STUDIES OF THE TRANSMISSION ROUTES OF THE \textit{Rickettsia} SYMBIONT IN \textit{Ixodes Pacificus} TICKS BY FLUORESCENT \textit{IN SITU} HYBRIDIZATION AND TRANSMISSION ELECTRON MICROSCOPY

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ABSTRACT

Studies of the Transmission Routes of the *Rickettsia* Symbiont in *Ixodes pacificus* Ticks by Fluorescent in Situ Hybridization and Transmission Electron Microscopy

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*Ixodes pacificus* is a primary vector of Lyme borreliosis and human anaplasmosis to people and animals in the Pacific states of the United States. This tick is also a host for a number of other bacterial species including members of the genus *Rickettsia*. Recently, our laboratory reported two new phylotypes (G021 and G022) of *Rickettsia* in *Ixodes pacificus* ticks based on PCR and sequence analysis. The prevalence, host specificity and biology of interaction of these new phylotypes with its tick host are a focus of analysis in the lab. As part of this overall goal, this study focused on the molecular detection of these new phylotypes of *Rickettsia* in the ovary and midgut of *Ixodes pacificus* ticks by *in situ* hybridization using *Rickettsia*-specific labeled probes and ultrastructural analysis. To identify the ideal medium for generating thin sections of detecting the intracellular bacteria without high fluorescence background, Technovit 8100, Unicryl and paraffin were used in *in situ* hybridization studies. The high background fluorescence of Technovit 8100 and Unicryl made it difficult to detect *Rickettsia* in these sections. Tissue embedded in paraffin gave lower autofluorescence. However, the signal generated by
fluorescently labeled probes was not high enough to generate convincing images. Therefore, Digoxigenin-tagged probes were used for the hybridization and the signal was amplified using Tyramide Signal Amplification reaction. Using this technique, *Rickettsia* bacteria were detected in the ovary of *Ixodes pacificus* ticks. The presence of the *Rickettsia* species in ovary and midgut was further confirmed by transmission electron microscopic analysis and PCR. Overall, the methods in this study can be used to understand transmission routes of this bacterial species in ticks.
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INTRODUCTION

Among the arthropod vectors of human diseases, ticks rank next to mosquitoes. However, ticks are the predominant arthropod vectors of human diseases in temperate regions of the world and the most common arthropod vector in the United States of America (Barbour and Fish, 1993; Nocton and Steer, 1995). Ticks are vectors of a number of different pathogens, including bacteria, viruses and other pathogens (Parola and Rault, 2001; Bratton and Corey, 2005; Mansfield et al., 2009; Randolph, 2011). However, the most common tick-borne diseases are bacterial diseases that include Lyme borreliosis, Rocky Mountain spotted fever, and anaplasmosis. In 2010, more than 22,500 confirmed cases of Lyme borreliosis alone have been reported in the US (http://www.cdc.gov/niosh/topics/tick-borne/). Even though Lyme borreliosis is the most common vector-borne disease in the United States, another major group of human pathogens transmitted by ticks are members of the genus *Rickettsia* (Parola and Rault, 2001).

More than a century ago Ricketts provided proof for the pathogenic basis of Rocky Mountain spotted fever (Ricketts, 1906). In honor of him, the pathogenic bacterium was named *Rickettsia*. This bacterial species belongs to a group of Gram-negative alphaproteobacteria that cannot be cultured in artificial media. These bacteria were divided into three major groups, spotted fever group (SFG), the typhus group (TG) and scrub typhus group (STG) (Raoult and Roux, 1997; Fournier and Raoult, 2009).
However, based on molecular data, the members STG have been reclassified to a separate genus *Orientia* (Tamra et al., 1995).

The members of the SFG are mostly associated with ticks; however, some are associated with fleas and mites. As the name implies, they cause spotted fever in humans. They have been shown to be able to polymerize actin for their motility within the host cells and can be seen inside the nuclei (Burgdorfer et al., 1968; Heinzen et al., 1993; Fournier and Raoult, 2009). The TG on the other hand are usually associated with body lice or fleas and cannot polymerize actin (Heinzen et al., 1993; Fournier and Raoult, 2009).

The term “*Rickettsia*” refers to arthropod-borne bacteria belonging to the genus *Rickettsia* within the family *Rickettsiaceae* in the order *Rickettsiales*, of the alphaproteobacteria. Rickettsiae are small aerobic coccobacilli. Because of the small size and varied morphology, it is not easy to identify them using light microscopic techniques. Because of the obligate intracellular biology of these bacteria, many of the cultural methods used for identification and characterization of bacterial species are not feasible for *Rickettsia*. Even though this hampered early attempts to classify *Rickettsia*-like organisms, whole genome analysis and development of refined polymerase chain reaction (PCR) methods have allowed refinement of the classification of *Rickettsia* and identification of new species and phylotypes (Balraj et al., 2009; Fournier and Raoult, 2009; Phan et al., 2011).

The order *Rickettsiales* is composed of the genera *Anaplasma, Ehrlichia, Neorickettsia, Orientia, Rickettsia*, and *Wolbachia* (Tamura et al., 1995; Dumler et al.,
Because of its significance in human diseases, a number of *Rickettsia* species have been identified and fairly well characterized. There are 25 validated species of *Rickettsia* and many more potential species now characterized only as amplicons from arthropod genomic DNA isolations. (Fournier and Raoult, 2009). A majority (16 of the 25 confirmed species) of *Rickettsia* species are recognized as human pathogens.

In recent years, the classification of *Rickettsia* has undergone significant changes (Roux and Raoult, 2000; Dumler et al., 2001; Fournier and Raoult, 2009). With advances in molecular tools, especially nucleic acid analysis tools, classifications based on morphology, antigenic and physiological characteristics were found to be unreliable. The 16S rRNA gene sequence was used to refine the classification and allowed some bacteria originally classified as *Rickettsia* to be reclassified into different genera (Weisburg et al., 1989; Roux et al., 1997). Even though 16S rRNA gene sequence analysis in some instances allowed reclassification of *Rickettsia* into different species or strains, identical sequence of many highly related, yet potentially different species made further classification within the genus difficult (Fournier et al., 2003; Fournier and Raoult, 2009). Standard molecular criteria used for classification of bacteria like DNA-DNA hybridization value, 16S rRNA sequence divergence of < 3% cannot be used for the classification of *Rickettsia*. As an example based on DNA-DNA hybridization value of >73%, it would not be possible to distinguish *R. rickettsii*, *R. conorii*, *R. sibirica*, and *R. montanensis* as distinct species. Similarly, the diversity of 16S rRNA sequence in all *Rickettsia* is less than 2% (Fournier and Raoult, 2009).
Classification of *Rickettsia* based on 16S rRNA gene sequence was further refined by the sequence analysis of additional genes like citrate synthase (*gltA*), the 17 KDA common antigen. The surface cell antigen family of proteins (*ompA, ompB, sca 1, sca 2, sca 4*) has been used to further refine the classification of this group of bacteria (Fournier, 1998; Roux et al., 1997; Anderson and Tzianabos, 1998; Roux and Raoult, 2000; Fournier and Raoult, 2009).

The genome of a number of species of *Rickettsia* has been sequenced (www.ncbi.nlm.nih.gov/Genbank/index.html). This has allowed the comparison of nearly identical genomic sequence data to identify those regions of the genome that can be potentially used to distinguish *Rickettsia* at the species and strain level (Merhej and Raoult, 2011). These types of analyses have allowed the identification of variable intergenic spacers as a reliable marker for species (Fournier et al., 2004). Using a combination of sequences from different variable intergenic spacers (technique called Multispacer Typing, MST), it is possible to identify not only different species, but also different strains of *Rickettsia* (Fournier et al., 2004; Fournier and Raoult, 2007). In spite of these advances in molecular tools to identify intracellular bacteria like *Rickettsia*, much work remains to be done in the cataloging and classification of these organisms.

Even though the incidence of disease reported due to *Rickettsia* species is not at the level of other tick-borne diseases like Lyme borreliosis in US, different species of *Rickettsia* have been reported from distinct regions of the country (Graf et al., 2008; http://www.cdc.gov/ticks/diseases/). Rocky Mountain Spotted Fever (RMSF), the most widely known disease associated with *Rickettsia*, is widely reported in the United States
rickettsial pox caused by *R. akari* is primarily reported from northeast parts of the United States and sporadic typhus caused by *R. rowazeki* is also reported from the eastern United States. *Rickettsia* species causes a number of diseases in different parts of the world (Richards, 2012). *R. conorii* that has been associated with spotted fever in Mediterranean and Israel, *R. sibirica* has been associated with Siberian tick typhus, *R. australis* has been associated with Queensland tick typhus and *R. japonica* has been associated with Japanese oriental spotted fever. In addition to these well-studied examples, there are many reports of other diseases potentially caused by *Rickettsia* from different parts of the world (Hendershot and Sexton, 2009; Jensenius et al., 2009).

