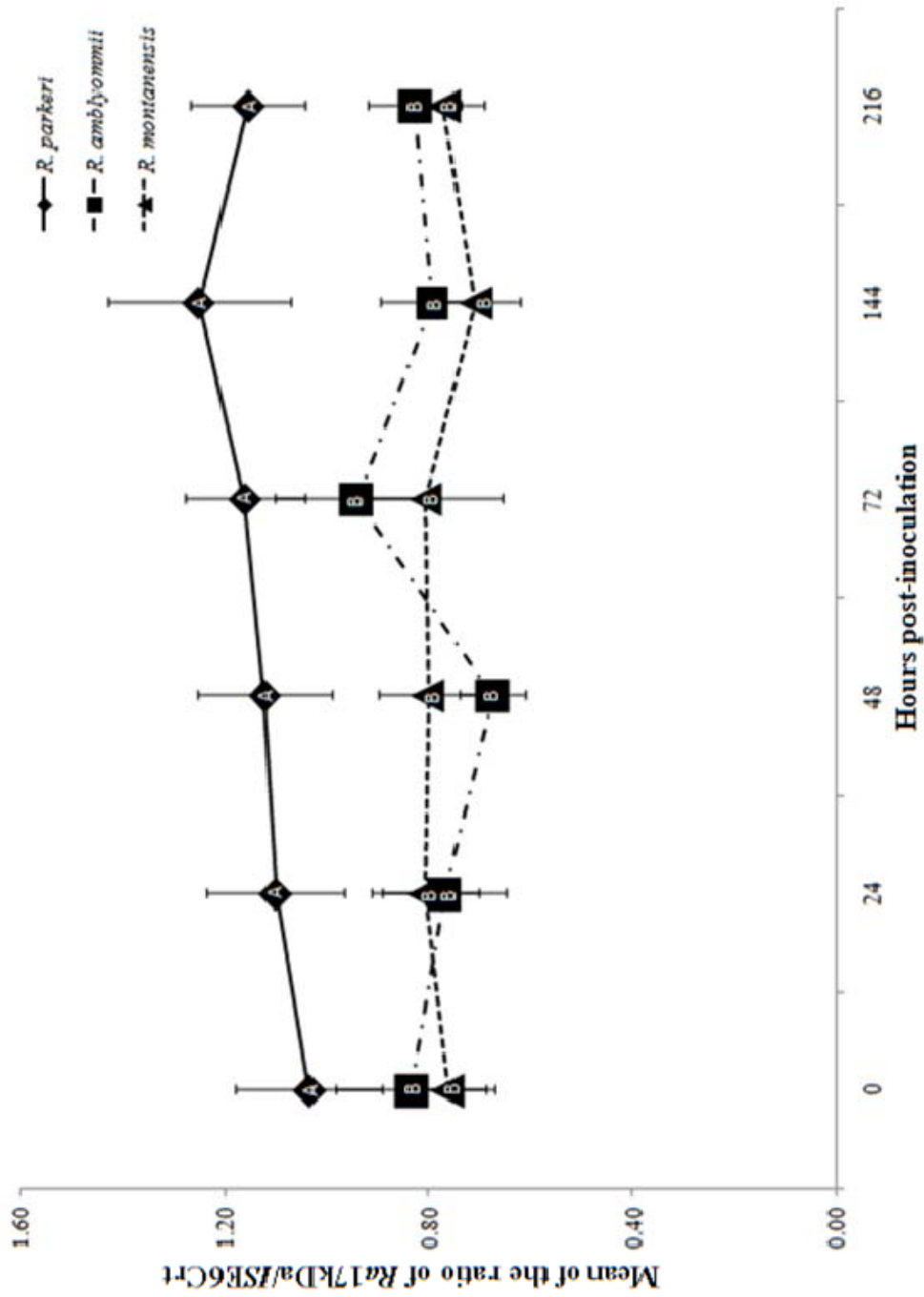
Figure 18. Growth of rickettsiae in ISE6 cells within each time point (hours post-inoculation). Means with the same letter inside the marker are not significantly different ($P > 0.05$; LSM).
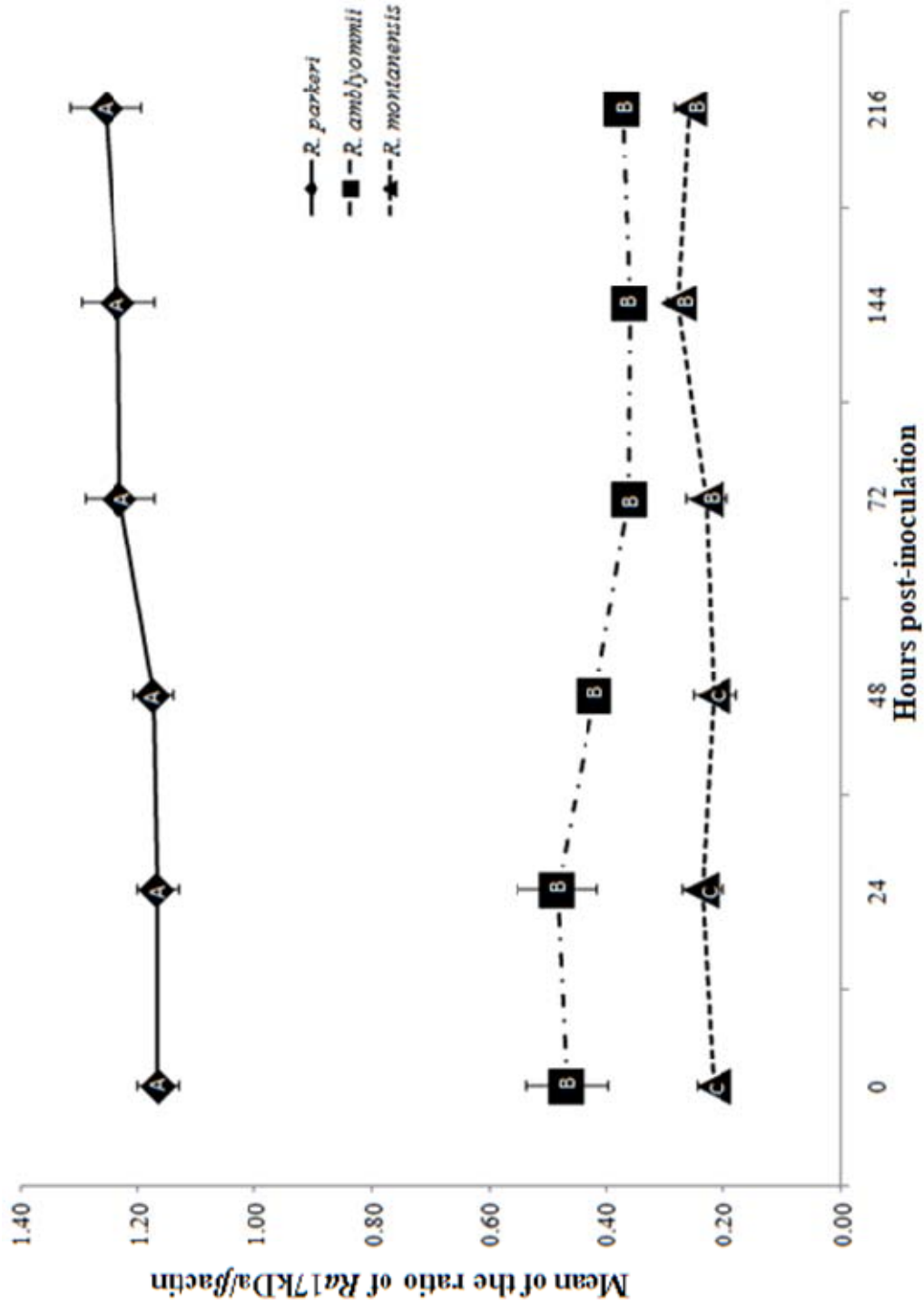
Figure 19. Growth of rickettsiae in Vero cells within each time point (hours post-inoculation). Means with the same letter