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Infection of *Ixodes scapularis* ticks with *Rickettsia monacensis* expressing green fluorescent protein: A model system

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Abstract

Ticks (Acari:Ixodidae) are ubiquitous hosts of rickettsiae (Rickettsiaceae:*Rickettsia*), obligate intracellular bacteria that occur as a continuum from nonpathogenic arthropod endosymbionts to virulent pathogens of both arthropod vectors and vertebrates. Visualization of rickettsiae in hosts has traditionally been limited to techniques utilizing fixed tissues. We report epifluorescence microscopy observations of unfixed tick tissues infected with a spotted fever group endosymbiont, *Rickettsia monacensis*, transformed to express green fluorescent protein (GFP). Fluorescent rickettsiae were readily visualized in tick tissues. In adult female, but not male, *Ixodes scapularis* infected by capillary feeding, *R. monacensis* disseminated from the gut and infected the salivary glands that are crucial to the role of ticks as vectors. The rickettsiae infected the respiratory tracheal system, a potential dissemination pathway and possible infection reservoir during tick molting. *R. monacensis* disseminated from the gut of capillary fed *I. scapularis* nymphs and was transstadially transmitted to adults. Larvae, infected by immersion, transstadially transmitted the rickettsiae to nymphs. Infected female *I. scapularis* did not transovarially transmit *R. monacensis* to progeny and the rickettsiae were not horizontally transmitted to a rabbit or hamsters. Survival of infected nymphal and adult *I. scapularis* did not differ from that of uninfected control ticks. *R. monacensis* did not disseminate from the gut of capillary fed adult female *Amblyomma americanum* (L.), or adult *Dermacentor variabilis* (Say) ticks of either sex. Infection of *I. scapularis* with *R. monacensis* expressing GFP provides a model system allowing visualization and study of live rickettsiae in unfixed tissues of an arthropod host.

Keywords

Ixodidae; *Rickettsia*; ticks; endosymbiont; GFP; live imaging; tracheal dissemination

1. Introduction

Ticks (Acari: Ixodida) are unique among blood feeding arthropods in that epithelial cells lining the midgut lumen phagocytose and digest the blood meal intracellularly. That process is presumably significant to the close association of ticks with obligate intracellular bacteria within the order Rickettsiales (Balashov, 1972; Munderloh and Kurtti, 1995). The genus *Rickettsia* includes spotted fever group (SFG) pathogens of vertebrate and tick hosts such as *Rickettsia rickettsii* and *Rickettsia conorii* (Niebylski et al., 1999; Santos et al., 2002), as well

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as nonpathogenic endosymbionts such as *Rickettsia peacockii* that are maintained in ticks by transovarial transmission (TOT) and are believed to prevent ovarian superinfection by pathogenic rickettsiae (Burgdorfer et al., 1981; Niebylski et al., 1997; Azad and Beard, 1998; Macaluso et al., 2002; de la Fuente et al., 2003). The physiological mechanisms that mediate rickettsial interactions with arthropod hosts are poorly understood. Rickettsiae are notoriously difficult research subjects due to their small size, slow growth, instability outside host cells and resistance to genetic manipulation. Moreover, the pioneering efforts of early 20th century researchers to establish tick host models of rickettsial infection dynamics (Harden, 1990) have been only minimally improved upon (Hayes and Burgdorfer, 1989). Recent advances in genetic manipulation of rickettsiae, coupled with development of improved host model systems, should lead to a better understanding of the physiology of rickettsial host range, tissue tropisms, transmission dynamics, pathogenesis versus symbiosis and superinfection resistance in arthropods.