In 2010, tick-borne diseases affected more than 22,500 people in United States (http://www.cdc.gov/niosh/topics/tick-borne/). Even though there are no reliable statistics, the economic cost amounts to millions of dollars. There are approximately 878 species of ticks known, belonging to four families in the class *Arachnida*: *Argasidae* (soft-bodied ticks), *Ixodes* (hard-bodied ticks), *Nutalliellidae* and *Laelapitade* (Anderson and Magnarelli, 2008). Of these, *Nutalliellidae* and *Laelapitade* are represented by only one species. Of the known 878 species of ticks, 222 species are reported to feed on humans, and 28 of them have been shown to harbor and transmit human pathogens (Anderson and Magnarelli, 2008). Of these, *Ixodes* species have been associated with a number of serious tick-borne human diseases such as Lyme borreliosis and Rocky Mountain spotted fever (Bratton and Corey, 2005).
Even though the black-legged deer tick, *Ixodes scapularis*, is the primary vector of Lyme borreliosis, in the eastern United States, *Ixodes pacificus* is the primary vector for the Lyme borreliosis in the western United States (Lane et al., 1991). The *I. pacificus* tick has been reported in the region from California to southern British Columbia (Furman and Loomis, 1984). Therefore, critical analysis of the biology of this tick is necessary to assess its potential in the dissemination of disease-causing pathogens in the western United States.

*Ixodes* ticks have four distinct life stages. Larvae emerge from the egg molt to the nymphal stage after a blood meal from a vertebrate host. Nymphs feed and molt to the next and final stage, the adult. After feeding once more, the adult female hard ticks lay one batch of thousands of eggs and then die (Figure 1). The life cycle of hard ticks are classified based on the number of hosts that they feed on during the three different feeding stages (http://www.dpd.cdc.gov). As an example, *Rhipicephalus microplus* is a one-host tick that feeds on cattle during all three stages of its life cycle (Rechav et al., 1997). Most of the *Ixodes* species on the other hand are three-host ticks (Oliver, 1989). Ticks remain associated with the host while feeding. Larvae that emerge by hatching will attach to a host and after completion of a blood meal drop off the host to the ground where they will molt and become nymphs. Nymphs attach to another host to complete its blood meal and like the larvae, the nymphs drop off to the ground. After molting, adults attach to a third host. After mating, the females complete their blood meal and drop off to the ground where they will lay eggs (Padgett and Lane, 2001).
Under favorable conditions, *I. pacificus* completes its life cycle in three years (Eisen and Lane, 2002). Nymph ticks reach peak activity during early spring and overlap with larval stages. Larval stages reach peak activity several weeks after the nymph stage reaches its peak. Adults are mostly active during the winter. However, they can be active during other seasons (Eisen and Lane, 2002).

![Figure 1. Developmental Stages of *Ixodes pacificus* ticks.](image)

*Ixodes* species are known to feed on a wide range of hosts. As an example, *I. pacificus* and *I. scapularis* are known to feed on more than 100 different host species in North America (Anderson, 1989). The most common host for *I. pacificus* is Columbian black-tailed deer and other medium to large sized animals, including dogs and horses. The nymphs have been shown to feed on small animals such as birds and lizards (Furman and Loomis, 1984). Larvae have been shown to feed on a variety of hosts including...

Although *Rickettsia* are best known as arthropod vectored pathogens, an ever-growing number of *Rickettsia* species without any known vertebrate pathogenicity would argue that this bacterial association with the arthropod hosts may be more of a symbiotic association (Fournier and Raoult, 2009). Since the pathogenicity of many *Rickettsia* sp. is not well understood, it is more appropriate to state that rickettsiae are probably endosymbionts with a beneficial, but not necessary always, association with its arthropod host. There is some argument in the literature that all *Rickettsia* are potentially pathogenic (Hechemy et al., 2006). However, this is highly controversial. Without further evidence, it is assumed that many of the *Rickettsia* sp. found associated with ticks are non-parasitic, potentially symbiotic and are beneficial to the arthropod host (Hechemy et al., 2006; Periman et al., 2006).

Symbiosis describes a close relationship between members of different species, such as the association between large multicellular organisms and microbes. Symbiotic bacteria are ubiquitous in animal hosts. They affect development, nutrition, reproduction and speciation, defense against natural enemies and immunity (Braendle et al., 2003; Koropatnick et al., 2004; Piel, 2002; Scarborough et al., 2005; Oliver et al., 2003). The mechanisms by which symbionts protect their hosts from natural enemies are diverse. Some produce substances with antimicrobial properties (Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992) while others produce toxic compounds (Lopanik et al., 2004; Piel, 2002).
The interaction between hosts and symbionts may not be clearly parasitic or symbiotic, but may lie anywhere in the continuum between parasitism and mutualistic symbiosis. Generally speaking, microbes at the parasitic end of the symbiosis continuum tend to transmit horizontally (horizontal transmission, HT) from one host to another, while those at the mutualistic end of the symbiosis continuum tend to transmit vertically (vertical transmission, VT) from parent to offspring (Tharll et al., 2006). In vertical transmission, the bacteria are transmitted from one generation to the next through the eggs. Therefore, the presence of symbiotic bacteria in the oocyte is usually an indication of vertical transmission. Horizontal transmission across different host lineages tends to facilitate the virulence of the associates, whereas vertical transmission through host generations tends to attenuate the virulence, potentially leading to commensalism and ultimately to mutualism (Dale and Moran, 2006).

The observation that a large number of Rickettsia sp. are found associated with different hard ticks would suggest that there is a special biological relationship between these two organisms. However, the nature of this relationship remains poorly understood. It has long been recognized that Rickettsia with no pathogenic effects (as well as other co-infecting micro-organisms) could affect the distribution and dynamics of pathogenic Rickettsia, with important public health consequences. As an example, Burgdorfer et al. reported that in the Bitterroot Valley of Montana, the prevalence of non-pathogenic Rickettsia species may be a limiting factor in the distribution of pathogenic R. rickettsii – the causative agent of Rocky Mountain Spotted Fever, because this symbiotic nonpathogenic Rickettsia outcompetes R. rickettsii via highly efficient vertical
transmission (Burgdorfer et al., 1981). They found that the disease was more prevalent in
the residents of the west side of the valley even though tick population was evenly
distributed on both the east and west side of the valley. Tick samples collected from the
west side of the valley had a high frequency of pathogenic *Rickettsia*, while those
collected from the east side had a high frequency of non-pathogenic species of *Rickettsia*.
This study would suggest a vertical transmission of *Rickettsia* (both pathogenic and non-
pathogenic) in these ticks with the established species somehow preventing other species
from infecting the host. This is consistent with the observation of competitive interactions
between *R. rhipicephali* and *R. montana* in *Dermacentor variabilis*, where the presence
of one symbiont inhibited vertical transmission of the other (Macaluso et al., 2002).

Even though the Bitterroot Valley study does not lend direct support for the
hypothesis that non-pathogenic species can outcompete pathogenic species,
understanding the biology of exclusion of one species of bacteria by the dominant
resident species would be critical in designing experiments to formulate an effective
biological control of pathogenic species of *Rickettsia*. Therefore, further studies are
needed to test the hypothesis as to whether non-pathogenic strains of the bacteria have
any direct effect on the prevalence and dissemination of pathogenic strains of bacteria.

Even though there are a few examples of *Rickettsia* that can potentially kill its
arthropod host, a majority of them are believed to have a beneficial effect for the host
(Azad and Beard, 1998). Based on published data, a majority of *Rickettsia sp.* associated
with ticks are non-pathogenic and are potentially vertically transmitted transovarially –
transfer of the bacterium from mother to the offspring through eggs (Azad and Beard,
It is clear that many *Rickettsia* sp. can co-inhabit the same host, but it is not known whether vertical transmission of one species would have any negative effect on the vertical transmission of another species of bacteria. It has been shown that different strains of *R. conorii* have different effects on the survival of *Rhipicephalus sanguineus* ticks (Levin et al., 2009). These types of studies point to the specific relationship between a bacterial species and its arthropod host.

Studies have shown that endosymbiotic bacteria in arthropods affect survival and reproduction of hosts (reproductive parasitism) where either they preferentially kill males or reduce the fitness of females without bacterial infection to enhance the dispersal of the bacterium (Bull, 1983; O’Neal et al., 1997). Perotti et al., reported that a mycetomic *Rickettsia* is essential for egg production in the book louse (Perotti et al., 2006). Experimentally reducing the number of intracellular bacteria by antibiotic treatment has a negative effect on the reproductive fitness of ticks (Zhong et al., 2007). These types of studies provide clear evidence for a symbiotic relationship between the bacterial species that inhabit the tick tissue and the host. Since different microorganisms inhabiting the same tick are competing for the same resources, it can be argued that competition could play a critical role in the prevalence and survival of these microorganisms, parasites, pathogens or non-pathogens.

The nature and the origin of symbiotic relationship between *Rickettsia* and its tick hosts remain poorly understood. Molecular identification of new phylotypes of *Rickettsia* in *I. pacificus* ticks collected from northern California (Phan et al., 2011) provides a unique opportunity to understand the interaction of *Rickettsia* (presumably a non-
pathogenic species), other intercellular bacteria and *I. pacificus* ticks. Of the two phylotypes identified, GO21 has high homology to *I. scapularis* endosymbiotic *Rickettsia* and GO22 appears to be a novel SFG *Rickettsia* (Phan et al., 2011). These two phylotypes have been shown to be present in 100% of the *I. pacificus* ticks collected from northern California and based on the PCR data of DNA isolated from eggs, appear to have 100% transmittance rate (Zhong, personal communication, 2012). Even though it would be difficult to determine the potential pathogenicity of the new phylotypes based on molecular characterization alone, understanding the transmission routes of the bacteria is necessary to understand the biology of these bacteria.