inside the marker are not significantly different ($P > 0.05$; LSM).
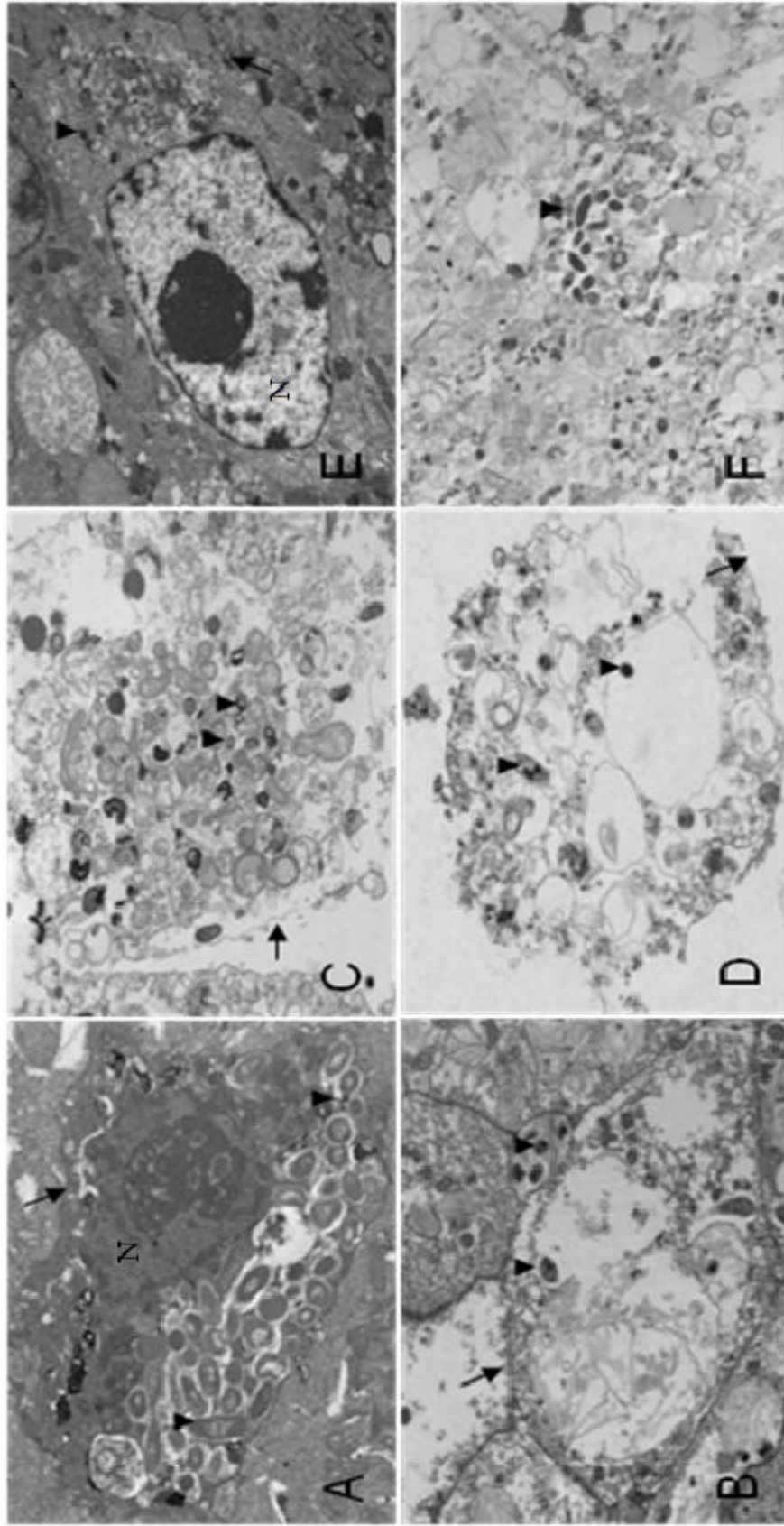
Figure 20. Transmission electron micrographs of Vero (A, C and E) and ISE6 cells (B, D and F) infected with *R. amblyommii* (A and B), *R. parkeri* (C and D) and *R. montanensis* (E and F). Arrows indicate cell membrane, arrows heads indicate microorganisms and (N) indicate nucleus; 10.000X.