Rickettsiologists have relied on stain-based light microscopy, transmission electron microscopy (TEM) and immunological detection methods to observe rickettsiae within host cells and tissues. Because those techniques detect fixed rickettsiae in fixed cells, they cannot fully define the interactions of rickettsiae and hosts, a task that will ultimately require live tissue techniques. Transformation of rickettsiae to express fluorescent markers will allow study of live rickettsiae within live host tissues, analogous to the advances achieved with such techniques in study of other arthropod-borne pathogens. For example, transformation with variants of the green fluorescent protein (GFP) reporter gene from the jellyfish, *Aequorea victoria* (Chalfie et al., 1994) allowed characterization of tissue distributions and infection dynamics of GFP-labeled arboviruses in mosquito hosts (Brault et al., 2004; Foy et al., 2004), of *Leishmania* protozoans in phlebotomine sand flies (Guevara et al., 2001) and of trypanosomes in tsetse flies (Bingle et al., 2001) and triatomine bugs (Guevara et al., 2005). Imaging of the movement of GFP-labeled malarial parasites in live mosquito hosts (Frischknecht et al., 2004; Vlachou et al., 2004) demonstrated the full power of this technology.

In the first reported studies of transgenic fluorescent bacteria in ticks, Ceraul and colleagues (2002) described evidence for encapsulation/nodulation of GFP-labeled *Escherichia coli* injected into *Dermacentor variabilis* ticks, while Matsuo and colleagues (2004) described clearance from the midgut of GFP-labeled *E. coli* ingested by *Ornithodoros moubata* ticks. However, *E. coli*, unlike rickettsiae, is not a normal constituent of the tick microflora and does not grow intracellularly. The recent development of electroporation techniques and transposon-based transformation vectors for rickettsiae (Rachek et al., 1998; Qin et al., 2004; Baldrige et al., 2005) has made their genetic manipulation a reality.

In this report, we describe the evaluation and selection of *Ixodes scapularis* from three North American tick species as a model host for *Rickettsia monacensis* transformed to express GFP (Baldrige et al., 2005). *R. monacensis* is a SFG rickettsial endosymbiont isolated from the European sheep tick, *Ixodes ricinus* (Simser et al., 2002). Phylogenetic analyses have shown that *R. monacensis* and related rickettsiae detected in *I. ricinus* populations by molecular means (Sreter-Lancz et al., 2005 and references therein) form a separate cluster within the SFG group distinct from its known pathogenic members (Ishikura et al., 2002, 2003; Kurtti et al., unpublished). *I. scapularis* is a member of the *I. ricinus* species complex (Keirans et al., 1999) and has been shown by phylogenetic analyses to be very closely related to *I. ricinus* (Fukunaga et al., 2000; Xu et al., 2003). The two ticks are morphologically and behaviorally similar and, along with *I. pacificus* and *I. persulcatus*, are the principal vectors of the Lyme Disease agent *Borrelia burgdorferi* and related *Borrelia* in the northern hemisphere (Keirans et al., 1999; Fukunaga et al., 2003).

We infected ticks by capillary feeding rickettsiae to adults and nymphs and by immersion of larvae in a rickettsial suspension. Fluorescent *R. monacensis* were readily visualized by epifluorescence microscopy (EFM) in dissected, unfixed tick tissues but could not be observed through the strongly autofluorescent tick cuticle. In adult female *I. scapularis*, but not in *Amblyomma americanum* or *Dermacentor variabilis*, *R. monacensis* disseminated from the gut and infected the salivary glands that mediate transmission to vertebrate hosts. *R. monacensis* was transstadially transmitted (TST) in *I. scapularis* and was maintained in apparently healthy adult ticks for up to 6 months. We did not obtain evidence for transovarial transmission (TOT), possibly due to superinfection resistance. *R. monacensis* infected the tick tracheal system that surrounds and penetrates tissues within the tick hemocoel. The tracheal system could potentially serve as a dissemination pathway and infection reservoir during the tick life cycle. Infection of *I. scapularis* with GFP-labeled *R. monacensis* provides a new model system for study of the interactions of a rickettsial endosymbiont with a tick host.