Ticks become infected with the bacteria when they feed on hosts carrying the bacteria or may be transmitted vertically, transovarially or transstadially. It has been shown that *Rickettsia* can be transmitted through all three mechanisms (Raoult and Roux, 1997). *Rickettsiae* have been shown to multiply in almost all tick tissue and fluids (Parola and Raoult, 2001). There are examples of bacteria that are transovarially transmitted, however are absent in the salivary glands (Niebylski et al., 1997). Even though as adults these ticks cannot transmit the disease to other hosts horizontally, it is possible that the bacteria can be transferred transstadially to other stages in the tick life cycle and to other hosts (Niebylski et al., 1997). Since not all *Ixodes* ticks transmit bacteria transstadially, it important to undertake such studies for each bacterial species (Parola and Raoult, 2001). As an example, not all *Ixodes* species that get infected with *B. burgdorferi*, the disease agent of Lyme borreliosis, transmit the bacteria transstadially (Parola and Raoult, 2001). Therefore, it is critical to understand the transmission routes of
bacteria in tick host tissue at all stages of development to assess the potential of a bacterial species to be transmitted to other hosts and it interaction with the host tissue.

It has also been reported that bacterial pathogens can become avirulent in tick hosts after repeated transovarial transmission (Lane, 1994). As an example, *B. duttonii* lose pathogenicity after repeated transovarial passage in *Ornithodoros moubata* (Lane, 1994). Even though the biology of this type of change in the biological properties of bacteria is not well understood, it has been shown that in *R. rickettsii*, loss of virulence, at least when tested in guinea pigs, appears to be due to physiological stress (Raoult and Roux, 1997). These types of data would suggest that to understand the relationship between a bacterial species and the host tissue, it is critical to undertake a detailed analysis of the prevalence of bacteria within the host tissue and the genetic changes that they undergo over a period of time.

A recent report has shown that different strains of *Wolbachia* have a significant effect on rate of apoptosis and cell survival in the germline cells of *Drosophila* (Zhukova and Kiseleva, 2012). This points to the significance of undertaking a detailed analysis of different strains of bacteria within the host tissue. Even though there is very convincing molecular data in support of the presence of new phylotypes of *Rickettsia* sp. in *I. pacificus* ticks (Phan et al. 2011), to understand the biological significance of these new phylotypes, it is necessary to undertake a detailed study of the distribution of the bacteria within the host tissue and their transmission routes. The molecular data has to be further supported by light microscopic confirmation of bacteria in different tissues within the tick host to initiate detailed biological interaction studies. Therefore, this study will focus on
further confirmation of the presence of specific strains of *Rickettsia* using *in situ* hybridization studies using probes to the 16S rRNA gene and the *ompA* genes. Different embedding media will be tried to establish the ideal conditions for *in situ* hybridization analysis of tick tissue. The *in situ* hybridization studies will be further confirmed by electron microscopic analysis and PCR verification using specific primers.
MATERIALS AND METHODS

Study areas and tick collection

Engorged female ticks collected from dogs at the Mendocino County Animal Shelter, Ukiah, California, over a period of ten months (September 2010 and June 2011) were used in this study. One hundred and twenty ticks collected during this time period were kept in veterinary specimen collection and transport vials (Thermo Scientific, Waltham, MA) for further identification. From this collection of ticks, eighty of them (30 engorged females, 30 adult female and 20 males) were identified as *Ixodes pacificus* using morphological features under a Leica MZ Apo dissecting microscope (Leica, Buffalo Grove, IL)(Furman and Loomis, 1984). From this group of *I. pacificus* ticks, twenty engorged ticks were dissected and the internal organs (midgut and ovary) were collected for light and electron microscopic analyses. Genomic DNA was extracted from eight of these samples (ticks 1-8) for polymerase chain reaction (PCR) using *Rickettsia* specific primers. The remaining ten engorged females were maintained in a glass desiccator at 25 and 90% relative humidity for oviposition and collection of eggs and larvae for further analysis. Unfortunately, eight of the ten died. The remaining two laid eggs that were used for further analysis.

Light microscopic analysis

Fluorescent *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their
natural environments. Fluorescence in situ hybridization detects nucleic acid sequences by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell (Wetmur, 1991).

One of the persistent problems in in-situ hybridization studies in bacteria using fluorescent dyes is the high autofluorescence background (Szollosi et al., 1995). After trying different fixatives from different commercial sources, freshly made buffered (pH 7.2) 4% paraformaldehyde (Thermo Fisher Scientific, Morris Plains, NJ) was found to give least amount of autofluorescence. Therefore, all tissue samples used for in situ hybridization studies were fixed in freshly prepared 4% buffered paraformaldehyde solution.

E. coli studies

To determine the ideal conditions and appropriate media for in situ hybridization studies to detect Rickettsia in tick tissue, E. coli embedded in 2% agarose were used as a test specimen. Diethyl pyrocarbonate (DEPC, Acros, Geel, Belgium) treated water was used for all solutions. One ml of overnight culture of E. coli in Luria Broth was transferred to a sterile 1.5 ml microcentrifuge tube and the bacterial cells were pelleted by centrifugation at 5,000 x g for 5 minutes in a microcentrifuge. After removing the growth medium, the pellet was resuspended in 250 µl of sterile phosphate buffered saline (pH 7.2, PBS). To bacterial suspension, 250 µl of fresh 4% paraformaldehyde in 1X PBS was added to the tube for a final formaldehyde concentration of 2%. The solutions were mixed by inversion and allowed to stand at room temperature for 1-2 hours and then
stored at 4 °C for 9, 12, 24 or 48 hours. Different fixation times were used to determine the ideal conditions for *in situ* hybridization. At the end of fixation, the samples were spun down 5,000 x g for 5 minutes and the supernatant was removed to a waste container for proper disposal. Fixed *E. coli* samples were washed in 1X PBS four times for 15 minutes each at room temperature and embedded in 2% agarose.

A drop of melted 2% agarose was mixed with 10-15 µl slurry of fixed *E. coli* on a clean glass slide. The agarose was allowed to solidify and regions of agarose with high concentration of bacteria were cut in to 4-5 mm blocks using a sterile razor blade (Fisher Scientific, Houston, TX). A graded series of 25-100% ethanol (25%, 50%, 70%, 90%, 100%) was used for dehydration with 30 minutes of incubation at room temperature for each stage. Dehydrated samples of *E. coli* in agar blocks were embedded in two different resins and paraffin. The resins used were Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) and Unicryl (Electron Microscopy Services, Hatfield, PA). A low melting paraffin wax, Histoplast LP (Thermo Scientific, Kalamazoo, MI), was also used for embedding.

Paraformaldehyde fixed and washed agar blocks with *E. coli* were embedded in Technovit 8100 by initially dehydrating the tissue in cold 100% acetone at 4°C for sixty minutes. The acetone solution was replaced three times to ensure that all of the moisture had been removed. After the final change, the tissue was allowed to stand in 100% acetone for one hour at 4°C. 100 ml of Technovit 8100 resin was mixed thoroughly with 0.6 g of hardner-I. To 15 ml of this mix, 0.5 ml of the second hardner (hardner II) was added and mixed thoroughly before using it for embedding. This resin mix was
transferred to a gelatin capsule (Ted Pella, Redding, CA) and the dehydrated agar blocks with *E. coli* from acetone were transferred to the resin using a sterile toothpick. The blocks were allowed to harden overnight at 4°C and used for thin sectioning using a Leica Ultracut ultramicrotome. (Leica, Buffalo grove, IL).

Similarly, the paraformaldehyde-fixed tissue samples were embedded in Unicryl by dehydrating in a graded series of ethanol (50%, 70%, 90%, 100% with 30 minutes of incubation at each stage. The dehydrated samples were infiltrated in 100% Unicryl by transferring the samples using a sterile toothpick and incubated in the resin overnight at room temperature in a closed gelatin capsule (Ted Pella, Redding, CA). The samples were transferred into fresh resin in a fresh gelatin capsule and the samples in 100% resin were transferred to the fresh resin using sterile toothpick and placed in a 60°C vacuum oven overnight for curing and hardening. The hardened capsules were removed from the oven and allowed to cool down to room temperature before sectioning using a Leica Ultracut ultramicrotome.

Agar blocks with *E. coli* for paraffin embedding were dehydrated in an ethanol series (50-100%) as described before. In a fume hood, the samples were transferred to 100% xylene and allowed to incubate for one hour at room temperature. At the end of the incubation period, the xylene solution was replaced by fresh xylene and allowed to incubate for an additional one hour. One half-inch square aluminum boats were prepared for infiltration of tissue with wax. Chips of highly purified low melting (melting point 50-54°C) Histoplast LP were melted in aluminum boats (four/sample) on a slide warmer kept at 60-65°C. The samples in pure xylene were transferred to a melted wax solution and
allowed to incubate for 30 minutes. The samples were then transferred to a fresh wax solution in a separate boat and incubated for an additional 30 minutes. The process was repeated two additional times. In the final step, the samples were arranged such that the small paraffin blocks for sectioning could be made. The boat with samples were removed from the slide warmer and allowed to solidify at room temperature. The wax around the tissue samples were cut into 5 mm blocks using a sharp razor blade for sectioning. If they were not sectioned immediately, these paraffin blocks with samples were stored at 4°C for future use.