associations, in this preliminary study, we established an *in vitro* model to investigate if replication is also associated with specificity in the tick-SFGR interaction. Mammalian (Vero) and tick (ISE6) cell lines were challenged in a reciprocal fashion with SFGR naturally associated with *Amblyomma* (*R. parkeri* and *R. amblyommii*) and *Dermacentor* (*R. montanesis*) ticks and the replication patterns of these SFGR in those host cells were assessed by qPCR, Cytospin®-stained slides of infected cells and transmission electron micrographs.

R. parkeri is the second most important tick-transmitted spotted fever in the Western Hemisphere (Bechah et al., 2008). It was first isolated in 1937 from *A. maculatum* ticks found on cattle in the Gulf Coast region of Texas (Parker et al., 1939). Once believed to be naturally maintained in a single tick species (Parker et al., 1939), *A. dubitatum* and *A. cajennense* (Pacheco et al., 2007) and *A. triste* (Silveira et al., 2007; Venzal et al., 2008a; 2008b), are now associated with *R. parkeri* infection in Latin America countries, such as Uruguay and Brazil. *R. montanensis* (formerly *R. montana*) is a SFGR of unrecognized pathogenicity and was first isolated from *D. variabilis* and *D. andersoni* ticks in eastern Montana (Bell et al., 1963). In the past 45 years, the presence of *R. montanensis* has been also reported in naturally infected *Dermacentor* ticks collected in several different United States states (Feng et al., 1980; Anderson et al., 1986; Breitschwerdt et al., 1988; Pretzman et al., 1990; Ammerman et al., 2004). The WB-8-2 agent was first isolated, at high levels, in 1974 during an unsuccessful attempt to associate *A. americanum* ticks with *R. rickettsii* infections in field-collected ticks from Arkansas, South Carolina and Tennessee (Burgdorfer et al., 1981b). Subsequently, molecular analysis of a SFGR isolated from field-collected *A. americanum* ticks (MOAa) in Missouri (Weller et al., 1998) was found to be closely related with WB-8-2. In fact, both WB-8-2 and MOAa strains are believed to represent the same SFGR (Paddock and Yabsley, 2007). The most comprehensive survey examining infection of *A. americanum* with *R. amblyommii* was performed between 1998 and