Materials and Methods

2.1 Rickettsiae

Rickettsia monacensis, isolated from *I. ricinus* ticks (Simser et al., 2002) and transformed with the GFPuv gene (Rmona658) (Baldrige et al., 2005), was grown in the *I. scapularis* ISE6 cell line maintained in L-15B300 medium (Munderloh et al., 1999). To isolate Rmona658 for infection of ticks, lysates of infected ISE6 cells were prepared as described (Baldrige et al., 2005) and centrifuged at 500 x g for 5 min to pellet remaining cells and large debris. The supernatants were sequentially passed through 5.0 and 2.7 μ m syringe filters to remove fine debris and rickettsiae were collected from filtrates by centrifugation at 18,400 x g, 4 °C for 5 min. Rickettsiae were resuspended in RPMI1640 medium supplemented with 10% fetal bovine serum for tick capillary feeding experiments or in L-15B300 medium for larval immersion.

2.2 Ticks and infection with rickettsiae

Ixodes scapularis ticks were collected from hunter-killed deer at Camp Ripley, MN. Engorged females were used to found a colony maintained under a 16 hr light/8 hr dark photoperiod at 22 °C in shell vials held in glass desiccators above a saturated aqueous solution of K₂SO₄ that provided a relative humidity of 97% (Kurti et al., 1996). *Dermacentor variabilis* ticks were collected by flagging near Forest Lake, MN. Blood fed adult and larval *Amblyomma americanum* ticks were collected from dogs near Montrose, IA.

To infect ticks by feeding, drawn-out glass microcapillary pipets loaded with RPMI medium containing Rmona658 at $2 - 6 \times 10^5/\mu$ l were placed over hypostomes of ticks immobilized in Petri dishes as described for infection with *Borrelia* (Korshus et al., 2004). Following 3 – 4 hr feeding at 34 °C, ticks were recovered into shell vials and returned to colony maintenance conditions. To infect larvae by immersion, ticks were placed in L-15B300 medium containing Rmona658 at $8 \times 10^6/\mu$ l, vortexed at medium speed for 5 s and incubated at 34 °C for 30 min. The larvae were surface disinfected by immersion in a 0.1% bleach solution for 2 min, washed in distilled H₂O and returned to colony maintenance conditions.

2.3 Tick feeding on animal hosts and tissue collection

For TST, TOT and horizontal transmission studies, larvae and nymphs were fed on hamsters and adults on a rabbit. Engorged ticks were surface disinfected and returned to colony maintenance conditions as above. Six weeks after feeding, blood was collected by cardiac puncture from hamsters killed by exposure to CO₂. Two weeks after feeding, rabbit liver, spleen, and heart tissue samples were collected. Approximately 0.5 g of each tissue were minced under sterile conditions and inoculated individually into tick ISE6 and monkey RF/6A cell cultures in 25 cm² flasks to attempt culture reisolation of rickettsiae. All animals were

maintained in facilities in accordance with NIH and University of Minnesota animal rearing regulations.

2.4 Epifluorescence microscopy (EFM)

To assess rickettsial infection of tick tissues by EFM, ticks were individually dissected and tissues were mounted on glass slides in Vectashield mounting medium (Vector Labs, Burlingame, CA.). Fluorescent rickettsiae were visualized on a Nikon Eclipse E400 microscope equipped with epifluorescent illumination, FITC and dual FITC/TexasRed filter sets and a DXM 1200 digital camera coupled to the ACT-1 imaging program (Nikon, Melville, NY).