A Leica Ultracut ultramicrotome was used to generate 0.5 µm sections resin embedded E. coli cells using a diamond knife (Diatome, Hartfield, PA). Individual sections were transferred to a drop of sterile DEPC treated water on clean specially treated “Probe on Plus” glass microscope slide (Fisher Scientific, Pittsburg, PA) using a single hair brush. The slides were placed on a hot plate to evaporate off the water so that the sections would stick to the slides. At least 20 slides with multiple sections were generated for each tissue sample block. At least one or two of these slides with tissue samples were stained with 1% methylene blue to ascertain the presence of tissue sample in the thin sections. Thin sections were covered with a drop of 1% methylene blue and gently heated by passing the slide through a Bunsen burner flame. After cooling the slide down to room temperature, the excess stain was removed in a gentle stream of sterile water. The slides were air-dried and examined using Zeiss Axiophot epifluorescent microscope (Carl Zeiss, Thorn wood, NY) with 40 X objective lens. Images were captured using a SPOT RT camera (SPOT Imaging Solutions, Sterling Heights, MI).
After verifying the presence of bacteria, the remaining section from the same block was further processed for different in-situ hybridization conditions to determine the ideal conditions with minimum autofluorescence.

Paraffin blocks with tissue were sectioned using microtome 820 (American Optical Company, Buffalo, NY). Ribbons of 5 µm thick sections were collected and mounted on clean Probe On Plus microscope glass slides. The sections were floated on 20-30 µl of DEPC treated sterile water and heated on a slide warmer at 45°C overnight. After ensuring that all the sections were adhered to the slides, the paraffin was removed by immersing the slides in a series of three coupling jars with 100% xylene solution for five minutes each in a fume hood. After removal of the paraffin wax, the sections were rehydrated by a graded series of ethanol in the reverse order (95-50% starting with 95% ethanol) and ending up in DEPC treated sterile water for 2 minutes each. Excess water was removed by allowing the slides to stand on ends in slide holder lined at the bottom with paper towel. These tissue samples on the slides were immediately processed for in-situ hybridization.

Tick dissection and tissue processing

Engorged ticks were dissected to isolate the midgut and ovary for DNA isolation and light and electron microscopic analyses. The protocol given for dissection of *Amblyoma* ticks was followed with slight modification (Edeards et al., 2009). All the tools used for dissection were sterilized. Ticks for dissection were surface sterilized with 70% Ethanol solution and were immobilized on a Petri plate with Histoplast LP paraffin. Using a hot spatula, wax was melted towards the center of the petri plate and using sterile
tweezers, the tick to be dissected was placed with ventral side down so that all the legs and ventral side of the body was in contact with the melted wax. The wax was allowed to solidify completely before starting the dissection. The ticks were dissected under a Leica MZ APO stereo microscope. The tick to be dissected was covered with few drops of sterile 1 X PBS (pH 7.2) and using sterile blades (Ted Pella, Redding, CA), the scutum was removed by making an incision at the anterior side of the tick and then cutting along the edge of the scutum all the way around the body. The dorsal exoskeleton was removed by lifting it off using sterile forceps and cutting it off using a sterile fine scissors. The internal organs were rinsed in fresh 1 X PBS and carefully removed into a sterile Petri dish with fresh 1 X PBS. Special care was taken not to damage the blood filled midgut from engorged females to avoid any contamination. The individual organs collected (midgut and ovary) were divided into three groups for light and electron microscopic analyses and DNA isolation. The samples for microscopic analysis were fixed as described below and the samples for DNA isolation were transferred to sterile 1.5 ml microcentrifuge tube and frozen immediately at -80°C.

Tick tissue sample preparation for in situ hybridization

Immediately after dissection, tissue samples in 1 X PBS (midgut and ovary) were cut into 3-4 mm pieces using sterile scissors and fixed by adding an equal volume of freshly prepared 4% buffered paraformaldehyde stock solution. The samples were fixed at room temperature for 1-2 hours before incubating at 4°C for an additional 24-48 hours. The fixed samples were washed in 1 X PBS four times for 15 minutes each. The tissue
samples were dehydrated using a 25-100% alcohol series (25, 50, 70, 90, 100) with 30 minutes of incubation between the stages. In some cases, the samples were kept at 4°C in 70% alcohol overnight. After dehydration, samples were embedded in Unicryl (from the E. coli study, this resin gave the least amount of autofluorescence) or paraffin wax as described before (page 3,4). Sections of embedded tissue were made as described before (page 4).

Ultrastructural analysis

To confirm the presence of bacterial cells with Rickettsia-like morphology at the ultrastructural level in different organs of the ticks, samples of dissected tissue samples were fixed in 2.5% buffered gluteraldehyde (Ted Pella, Redding, CA) in 100mM cacodylate buffer (pH 7.4). The tissue samples were allowed to fix for 2 hours at room temperature with continuous agitation and fixed for an additional 24-48 hours at 4°C. The fixed samples were washed three times in 1 X PBS for 30 minutes each. The samples were post-fixed in 1% osmium tetroxide in water for 2 hours at room temperature. The samples were again washed in 1 X PBS as described before and then dehydrated using a graded series of ethanol (25%, 50%, 70%, 90%, 100%) by incubation in each solution for 30-60 minutes. Samples in 100% ethanol were removed to a fresh tube with 100% ethanol and allowed to incubate for an additional 1 hour.

The dehydrated samples were embedded in Quetol following manufacturer’s directions (Quetol-ERL 4221, Ted Pella, Redding, CA). This included incubation in a graded series of ethanol – resin mixture starting with 50% resin and ending up in 100%
resin (50%, 75%, 100%). The tissue samples were incubated at each step for at least 2 hours. In the final step, the samples were removed to another tube with 100% resin and allowed to incubate overnight with continuous agitation on a rocker. The samples were placed in “00” gelatin capsules (Ted Pella, Redding, CA) and filled with fresh resin and cured at 60°C overnight in a vacuum oven.

The blocks of tissue were removed from the 60°C oven and allowed to cool down to room temperature before sectioning. The presence of tissues in the embedded resin blocks was initially determined by generating 0.5-1 µm thick sections using a Leica Thincut ultramicrotome (Leica, Buffalo grove, Il) fitted with a diamond knife. Sections were mounted on a clean glass slide, stained with 1% methylene blue, and examined under the 20 X objective of a compound microscope. After confirming the presence of tissue sample in the resin block, 0.07 µm thin sections were generated using the ultramicrotome fitted with a diamond knife and mounted on 200 mesh pre-cleaned copper grids. The sections were stained with 1% uranium acetate and 1% lead citrate. The sections were washed thoroughly in double distilled water after each staining step. Air dried samples were examined using a Phillips EM 208S transmission electron microscope (Phillips USA, Hillsboro, OR) operating at 60 kV. Images captured on photographic film were developed and printed. Thin sections for electron microscopic analyses were generated from 4-5 resin blocks for each tissue sample.
Genomic DNA Isolation

Genomic DNA was isolated from the tissue samples that were used for light and electron microscopic analyses to validate the microscopic data by PCR amplification of rickettsial specific genes. Tissue samples stored at -80°C were transferred to a container with liquid nitrogen and frozen tissue samples were ground to a fine powder to break up the cells using a sterile disposable pestle (Fisher Scientific, Houston, TX). Genomic DNA from these finely ground tissue samples was isolated using a Qiagen DNAeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Tissue samples were re-suspended in the buffer provided and were incubated in 1mg/ml proteinase K (Sigma, St. Louis, MI) for 60 minutes at 50°C. The protocol that came with the kit was followed for the rest of the steps for isolation of DNA. Isolated DNA was eluted from the DNA binding column using sterile nuclease free water. The concentration of the DNA isolate was determined using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE) and stored at -20°C for further analysis. In the control extraction, the tissue samples were replaced with sterile nuclease free water.

PCR analysis of Genomic DNA for detection of *Rickettsia*

The presence of the *Rickettsia* species in engorged female *I. pacificus* ticks, and eggs were detected by polymerase chain reaction (PCR) analysis. DNA isolated from different ticks organs and subsequently used to detect the presence of the *Rickettsia* species by using primers and probes of the rickettsial *ompA* gene and *16s rRNA* gene. All
of the primers used in this study were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Two sets of primers (Table 1) were used for the detection of *Rickettsia* in the genomic DNA samples isolated from different ticks and tissue samples.

These broad-spectrum primers targeted to conserved regions of the 16S rRNA gene and the rickettsial outer membrane protein A gene (*ompA*). Twenty five microliter PCR reactions included 12.5 µl of 2x PCR mix (Promega Corporation, Madison, WI), 0.4 µM of RP F 190-70, 0.4 µM of RP R 190-701, appropriate volume of template DNA of *I. pacificus* (10-15 ng) and nuclease free water. Positive reaction contained DNA with *Rickettsia* positive *I. pacificus* genome replaced the genomic DNA isolates as templates. An equal volume of mock DNA extracts without any tissue was used as one of the negative controls. In the second negative control, nuclease free double distilled sterile water was used as the template.
Table 1. Primers Used for PCR Detection of *Rickettsia*

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Primer gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rick ompA F 190-70</td>
<td>ompA</td>
<td>SFG</td>
<td>ATGGCGAATATTCTCCAAAA</td>
<td></td>
</tr>
<tr>
<td>Rick ompA R 190-701</td>
<td>ompA</td>
<td>SFG</td>
<td>GTTCCGTTAATGGCAGCATCT</td>
<td></td>
</tr>
<tr>
<td>Rickettsia fRNA F</td>
<td>16s rRNA</td>
<td>SFG</td>
<td>TAAGGAGGTAATCCAGCC</td>
<td></td>
</tr>
<tr>
<td>Rickettsia rRNA R</td>
<td>16s rRNA</td>
<td>SFG</td>
<td>CCTGCTCAGAACGAA</td>
<td></td>
</tr>
</tbody>
</table>
The \textit{ompA} gene was amplified for thirty-five cycles using the following parameters: melting at 92°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds. Similarly, the 16S rRNA gene was amplified for thirty-five cycles using the following parameters: melting at 92°C for 30 seconds, annealing at 52°C for 1 minute, extension at 72°C for 2 minutes. The PCR products were analyzed on a 1% agarose gel. Fifteen \textmu l of the PCR reaction was mixed with 3 \textmu l of 6x loading dye (Promega, Madison, WI) and was loaded on the gel. A sample of 1 kb DNA ladder (Promega, Madison, WI) was also loaded on each gel. The products were visualized by staining with ethidium bromide staining and imaged using Alpha Innotech Gel Documentation system (Alpha Innotech, Santa Clara, CA).