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2005 in nine states (Mixson et al., 2006). *R. amblyommii* has also been identified in other

countries, particularly in Brazil, Argentina and French Guyana where *A. cajennese* (Labruna et

al., 2004b); *Amblyomma coelebs* (Labruna et al., 2004a; Parola et al., 2007), and *A. neumanni*

(Labruna et al., 2007b) are the major vectors. Considering the pathogenic nature of this

microorganism, a lot of speculation based on serological evidences (Dasch et al., 1993; Sanchez

et al.,1992; Labruna et al., 2007b; Apperson et al., 2008; Saito et al., 2008), epidemiological

studies (Mixson et al., 2006) and molecular analyses (Billeter et al., 2007) suggest that this

SFGR might be the cause of mild-illness in humans, however the lack of classical RMSF clinical

signs (Sanchez et al.,1992), and the inability to replicate the infection in animal models

(Burgdorfer et al., 1981b) coupled with the failure to isolate the bacterium from human patients

previously exposed to infected *A. americanum* ticks (Billeter et al., 2007), make the inclusion of

this *Rickettsia* in the group of tick-transmitted spotted fever difficult (Walker and Ismail, 2008).

Therefore, to the best of our knowledge *R. amblyommii* is still considered as a SFGR of

unrecognized pathogenicity.

In the literature, *R. parkeri*, *R. montanensis* and *R. amblyommii* have been maintained

mainly in Vero cells (Macaluso et al., 2001; Labruna et al., 2004b; Sangioni et al., 2005) while

ISE6 cells have been utilized to study infectivity of the human granulocytic ehrlichiosis agents

and to characterize the growth of *Rickettsia felis* (Munderloh et al., 1999; Pornwiroon et al.,

2006; Sunyakumthorn et al., 2008). Even though *R. amblyommii* was brought to our laboratory in

ISE6-infected cells, no previous reports have documented the use of this tick cell line to maintain

R. parkeri and *R. montanensis* infections.

In this study, rickettsiae were inoculated at equal amounts in different host cells in a ratio

of 0.78 *Rickettsia*/host cell in accordance with the overall finds of the mean of ratio of *glt*A/MIF

(0.71) and *Ra*17kDa/*Aa*MIF (0.78) in adult *A. americanum* ticks reported in previous studies (Zhong et al., 2007; Zanetti et al., 2008).