2.5 Polymerase Chain Reaction (PCR) assays

To assess rickettsial infection of tick tissues by PCR, DNA template samples were prepared from unengorged larvae and nymphs homogenized in 1.5 ml microfuge tubes containing 50 µl of STE buffer supplemented with proteinase K as described by Noda et. al. (1997). We used the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN.) according to the manufacturer's protocol to prepare DNA from adult ticks and rabbit tissue samples (tissue protocol) or from cultured cells (cell protocol). All PCR assays used Taq polymerase (Promega, Madison, WI) in 50 µl reactions with the manufacturer's suggested buffer and nucleotide concentrations. Primers were from Integrated DNA Technologies (Coralville, IA) and sequences are shown in Table 1. Presence of rickettsial DNA in tissue and cultured cell extracts was detected with the primer pair RpCS.877p and RpCS.1273r yielding a 381 bp product specific for the rickettsial citrate synthase gene (*gltA*) and primer pair 190-70p and 190-602n yielding a 532 bp product specific for the SFG rickettsial *ompA* gene (Regnery et al., 1991). Cycling conditions for both primer pairs were as described (Baldrige et al., 2004). Presence of Rmona658 DNA in samples was detected with primer pair CATF and CATRC yielding a 660 bp product specific for the chloramphenicol acetyltransferase gene and primer pair GFPuvF and GFPuvR yielding a 720 bp product specific for the green fluorescent protein gene. Cycling conditions for both primer pairs were as described (Baldrige et al. 2005). In some cases, GFP and CAT PCR reaction products were re-amplified (annealing temperature of 52 °C) with nested primer pairs nGFPuvF/nGFPuvRC and nCATF/nCATR yielding 400 and 355 bp products, respectively. As a control for presence of intact PCR-amplifiable DNA and absence of PCR inhibitors in tissue extracts we used primer pair 16S+1 and 16S-1 specific for a 460 bp tick mitochondrial 16S rRNA gene product (Black and Piesman, 1994). Cycling conditions were: one cycle of 95 °C for 5 min, 10 cycles of 92 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min 30 sec, 32 cycles of 92 °C for 1 min, 54 °C for 35 sec, 72 °C for 1 min 30 sec, followed by a final 72 °C, 7 min cycle.

2.5 Southern blot and Western immunoblot assays

We confirmed the validity of nested primer PCR reactions indicating presence of Rmona658 in tissue extracts by Southern blot analysis. Aliquots of first round PCR reactions used as nested PCR reaction templates were electrophoresed, blotted and hybridized with digoxigenin-labeled probes as described (Baldrige et al., 2005). Hamsters used to blood feed Rmona658-infected nymphs were analyzed for evidence of infection with Rmona658 by Western immunoblotting. Purified *R. monacensis* protein extracts were prepared and blotted as described (Baldrige et al., 2005). Sera from hamsters used to blood feed Rmona658-infected ticks were diluted 100-fold in PBS with 3% bovine serum albumin and incubated with the blots overnight. Hamster antibodies bound to rickettsial proteins were detected with anti-hamster goat secondary IgG antibody complexed to horseradish peroxidase as described (Baldrige et al., 2005).

3. Results

3. 1 Initial assessment of *Amblyomma*, *Dermacentor* and *Ixodes* ticks as Rmona658 hosts

To initiate development of a model system for study of interactions of Rmona658 with a tick host by EFM, we capillary fed Rmona658 to adult *Amblyomma*, *Dermacentor* and *Ixodes* tick species known to host SFG rickettsiae in N. America. Preliminary work showed that the optical limits of EFM and the combined effects of strong fluorescence from the tick cuticle and guanine accumulated as a nitrogenous waste product within ticks prevented imaging of Rmona658 in intact ticks. All results described below were obtained from analysis of dissected tick tissues.

Three adult female *A. americanum* ticks collected as partially engorged ticks from a dog each ingested approximately 1.5 µl of medium containing Rmona658. Live single ticks were dissected and examined at 1, 2 and 3 weeks post-capillary feeding (PCF). At 1 week PCF, fluorescent Rmona658 were abundant throughout the midgut versus an estimated 10 – 100-fold fewer but still widely distributed rickettsiae in 2 week PCF gut. In 3 week PCF gut tissue, rickettsiae were rare and occurred in isolated areas (Fig. 1A and B). Rmona658 were not observed in any other tissue. Four of ten nymphs that had recently molted from larvae engorged with dog blood survived and were examined at 1 week PCF. Rmona658 were visible in midgut tissue only of 1 nymph. The results suggested that Rmona658 replicated poorly within and did not disseminate from the gut of partially fed adult or unfed nymphal *A. americanum*.