\textbf{Giemsa Staining}

In order to detect the presence of intracellular gram negative bacteria in tick tissue samples, thin sections were stained with Giemsa stain (Invitrogen, Gand Island, NY). The A-T rich bacterial DNA will give the cells a pink color that can be easily distinguished from the deep purple color of the host nuclear DNA (Saxena, 2010). Tissue samples that were used for generating thin sections for transmission electron microscopic (TEM) analysis were stained with Giemsa stain. A 5\% solution of Giemsa in sodium phosphate buffer (pH 6.5) was applied to thin sections and allowed to stand for 30 minutes at 37°C. The excess stain was washed off in sterile water, and the slides were air dried before examination under the light microscope.
Preparation of eggs for light and electron microscopic analysis

To detect the presence of *Rickettsia* in fully developed eggs, between 100-150 eggs were prepared for light microscopic and electron microscopic analysis. The eggs were initially fixed in 2% buffered freshly prepared paraformaldehyde for light microscopic analysis and 2.5% buffered (100 mM cacodylate buffer, pH 7.4) gluteraldehyde for TEM studies. After fixing the eggs at room temperature for 2 hours, they were transferred to 4°C for an additional 12 hours. The fixed eggs were washed four times in 1X PBS for a total of 60 minutes. The washed eggs were treated with 1 µg/ml *Brugia malayi* chitinase, (New England Biolabs, Ipswich, MA) in a phosphate buffer (200 mM sodium phosphate, 200 mM NaCl, 100 mM EDTA, 500 µg/ml BSA) for eight hours at 37°C. The chitinase treated eggs were washed in 1 X PBS for 15 minutes and fixed again in paraformaldehyde or gluteraldehyde as described above for an additional 24 hours. The fixed eggs were washed in 1 X PBS for 60 minutes as described above and dehydrated in a 25-100% ethanol series. The dehydrated eggs were embedded in Unicryl or paraffin for light microscopic analysis or in Quetol-ERL 4221 for transmission electron microscopic studies. Thin sections of embedded eggs were made and mounted on glass slides or 200 mesh copper grids as described before.

*In situ* Hybridization

Bacterial cells in thin sections were directly visualized by *in situ* hybridization. Glass slides with resin sections were used directly for hybridization. Paraffin thin
sections were first treated to remove the paraffin before the hybridization protocol. To remove the paraffin, the slides with thin sections were heated to 60-65°C to for 30 minutes in a fume hood, transferred to warm xylene (~50°C) in a Coplin jar and allowed to stand for five minutes in an incubator kept at 50°C. At the end of the incubation, in a fume hood, the slides were rehydrated in a reverse series of alcohol (95-50%) ending in DEPC treated water. The slides were incubated in each solution for 5 minutes. At the end of the rehydration protocol, the sections were ready for the hybridization protocol.

**In situ** Hybridization of *E. coli* test samples

To establish the appropriate *in situ* hybridization conditions and the least amount of background autofluorescence, thin sections with *E. coli* were hybridized to fluorescently labeled EUB-Alexa568 probes (Table 2) designed to bind to all eubacteria. Hybridization was done at 37°C in a 0.1 M Tris buffer (pH 7.2) with 0.9 M NaCl, 0.1% SDS, 0.1% Poly A DNA (Roche Diagnostics, Indianapolis, IN) in 50% deionized fresh formamide (Amersco, Solon, OH), in a moist closed chamber. A 150 mm petri plate was lined with filter paper and saturated it with DEPC treated water. The slides with sections (sections facing up) were placed on glass rods so that they were not in contact with the moist filter paper. 10-20 ml of hybridization buffer was equilibrated to 37°C for 1 hour. The sections were pre-incubated in the pre-warmed hybridization buffer for 2 hours at 37°C by adding about 100 µl such that all the sections are completely covered by the buffer. At the end of two hours, the hybridization buffer was replaced by hybridization buffer with 50 ng/µl fluorescent labeled probes and incubated for 24-48 hours at 37°C. At
the end of incubation period, the sections were washed in wash buffer containing 0.1 M Tris buffer (pH 7.2) with 0.1 M NaCl and 0.01% SDS for 20 minutes, three times at 50°C. 10 µg/ml of 4’,6- diamidino-2- phenylindole (DAPI) was included in the final wash. After a final rinse in wash buffer the excess liquid was removed and about 10 µl of Prolong (Invitrogen, Eugene, OR) was placed on the top of the sections and a 22 x 22 mm #1 cover slip was lowered gently to avoid any air bubbles. The slides were examined under different objectives using a Zeiss Axiphoto epifluorescent microscope. The images were captured using a SPOT RT camera using SPOT advanced image capture software (SPOT Imaging Solutions, Sterling Heights, MI). Each in-situ hybridization experiment was done in duplicate and with both forward and reverse primers. In addition, there was always a negative control without any probes. These experiments were repeated at least 10-15 times with slight variations in the hybridization times and washing conditions.

In-situ hybridization of tick tissue samples

For direct visualization of Rickettsia in tick tissue, thin sections of tick tissue samples were hybridized to ompA probes or 16S rRNA probes. These studies were completed in two different phases. In the first phase of the study, oligonucleotide probes conjugated with fluorescent dyes were used for the direct detection of bacteria. In the second phase of the study, Digoxigenin (DIG) labeled probes were used and the signals were amplified using a Tyramide Signal Amplification (TSA) protocol.

In the first phase of the study, two different oligonucleotide probes, EUB-Alexa568 and Rick-Alexa488 (Table 2), a broad spectrum probe that would detect all
eubacteria and a very specific probe targeted to *Rickettsia*, were used. One probe was targeted to a highly conserved region of the 16S rRNA gene of all eubacteria and is named EUB probe. The second more specific probe is targeted to *Rickettsia*-specific regions of the 16S rRNA gene and is named “Rick” probe (Table 2). The EUB probes were conjugated with Alexa 568 fluorophores (Invitrogen, Eugene, OR) and the “Rick” probes were conjugated to Alexa 488 (Invitrogen, Eugene, OR).

The conditions that were used for the hybridization of *E. coli* samples were used in these experiments, except the hybridization was done at 50°C. The tissue samples were washed as described earlier and mounted tissue samples were examined using Zeiss Axiophot epifluorescent microscope. Hybridization with sense strand probes and no probes served as controls. Each hybridization experiment was done in duplicate and the experiment was repeated more than 30 times.
Table 2. Probes Used for *in-situ* Hybridization

<table>
<thead>
<tr>
<th>Oligonucleotide Probe</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB-Alexa 568</td>
<td>16S rRNA</td>
<td><em>E.coli</em></td>
<td>5’GCTGCCTCCCGRAGGAGT3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Anti sense)</td>
</tr>
<tr>
<td>EUB- Alexa 568</td>
<td>16S rRNA</td>
<td><em>E.coli</em></td>
<td>5’GCAGCCACCCGTAAGGTGT3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Sense)</td>
</tr>
<tr>
<td>Rick- Alexa 488</td>
<td>16S rRNA</td>
<td>SFG</td>
<td>5’ CCATCATCCCCTACTACA3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Anti-sense)</td>
</tr>
<tr>
<td>Rick- Alexa 488</td>
<td>16S rRNA</td>
<td>SFG</td>
<td>5’ CTCACCACCTTCAGGTA3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Sense)</td>
</tr>
</tbody>
</table>

Tyramide Signal Amplification (TSA) Protocol

Since the signal generated using fluorescently labeled probes was not high enough to clearly detect bacteria clearly from the background, the TSA protocol was used for clear detection of bacteria from the background. In this protocol, in place of the fluorescently labeled probes, Digoxigenin (DIG) labeled probes were used.

*Rickettsia* specific ompA and 16S rRNA probes were generated by an asymmetric PCR reaction using DIG nucleotides (Roche, Indianapolis, IN). The templates for the asymmetric PCR reaction were generated by PCR amplification of *ompA* and 16S rRNA gene from *I. pacificus* genomic DNA isolates using the primers and conditions that were described earlier (page 26). Since the template concentration needed for the asymmetric PCR reaction was at least 200 ng/µl, the reaction volume was adjusted to 50 µl and the
number of cycles was increased to 40. A 5 µl sample of the PCR products was run on 1% agarose gel to verify proper amplification. After confirming proper amplification of DNA, the remaining PCR products were purified using a PCR clean up kit (Promega, Madison, WI). To the remaining 45 µl of the PCR products, an equal volume of membrane binding solution was added and the mixture was loaded on to the DNA binding mini-column assembly. The column with the sample was centrifuged at 16,000 x g for one minute. After discarding the flow-through, the column was washed with 700 µl of column wash buffer and centrifuged again to remove the wash buffer. The flow through was discarded and washed again with 500 µl of wash buffer. After centrifugation, from the dry column DNA was eluted in 50µl of nuclease free water. The concentration of DNA was determined using a Nanodrop Spectrophotometer. If the desired concentration of 200 ng/µl was not obtained, the purified DNA products were concentrated in a vacuum centrifuge (Savant, Hicksville, NY) until the desired concentration was reached.