The first data collection was performed 3 hours post-inoculation after we changed the medium (0 hours), suggesting that, in this study, the establishment of rickettsial infection in Vero cells occurs faster than in ISE6 cells, because the number of infected cells was significantly greater in Vero-infected cells during the first 24 hours of experiment, however when the ratio of *Ra*17kDa/*ß*actin is compared with the ratio of *Ra*17kDa/*IS*Crt during the first hours post-inoculation considerable variability is observed in *R. amblyommi* and *R. montanensis* infected cells, because only an average of 0.31 *Ra*17kDa/*ß*actin were observed during the first 3 hours of experiment, which for instance, was lower than the average ratio observed for the same rickettsiae species at the same time point in ISE6-infected cells (0.80), indicating, therefore, that for those two *Rickettisa* species infection might occur first in Vero cells, but replication inside the cells is usually 2.58-fold greater in ISE6 cells infected with *R. amblyommii* and *R. montanensis*.

The qPCR assay generated in this study, demonstrated to be highly efficacious to quantiate single copy genes of both *Rickettsia* (Zanetti et al., 2008) and host cells (Ohno et al., 2003; Xu et al., 2005). The quantity for *ß*actin gene ranged from 10^5 to 10^6 copies, while the quantity for *IS*Crt gene ranged from 10^6 to 10^9 copies. Considering the enumeration of the *Ra*17kDa gene, quantity ranged from 10 to 10^6 copies in Vero cells, and 10^3 to 10^{10} copies in ISE6 cells. According to previous reports, the number of copies of SFGR in Vero cells was an average of 10^6 copies of *Omp*A gene, slighltly compared with the quantity presented in our study (Eremeeva et al., 2003). Conversely, the number of *Ra*17kDa copies observed in ISE6 cells infected with SFGR was literally 10^6-fold greater than the average quantity observed in *A. americanum* eggs infected with *R. amblyommii* (Zanetti et al., 2008).

In this study, our major goal was to investigate the replication patterns of different SFGR in atypical host cells; therefore, we normalized the number of cells used to calculate the ratio of *Rickettsia*/host cell throughout the *in vitro* experiment (Xu et al., 2006). Rickettsial regulation upon persistent host cell infection has been demonstrated in tick host cells. The growth of *D. andersoni* cells slows in *R. peacockii*-constitutively infected cells (Kurtti et al., 2005). Changes in the rickettsial conformation have been observed in ISE6 cells infected with *R. felis*, with minimal implications to the survival of infected cells (Sunyakumthorn et al., 2008). Throughout the *in vitro* experiment, the ratio of *Rickettsia* per host cell considerably differs within cell lines, especially in cells infected with *R. amblyommii* and *R. montanensis*. No differences were observed between *R. parkeri*-infected cells, but it is interesting to note that the ratio of *R. amblyommii* and *R. montanensis* per Vero cell was significantly lower than that observed in ISE cells. In normalized cells, fluctuation in the replication rates of these two SFGR when compared with the seeding quantity (3 hours post-inoculation) could be related with the interactions and the citophathic effects of those SFGR in different host cells but further investigations should be the performed to fully address this assumption. *A. americanum* eggs infected with *R. amblyommii* were observed to have a ratio of *Ra*17kDa/*Aa*MIF 1.3-fold lower than the average observed in ISE6 cells infected with both *R. amblyommii* and *R. montanensis* (0.79), and 1.35-fold lower than the ratio of *R. amblyommii* per ISE6 cells alone (Zanetti et al., 2008), indicating, therefore, that *R. amblyommii* can alter its replication patterns in different embryonic tick cells.

The *in vitro* model presented here is a useful method to investigate host specificity among SFGR. The possibility to assess rickettisal replication in different embryonic tick cell lines could offer important clues for the understanding of SFGR transmission to post-embryonic tick stages and the high rates of mortality observed in tick vectors related with some *Rickettsia* species

(Niebylski et al., 1999). The ultrastructural characteristics observed after 216 hours post-inoculation have a valuable importance to understand the character pathogenic and SFGR-host specificity. As a recognized pathogenic SFGR, *R. parkeri* infection was highly critical to the survival of both cell lines, indicating, therefore, that cell death is a consequence of infection in mammalian cells, while the mortality of the ISE6 cells observed after 216 hours of experiment in all SFGR investigated remains to be elucidated because the expansion of this model to other tick embryonic cell lines must be performed to fully characterize the different patterns of infection in SFGR.