Of 47 unfed mixed sex adult *D. variabilis* collected by dragging, 45 survived 2 days PCF. At 1 week PCF, all remaining ticks were alive and of 7 examined, fluorescent Rmona658 that were primarily intracellular were observed in midguts only of 1 female and 1 male. At 2 weeks PCF, 2 ticks were dead and of 13 live ticks examined, 8 females and 2 males contained Rmona658 in midguts only. At 4 weeks PCF, 12 ticks were dead and 6 females and 3 males among the 11 survivors contained Rmona658 in midgut tissue only. We noted extensive tissue damage and presence of many extracellular rickettsiae in midguts of the most heavily infected ticks. In contrast to the *Amblyomma* ticks, the relative abundance of Rmona658 in *Dermacentor* midguts increased with time and replicating (doublet forms) as well as dense clusters of rickettsiae were common in the 2 and 4 week PCF ticks (Fig. 1C and D). The results suggested that Rmona658 replicated within and probably damaged the midgut of adult *D. variabilis* but did not disseminate to other tissues.

Of 15 unfed mixed sex adult *I. scapularis* collected from deer, 14 survived at two days PCF. At 1 week PCF, 7 of 11 survivors were examined and fluorescent Rmona658 were observed in midguts of 3 males and 3 of 4 females (Fig. 1E). At 2 weeks PCF, midguts of 2 of the 4 remaining females contained abundant Rmona658 that often occurred as replicating forms and in dense clusters (Fig. 1F and H). We observed some particularly dense clusters of Rmona658 in close proximity to tracheae associated with the gut (Fig. 1F). Areas of the gut containing high concentrations of hematin granules from blood ingested and digested during the nymphal stage contained fewer Rmona658 relative to gut tissue containing less hematin (Fig. 1G and H). We observed Rmona658 in salivary gland acini of a single female tick at 1 week PCF and in a second female at 2 weeks PCF (Fig. 1I and J). The rickettsiae were widely distributed within the acini as single and replicating doublet form cells. The dense clusters of Rmona658 observed in midguts were absent. In both of the 2 week PCF female ticks with midgut infections, we observed Rmona658 infection of the tracheal network. The rickettsiae were present in large tracheae with chitinous taenidia secreted by the surrounding epithelial cells and in small tracheoles without taenidia (Fig. 1K and L). In both cases dividing cell forms and clusters of rickettsiae were present, indicating active replication in epithelial cells of the tracheal network. Rmona658 were not observed in any other tissue.

We also assessed potential pathogenicity of Rmona658 capillary feeding dose to unfed male *I. scapularis* collected from deer. We capillary fed four groups of 26 males each with medium containing 0, or 5.6×10^3 , 10^4 or 10^5 rickettsiae per μl . An additional group of 20 ticks was included as a no treatment control. Over a period of 3 weeks, ticks died at approximately the same rate in all treatment groups. At 3 weeks PCF, the mean mortality was 74.6% and ANOVA indicated no significant differences between groups ($P = 0.995$). Infection rates, determined by PCR using CATF and CATRC primers, of dead and surviving ticks fed at the 10^3 , 10^4 and 10^5 concentrations were 100, 88 and 100 %, respectively (data not shown). EFM examination of tissues from the 26 surviving rickettsiae-fed males demonstrated Rmona658, including dividing forms, in healthy midguts of 23 ticks and within gut-associated tracheae of 1 of those ticks (data not shown). Rmona658 were not found in any other tissue.

Our preliminary results demonstrated that EFM could be used to visually monitor Rmona658 infection in unfixed tissues of dissected ticks. In unfed adult female *I. scapularis* infected with Rmona658 by capillary feeding, the rickettsiae were capable of disseminating from the midgut to infect the tracheal system and salivary glands. Rmona658 did not disseminate from the midgut of unfed adult male *I. scapularis*, unfed adult *D. variabilis* of either sex or partially fed adult female *A. americanum* under our test conditions. We selected *I. scapularis* for further experiments to evaluate the tick's potential as a model host for *R. monacensis* expressing GFP.