To generate DIG labeled probes, 200 ng/µl of ompA or 16S rRNA purified PCR products were used as template for asymmetric PCR reaction. Both sense and anti-sense probes were generated for each gene. Each 25 µl reaction mixture had 4 µl of template DNA, 2.5 µl of 10X Taq buffer (Promega, Madison, WI) 0.4 µM of one of the four primers (rick ompa R 190-701, rick ompa F 190-70, Rickettsia fRNA forward, Rickettsia rRNA reverse), 1µl of Taq polymerase, 5 µl 5 X PCR buffer and 11.5 µl of nuclease free sterile water. The thermal cycler was programmed for 35 cycles with 30 seconds each of melting, annealing and extension at temperatures 95°C, 55°C and 72°C respectively and a
final extension time of 7 minutes at 72°C. The PCR products (25 µl) were transferred to a fresh sterile 1.5 ml microcentrifuge tube with 75 µl of sterile nuclease free water. To the diluted PCR products, 10 µl of sterile 1 M sodium chloride (NaCl) was added, and then 300 µl of 100% cold ethanol. The contents were mixed thoroughly and chilled at -20°C for at least two hours. The DIG-labeled DNA was pelleted by centrifugation at 16,000 x g in a microcentrifuge for 30 minutes. The supernatant was poured off gently, the visible pellet was rinsed in 70% ethanol and the pellet was air dried before it was re-suspended in 400µl of hybridization buffer. The hybridization buffer had 5 x saline sodium citrate (SSC), 50% deionized formamide (Amersco, Solon, OH), 100 µg/ml autoclaved herring sperm DNA (Sigma-Aldrich, Carlsbad, CA), 50µg/ml Heparin (Sigma-Aldrich, Carlsbad, CA) and 0.1% Tween-20 (Arcos, NJ). The DNA probe in the hybridization buffer was heated to 90°C for one hour in a heating block to generate short single stranded fragments. The concentration of DNA was determined using a Nanodrop spectrophotometer blanked with the hybridization buffer. The probe was aliquoted into 20 µl and stored at -80°C.

While the samples were pre-hybridizing in hybridization buffer for 2 hours, the nucleic acid probe was removed from -80°C and placed in heating block kept at 90°C. After the pre-hybridization was completed the hybridization buffer was removed and replaced by the denatured probes and allowed to hybridize for 24-48 hours at 37°C in the moist chamber in an incubator. At the end of the incubation period, the excess and non-specifically bound probes were removed by washing at 50°C in PBS with 0.1 % Tween-20 (PBTw) for 45 minutes (three changes of 15 minutes each).
A TSA kit from Invitrogen was used for the detection and amplification of the signal (Invitrogen, Eugene, OR). The manufacturer’s directions were used in the detection protocol. The tissue sections hybridized with DIG labeled probes were blocked by incubating for 15 minutes in PBTw with 0.5 mg/ml Bovine Serum Albumin (BSA) at 48°C. The sections were then incubated overnight in Anti-DIG primary antibody (1:1000 dilution in PBTw with 0.5 mg/ml BSA at 4°C. The sections were washed for 6 minutes (three changes of 2 minutes each) in PBTw and then a prolonged wash for 60 minutes (three changes of 20 minutes each) in PBTw with 0.5 mg/ml BSA. A blocking solution for the secondary antibody was made by dissolving 10 mg of component D in one ml of 1X PBS and making appropriate dilutions to give 0.2% solution in PBTw. A 1:100 dilution of secondary antibody, Anti mouse anti DIG antibody (Invitrogen, Eugene, OR) was made by diluting the stock secondary antibody solution in the blocking buffer. After the final wash to remove any non-specifically bound primary antibody, the sections were covered with secondary antibody and incubated at room temperature for two hours in a diffused light condition. At the end of the incubation period, the sections were washed for 45 minutes in PBTw (three changes of 15 minutes each). While waiting for the final wash to be completed, 6 µl of stock Tyramide solution was diluted in the working amplification buffer. The working amplification buffer was made by diluting hydrogen peroxide (H₂O₂) in 200 µl of Amplification buffer and then making a further 1:100 dilution of this in the Amplification buffer. At the end of the final wash, the sections were treated with diluted Tyramide solution for 20 minutes at room temperature in diffused light conditions. The slides were then washed in PBTw for 12 minutes (3 changes of 4
minutes each) and a drop of Prolong Gold mounting medium with DAPI (Invitrogen, Eugene, OR) was placed on the sections and a clean 22 x 22 mm glass cover slip was lowered avoiding any air bubbles. The slides were incubated at 4°C overnight and examined using Zeiss Axiophot epi-fluorescent microscope. The images were captured using an SPOT RT camera using a PCI image capture program. All hybridizations were done in duplicate. Sense probes served as one of the controls. The primary antibody was left out in another control reaction to detect any non-specific TSA amplification.
RESULTS

Genomic DNA was successfully isolated from the midgut and ovary tissues of eight engorged female *Ixodes pacificus* ticks. In addition, genomic DNA was isolated from two clutches of eggs. 10-15 eggs from a clutch of eggs were used for the isolation of the DNA. Ten to fifteen nanograms of isolated genomic DNA were used for PCR amplification of *Rickettsia* specific *ompA* and 16S rRNA genes.

Both *ompA* and 16S rRNA amplicons were successfully amplified from all genomic DNA isolates (Table 3). Some examples of agarose gel electrophoresis analysis of PCR amplified *Rickettsia* specific amplicons are shown in Figures 2-4. Using rick ompa F 190-70 and rick ompa R 190-701 primers, a 500 bp *ompA* gene DNA fragment was amplified from genomic DNA isolated from both midgut and ovary tissue (Figure 2). The results obtained are consistent with the expected size of the 500 bp amplicon. There was no amplification in mock extractions or PCR negative controls (Figure 2). The 500 bp *ompA* specific fragments were also amplified from genomic DNA isolates from eggs (Figure 3). Similarly, using Rickettsia rRNA F and Rickettsia rRNA R primers, a 1,460 bp 16S rRNA fragment was amplified from all sixteen genomic DNA isolates. The mock extraction and PCR control failed to amplify any DNA (Figure 4).

Conditions for detecting bacterial cells using the Giemsa staining protocol were first established in thin sections of *E. coli* samples. Rod shaped cells were visible in the thin sections of Unicryl embedded *E. coli* cells stained with Giemsa (Figure 5).

Unfortunately, using the same protocol, bacterial cells were not clearly distinguishable in
Unicryl embedded thin sections of tick tissues because of the high background (data not shown). However, Giemsa staining of egg smears showed many clusters of coccobacilli in the eggs examined (Figure 6).

*E. coli* cells were used to determine the ideal embedding medium for fluorescence *in situ* hybridization studies. *E. coli* cells embedded in Unicryl resin hybridized with eubacterial FITC conjugated probes gave excellent signal to noise ratio with relatively low background fluorescence (Figure 7). However, Technovit 8100 resin gave very high background fluorescence, making it difficult to clearly distinguish the signal from the noise (data not shown). Similar studies of *E. coli* cells embedded in paraffin and hybridized to FITC conjugated eubacterial probes gave even lower background fluorescence when compared to Unicryl embedded cells (Figure 8).

Thin sections of tick tissue embedded in Unicryl resin were used for *in situ* hybridization studies using *Rickettsia* specific 16S rRNA probes conjugated to Cy-3 fluorescent dye. The background fluorescence was too high to distinguish bacterial cells from the noise (data not shown). To decrease the amount of auto-fluorescence at the excitation energy for Cy-3 of 550 nm, *Rickettsia* 16S rRNA probes conjugated to Alexa 488 were also used for *in situ* hybridization of Unicryl embedded tick tissue. Even though the background of auto-fluorescence was not as high as that of the Cy-3 probe, bacterial cells were not clearly visible in these samples (data not shown). Therefore, all further experiments were done with paraffin embedded tick tissues. Even though paraffin embedded tick tissue
samples had the lowest autofluorescence background, bacterial cells were not distinguishable from the background noise in many sections (data not shown).

To detect the presence of rickettsiae in tick tissues, DIG-labeled probes of *Rickettsia* specific *ompA* and 16S rRNA genes were generated for *in situ* hybridization experiments. Asymmetric PCR amplification of 800 ng of *ompA* amplicons yielded 10-15 ng/µl *ompA* specific DIG labeled sense and anti-sense probes. Similarly, asymmetric PCR of the 16S rRNA gene amplicon yielded about 15 ng/µl of DIG labeled sense and anti-sense probes. Using *ompA* specific anti-sense DIG labeled probes, bacterial cells in the ovary tissue were visible above the background fluorescence (Figures 9A and 10A). These signals co-localized to DAPI stained intracellular bacterial cells (Figures 9B and 10B). A majority of the DAPI positive spots co-localized with the TSA amplified signal. However, the density of TSA amplified signals varied from cell to cell (Figure 9A and 10A). As seen in Figures 9C and 10C, the sense probes failed to produce any signal above the background fluorescence. Even though many TSA positive cells were observed in the tick tissue hybridized with 16S rRNA DIG probes, the noise level was too high to draw any conclusion (data not shown).