Chapter 4. Conclusions

In order to one understand the roles that pathogenic and nonpathogenic SFGR play within the tick host in relation to the epidemiology of tick-borne rickettsioses, the mechanisms of rickettsial infection within the tick must first be characterized. In this study, a qPCR assay was used as a tool to investigate the tick-SFGR interactions by accessing the ratio of *Rickettsia* per tick cell during tick feeding events and in atypical host cells. With these studies, we were basically interested to test if (1) the ratio of *Rickettsia* per tick cell during tick feeding and transovarial/transtadial transmissions in *A. americanum* ticks infected with unrecognized rickettsiae is directly correlated to the significance of the horizontal versus vertical route of transmission in SFGR; and if (2) the specificity of associations among rickettsiae and a particular tick species is correlated with replication under laboratory conditions.

The significance of these studies is that quantitative real-time PCR (qPCR) was successfully used to quantitate the replication of *Rickettsia* in naturally infected *A. americanum* ticks and in both mammalian and tick cell lines. Particularly, these qPCR assays were demonstrated to be efficient to assess the rickettsial burden inside the cell in *A. americanum* tissues during tick feeding and vertical transmission; and in Vero and ISE6 cells during rickettsial infection.

The internal-control plasmids generated to quantify the gene copy numbers of both rickettsiae (17-kDa antigen gene) and host cells (*Aa*MIF, *IS*E6Crt, and *ß*actin genes) were validated in these studies, therefore, these assays yielded the enumeration of as low as 10 copies of each *Rickettsia* species and host cells genes.

R. amblyommii infection in naturally infected *A. americanum* ticks was observed to remain relatively constant and at lower levels during blood acquisition and in post-embryonic tick life stages. In general, the ratio of *R. amblyommii* 17-kDa copy number per *A. americanum*

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gene (*AaMIF*) in adult female ticks was greater in salivary gland samples, followed by gut and ovary samples, but throughout the entire tick feeding experiment remained lower than one copy of *Rickettsia* per tick cell. Moreover, no significance differences were observed in the ratio of *R. amblyommii* 17-kDa copy number per *A. americanum* gene (*AaMIF*) in the immature stages, suggesting a physiological balance in the transovarial and transtadial transmissions of this SFGR in this tick host.

Considerably variability in the rickettsial replication rates were observed in the *in vitro* experiment developed to assess specificity of SFGR in different host cells. Replication patterns in Vero and ISE6 cells infected with non-pathogenic SFGR (*R. amblyommii* and *R. montanensis*) differed considerably after inoculation, when, reciprocally, compared with each other. However, comparable levels of replication in both cell lines infected with pathogenic *Rickettsia* (*R. parkeri*) were observed throughout the same experiment timeline. Therefore, this preliminary *in vitro* study offers additional information regarding specificity among SFGR.

In summary, qPCR was validated to understand the kinetics of SFGR in different hosts. The expansion of the use of qPCR to characterize the growth of recognized pathogenic SFGR in *A. americanum* ticks and to determinate the replication rates of these SFGR in different tick cell lines, under the same circumstances, could serve as a valuable approach to help elucidate the pathogenic nature of SFGR and to better understand the intimate relationship that some SFGR hold with tick species of different genera.

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Vita

Dr. Andre S. Zanetti was born to Mr. Jose Americo Zanetti and Mrs. Marilene C. Serrano Zanetti in 1980. He was accepted into the School of Veterinary Medicine at Sao Paulo State University (Unesp/Jaboticabal/Brazil) in 2001. After having received his Doctor of Veterinary Medicine degree in 2006 he came to the School of Veterinary Medicine at Louisiana State University in Baton Rouge to work as a D.V.M-postdoc researcher for one year before he was accepted into the Graduate School of the Louisiana State University to pursue his Master of Science degree in veterinary medical sciences in the department of Pathobiological Sciences School of Veterinary Medicine. He has a particular interest in clinical diagnoses and applied medical sciences and he plans to pursue a career as a veterinary clinical pathologist to teach fundamental concepts of clinical pathology to veterinary students and to establish novel methods that will improve our understanding of disease pathology in veterinary as well as in comparative medicine. He is a former water polo player and nowadays his major sport activities are tennis, weight lifting and swimming.