Transmission electron microscopic analysis of tick ovary tissue samples showed clusters of bacterial cells within the developing oocytes (Figure 11, 12). The microorganisms appeared rod shaped with a distinct inner and outer membrane with an average length and width of 1.5 by 0.5 µm. The clustered appearance of the bacteria seen in many sections is consistent with the light microscopic data using both Giemsa staining and *in situ* hybridization studies. The bacterial cells were the size of the mitochondria;
however mitochondria can be easily distinguished because of the presence of a cisternal membrane.
Table 3: Summary of PCR Amplification Data

<table>
<thead>
<tr>
<th>Tick</th>
<th>Tissue</th>
<th>ompA gene</th>
<th>16S rRNA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tick 1</td>
<td>Ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
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<tr>
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<td>Ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>Ovary</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tick 4</td>
<td>Ovary</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
<td></td>
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<td>+</td>
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<tr>
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<td>Ovary</td>
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<tr>
<td></td>
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<tr>
<td>Tick 9</td>
<td>Eggs</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tick 10</td>
<td>Eggs</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 2. An example of a 0.8% agarose gel image of *Rickettsia* specific *ompA* gene amplicon using DNA templates isolated from ovary and midgut tissues of four different ticks, using rick ompa F 190-70 and rick ompa R 190-701 primers. In the lane marked MW std., Promega 1 kb benchtop ladder was loaded according to the manufacturer’s directions. Appropriate molecular weight standards are labeled. Lanes marked 2-5 are *ompA* amplicon from genomic DNA isolated from the ovary tissue and lanes 6-9 are *ompA* amplicon from genomic DNA isolated from the midgut tissue. Lane 10 is the negative control. Lane 11 is the positive control of *ompA* amplicon generated from DNA isolates with known *Rickettsia* genome.
Figure 3. An example of a 0.8% agarose gel image of *Rickettsia* specific *ompA* gene amplification product using DNA isolates from eggs. In the lane marked MW std., Promega 1 kb benchtop ladder was loaded according to the manufacturer’s directions. The expected size of the MW markers is shown. Lanes marked 2, 4 and 6 are amplified products from genomic DNA isolated from eggs. The lane marked 8 is a negative control without any template. The lane marked 9 is positive control using genomic DNA template with the known *Rickettsia* genome. No samples were loaded in lanes 3 and 5.
Figure 4. Agarose gel image of *Rickettsia* specific amplicon of 16S rRNA gene amplified from genomic DNA isolations using primers *Rickettsia* fRNA forward and *Rickettsia* rRNA reverse. In the lane marked MW std., Promega 1 kb benchtop ladder was loaded according to the manufacturer’s directions. The expected size of the MW markers is labeled. Lanes marked 1, 3, 5, 7 and 9 are amplified products from genomic DNA isolated from ovary tissue and lanes 2, 4, 6 and 8 are amplified products from genomic DNA isolated from the midgut. The lane marked 11 is a negative control. The expected size of the 16S rRNA gene amplicon is 1,460 bp.
Figure 5. Light microscopic image of thin section of *E. coli* embedded in agarose and stained with 2.5 µg/ml Giemsa. *E. coli* cells in agarose were embedded in Unicryl resin and thin sections were stained with Giemsa. The arrows point to dark purple rod shaped cells. All the darkly stained rod shaped cells appear to be of the same size.
Figure 6. Light microscopic image of an egg smear on a sterile glass slide stained with Giemsa. The deep purple stained cells appear to be clusters and are coccobacilli (arrow). These cells appear to be dispersed throughout the egg cytoplasm. The large slightly denser structure is the nucleus (N) and the dark small circles (O) are believed to be oil droplets. A cluster with bacteria is enlarged in the upper right corner.
Figure 7. Fluorescent microscopic image of thin sections of *E. coli* cells embedded in Unicryl resin hybridized to FITC labeled EUB anti-sense probes. The bright green rod shaped bacterial cells were clearly visible above the background.
Figure 8. Fluorescent microscopic image of thin sections of paraffin embedded *E. coli* cells. FITC conjugated eubacterial (EUB anti-sense) probes were hybridized at 37°C for 24 hours and washed at 50°C (8A). Cells were counter stained with DAPI (8B, 8D) and EUB sense probe (8C).
Fig. 9
Fig. 9
Figure 9. Fluorescence microscopic image of thin sections of *Ixodes pacificus* ovary tissue embedded in paraffin. Ovary tissue samples from *I. pacificus* were hybridized with the *Rickettsia ompA* anti-sense probe at 48°C for 40 hours and washed at 48°C for two hours. Hybridized DIG-labeled probes were detected using anti-dig antibodies and the signal was amplified using the tyramide signal amplification (TSA) protocol. The signal of cells hybridized to the probes was clearly visible above the background on the fringes of the cells (arrow) (Figure 9A). Only a small fraction of the DAPI positive cells hybridized to *ompA* probes (Figure 9B). Ovary tissues were hybridized to *Rickettsia ompA* sense probes as a control (Figure 9C). Fluorescent image of the same section counter stained with DAPI showing DAPI positive bacterial cells and host nuclei. (Figure 9D). High background fluorescence was also observed in control experiments without any probes (Figure 9E and 9F).
Fig. 10
Figure 10. Fluorescent micrographs of a thin section of paraffin embedded *Ixodes pacificus* ovary tissue. Ovary tissue samples from *I. pacificus* were hybridized to the probes at 48°C for 40 hours and washed at 48°C for two hours. The signal of DIG labeled probes detected with anti-DIG antibody was amplified using TSA. The ovary tissue was hybridized to DIG labeled *Rickettsia ompA* anti-sense probes (Fig. 10A). Brightly red coccobacilli were clearly visible over the background (arrow). Fluorescent image of the same section counter stained with DAPI showing DAPI positive bacterial cells and host nuclei (Fig. 10B). Fluorescent image of a thin section of paraffin embedded *Ixodes pacificus* ovary tissue hybridized to *Rickettsia ompA* sense probes as a control (Figure 10C). Fluorescent image of the same section counter stained with DAPI showing DAPI positive bacterial cells and host nuclei (Figure 10D). Fluorescent image of thin sections of paraffin embedded *Ixodes pacificus* ovary control without any probes (Figure 10E). Fluorescent image of the same section stained with DAPI showing DAPI positive bacterial cells (arrow) and host nuclei (Figure 10F). High background fluorescence was also observed in control experiments without any probes (Figure 10E and 10F).
Figure 11. Transmission electron microscopic image of *Ixodes pacificus* ovary tissue showing rod shaped bacterial cells with double membranes. Clusters of bacterial cells were observed (arrow). A double membrane, characteristic of gram-negative bacteria is clearly visible in many longitudinal sections of the bacteria. The darkly stained objects are believed to be lipid droplets. The thick outer layer of the egg can be seen (O).
Figure 12. Transmission Electron micrograph of a thin section of *Ixodes pacificus* ovary tissue showing a developing egg with gram-negative bacteria (12A). In the longitudinal sections, the double membrane of the bacteria was clearly visible (black arrows). Tangential sections of a group of bacterial cells also were seen (white arrow). The outer thin membrane of the oocytes (O) was clearly visible. A single bacterial cell is enlarged to show the double membrane (blue arrows), a characteristic of gram-negative bacteria (12B).
DISCUSSION

This study was undertaken to directly visualize *Rickettsia* using fluorescence *in-situ* hybridization. *Rickettsia* were shown to be present in the developing oocytes by *in situ* hybridization (Figure 9A and 10A) and by PCR (Table 3). These results were further confirmed by transmission electron microscopic analysis (Figures 11 and 12).

It is important to study “non-pathogenic” strains of *Rickettsia*. The *Rickettsia* genomes vary in size from 1.1 -1.5 MB of DNA (Merhej and Rault, 2010). This range in size of these closely related bacterial species is believed to be either due to loss or gain of genetic information (Anderson et al., 1998; Wolf et al., 1999; Ogata et al., 2001; Blanc et al., 2007; Bechah et al., 2010). Horizontal transfer of genetic information in *Rickettsia* has been reported (Wolf et al., 1999). These results would suggest that the *Rickettsia* genome in a host tissue has the potential to undergo genetic changes that can potentially make a pathogenic strain non-pathogenic due to loss of a virulence gene. Studies on *R. prowazekii* lend further support to this argument (Bechah et al., 2010). However, one can also argue that a non-pathogenic strain of *Rickettsia* would have the potential to be pathogenic due to horizontal gene transfer. Even though there are no reported examples of such horizontal transfer of virulence factor in *Rickettsia*, the prevalence of horizontal gene transfer in *Rickettsia* would argue that any strain of *Rickettsia* would have the potential to become pathogenic due to horizontal gene transfer (Georgiades et al., 2011).

It has been shown that the virulence factor of *R. prowazekii* could be due to surface protein expression and protein methylation patterns, potentially due to adaptive mutations (Bechah et al., 2010). Therefore, a detailed analysis of new strains of *Rickettsia* in a host
with wide distribution in the Pacific Northwest of the US could be used to assess its potential virulence and pathogenicity.

It has been reported that the *Rickettsia* genome can undergo extensive shuffling due to transposon elements (Felsheim et al., 2009). In some cases, such genetic reorganization could lead to the formation of a new species of *Rickettsia* as in the case of the pathogenic strain, *R. rickettsii*, and the non-pathogenic strain, *R. peacockii* (Felsheim et al., 2009). Therefore, it is not surprising that the classification of different species and strains of *Rickettsia* based on one or two molecular characteristics can be extremely difficult.

Identification of *Rickettsia* solely on the basis of rRNA sequence is difficult. Therefore, many other molecular markers like the *ompA*, *ompB* gene, etc. are routinely used in conjunction with highly conserved regions of the 16S rRNA gene for the molecular identification of *Rickettsia* (Fournier and Raoult, 2009). PCR based analysis using *Rickettsia* specific primers to these genes can provide good evidence for the presence of bacteria in the ticks and potentially a rough estimate of the density of bacteria per host cell. However, direct evidence using light microscopic methods is necessary to confirm such molecular data. Fluorescent *in-situ* hybridization analysis is one of the accepted protocols for visual confirmation of bacterial cells within the host cells (Svendsen et al., 2009).

PCR based assays are routinely used for the identification of microorganisms from tick tissue (Sparagono et al., 1999). Comparative genomic analyses of *Rickettsia* genomes have allowed the identification of molecular differences between closely related
strains and the potential to develop molecular tools to detect them (Chuang et al., 2004). Since these types of analyses are simply detecting the presence of DNA in host tissue and from that inferring the presence of microorganisms, any contamination of the DNA isolation protocol could result in false positives. In addition, the presence of bacterial DNA in the hemolymph of the tick could be a source of contamination. Therefore, any molecular data should be further verified by direct visualization of microorganisms using \textit{in situ} hybridization with gene specific probes.

Since \textit{Rickettsiae} are obligate intracellular bacteria, thin sections of tissue were used for \textit{in-situ} hybridization studies. Therefore, one of the objectives of this study was to establish the ideal conditions for generating thin sections that would generate sufficient fluorescent \textit{in situ} hybridization signals for clear identification of the bacteria within the host tissue. Three different embedding media, two resins and paraffin wax, were tried using \textit{E. coli} as test samples to establish the appropriate conditions for \textit{in situ} hybridization analysis using fluorescently labeled eubacterial rRNA probes. Of the two resins tried, the Technovit 8100 resin gave a very high background fluorescence that made it very difficult to clearly distinguish the signal from the background. Even though this resin has been successfully used in immunocytochemical studies, in this analysis it gave a very high background using both FITC and CY-3 fluorescent dyes (Takechi et al., 1999; De Jonge, 2005). Unicryl is single component resin that has been shown to be an effective medium for \textit{in situ} hybridization studies (Pantoja and Lightner, 2001). This resin has the added advantage that the same tissue sample can be processed for ultrastructural analysis (Pantoja and Lightner, 2001). Unicryl resin gave excellent signal
to noise ratio using *E. coli* cells (Figure 6). Unfortunately, when tick tissue samples were used for the analysis using Unicryl, the background fluorescence was too high to distinguish the signal from the background (data not shown). Therefore, paraffin was chosen as embedding medium for *in-situ* hybridization analysis. Even though paraffin was the medium of choice for *in situ* hybridization studies, this necessitated the use of a different resin (Quetol-ERL 4221) for transmission electron microscopic analysis (Watters and Bartlett, 2002; Zordan, 2011).

*In situ* hybridization experiments using *E. coli* cells embedded in paraffin gave excellent signal to noise ratio (Figure 7). As expected, the background fluorescence of thin sections of tick tissue embedded in paraffin was considerably less than those embedded in Unicryl (data not shown). Even though fluorescently labeled probes have been used for the detection of *Rickettsia* in tissue culture cells, the high noise to signal ratio in tick tissue samples made it very difficult to detect bacterial cells (Svendsen et al., 2009). Amplification of signals using TSA is a well-established technique for detection of transcripts form single copy genes (Zwirglmayer, 2005; Zordan, 2011; Kawakami, 2012). To resolve the low signals generated by fluorescently labeled oligonucleotide probes, DIG labeled probes were generated. Since these highly specific probes to *Rickettsia ompA* and the 16S rRNA gene would have multiple nucleotides with DIG, the signal generated by primary and secondary antibodies to DIG would be considerably higher than those generated by fluorescent oligonucleotide probes (Kessler et al., 1990). Amplification of the antibody signal further by TSA would further increase the signal to noise ratio (Zwirglmayer, 2005; Zordan, 2011; Kawakami, 2012).
As shown in the figures 8A and 9A, in sections of I. pacificus ovary tissue, clusters of bacterial cells hybridized to Rickettsia specific ompA probes were clearly distinguishable from the background. Since these signals were generated only with anti-sense probes and co-localized to DAPI positive signals (Figures 8B and 9B), it can be argued that the Rickettsia specific signals represent bacterial cells and therefore, Rickettsia. The sense probes and negative controls failed to give any detectable signals (Figures 9C,D,E,F, and 10 C,D,E,F).

Based on the in situ hybridization analysis, the density of Rickettsia observed in individual cells varied considerably from tissue sample to tissue sample and from cell to cell (Figures 9, 10). However, based on the DAPI staining data, it appears that there is not a marked difference in the total number of bacteria/cell. This is consistent with the observation that tick cells host a variety of bacterial cells (Andreotti et al., 2011). Even though there are some clear examples where bacterial association can give the arthropod host a reproductive advantage (Perlman et al., 2006), details of the biological interactions between host cell and the resident bacteria are not well understood.

The in-situ hybridization results were further confirmed by transmission electron microscopic analysis (Figures 11 and 12). These results clearly show the presence of rod-shaped bacteria with the characteristic double membrane of gram-negative bacteria (Figure 11). Similar to the observations that were made using in situ hybridization analysis, many of these cells appear to be in clusters (Figures 9 and 10).

The functional significance of observed clusters of bacteria is not clear at this time. However, it can be argued that they may be indicative of cells undergoing active
cell division. This would not be a surprising finding since \textit{Rickettsia} can be maintained in tissue culture systems (Ammerman et al., 2008). The differences in density of \textit{Rickettsia} cells observed between different oocytes within a single ovary would suggest that the mechanism for translocation of bacteria to developing oocytes may be random. However, this could influence the dissemination of \textit{Rickettsia} in a tick population. Even though the presence of \textit{Rickettsia} was confirmed by PCR analysis in all eight ticks tested in this study, high densities \textit{Rickettsia} were detected in only three of the tick ovary samples. This would be consistent with the argument that distribution of \textit{Rickettsia} in host tissue appears to be random. However, with such small sample size, it would hard to draw any firm conclusions about the distribution of these phylotypes within a population. Further studies with a larger sample size are needed to reach any conclusions.

This study has firmly established the presence of new phylotypes of \textit{Rickettsia} in \textit{I. pacificus} ticks. However, much work remains to be done to understand the transmission of these bacteria and their prevalence in the \textit{I. pacificus} tick population. The presence of \textit{Rickettsia} in developing oocytes provides good evidence that these bacteria are transmitted transovarially to the next generation. The presence of \textit{Rickettsia} in all eight ticks examined in this study would suggest that this mode of transmission is very efficient. However, this can only be confirmed by undertaking a detailed analysis of the transstadial mode of transmission. In addition, individual eggs from a clutch of eggs should be tested for the presence of bacteria to determine the efficiency of transmission through oocytes. More critical is to establish whether this is the only mode of transmission of these bacteria. A report of these phylotypes in dogs would suggest
horizontal transmission of these bacteria (Zhong, personal communication, 2012). Therefore, detection of these phylotypes in the salivary glands of *I. pacificus* ticks could provide evidence for horizontal transfer of the bacteria to other hosts.

In conclusion, this study has shown that paraffin embedded tick tissue samples gave the best *in situ* hybridization results. TSA amplification of signals of DIG labeled probes generated good signal to noise ratio for clear detection of bacteria within the tick tissue. *In situ* hybridization and transmission electron microscopic data have confirmed the presence of *Rickettsia* in the developing oocytes of engorged *I. pacificus* ticks.
LITERATURE CITED


Ngwamidiba, M., G. Blanc, D. Raoult, and P.E. Fournier. 2006. Sca1, a previously undescribed paralog from autotransporter protein-encoding genes in Rickettsia species. BMC. Microbiol. 6: 12.


Silverman, D.J., C.L. Wiseman, and J.R.A. Waddell. 1980. In vitro studies of 
*Rickettsia*-host cell interactions: ultrastructural study of *Rickettsia prowazekii*-infected 

in situ hybridization technique for detection of *Rickettsia* spp. in archival samples. J. 


Szollosi, Saxena, R. 2010. Role of special histochemical stains in staining 

RNA hybridization using technovit resin in *Arabidopsis thaliana*. Plant. Mol. Biol. 17: 
43-51.

*Rickettsia tsutsugamushi* in a new genus, *Orientia* gen. nov., as *Orientia tsutsugamushi* 

Koropatnick, T.A., J.T. Engle, M.A. Apicella, E.V. Stabb, W.E. Goldman, and 
M.J. McFall-Ngai. 2004. Microbial factor-mediated development in a host-bacterial 
mutualism. Science 306: 1186-1188

Thrall, P.H., M.E. Hochberg, J.J. Burdon, and J.D. Bever. 2006. Coevolution of 
symbiotic mutualists and parasites in a community context. Trends. Ecol. Evol. 22: 120- 
126.