3. 2 Infection of nymphs and transstadial transmission (TST) to adults

To test for Rmona658 infection of *I. scapularis* nymphs and TST to adults, we capillary fed 95 laboratory colony F1 nymphs with medium containing Rmona658. At 2 days PCF, 67 nymphs (71%) survived and 45 were examined by EFM at 5 – 7 days PCF. In 10 nymphs, Rmona658 were not observed and in 11 they were present only in the midgut. In 24 nymphs, Rmona658 were present as replicating forms in both midgut (Fig. 2A) and salivary glands or tracheae, indicating an infection rate of 52% in nymphs sacrificed for assay and a disseminated infection in 69% of infected nymphs. All infected tissues appeared healthy. We infested a hamster with the remaining 22 nymphs and recovered 11 engorged nymphs of which 10 survived and molted into morphologically normal adult ticks approximately 6 weeks later. At 4 days post-eclosion, the adults were examined by EFM for evidence of TST. Rmona658 were observed in healthy midguts of 2 of 4 males, in 4 of 6 females and in salivary glands and tracheal tissue of one of the infected females, indicating a 60% TST infection rate. Rmona658 in the salivary glands were again widely dispersed in the acini as single and replicating doublet form cells (Fig. 2B). We also observed Rmona658 infection of salivary gland ducts as single and replicating doublet cells (Fig. 2C) and as dense clusters of replicating cells (Fig. 2D and E). In complexes of midgut associated with tracheae, we observed Rmona658 as massive clusters of replicating cells (Fig. 2F). The results demonstrated disseminated Rmona658 infection in *I. scapularis* nymphs infected by capillary feeding, TST to both male and female adult ticks, and disseminated infection in an adult female tick infected by TST.

To further test for infection mortality as well as long-term infection maintenance, we capillary fed 100 laboratory colony F1 nymphs with Rmona658 in medium and 52 control nymphs with medium only. Two days PCF, 69 (69%) of Rmona658-fed and 37 (71%) of control nymphs survived, indicating no immediate infection induced mortality. At 3 weeks PCF, 35 (51%) of Rmona658-fed and 20 (54%) control nymphs survived, again indicating no Rmona658 induced mortality. Hamsters were infested with the nymphs. After 1 week, 27 (77%) Rmona658 fed and 14 (70%) control engorged nymphs were recovered. All molted to morphologically normal adults after approximately 1 month. After 6 months, 7 male and 7 female Rmona658 adults (52%) compared to 5 male and 4 female control adults (64%) survived. Rmona658 were observed by EFM in healthy midguts of 1 male (Fig. 2G) and 1 female tick (Fig. 2H) and in salivary glands of the female. In both ticks, Rmona658 tended to occur in the midguts as clusters

rather than as the widely dispersed single and double cell forms typical of initial infections in adult ticks (Fig. 1H). The tracheal system of the female, but not the male tick, was massively infected with Rmona658 occurring in main and lateral trunk tracheae and in tracheoles (Fig. 2I and J). To further assess the rate of TST to the 14 adult ticks surviving from Rmona658 fed nymphs, tissue extracts were used as template for PCR assays. Using primers specific for the tick mitochondrial 16S rRNA gene, the predicted 460 bp product was obtained from extracts of all 14 adults (Fig. 3A, lanes 1 – 14), demonstrating presence of PCR competent DNA. Using Rmona658-specific GFP primers, only the EFM-positive female tick described above yielded a predicted first-round PCR 720 bp product (data not shown). However, nested PCR primer reactions using first round reactions as template yielded the predicted 400 bp product from the EFM-positive female and male ticks above and additional female and male ticks (Fig. 3B, lanes 3, 4, 8 and 9). Southern blot analysis of first round GFP PCR reactions confirmed results of the nested PCR analysis and indicated presence of Rmona658 in a third female tick (Fig. 3C, lanes 3, 4, 8, 9 and 10). In sum, the results indicated little or no lethal effect of capillary fed Rmona658 on *I. scapularis* nymphs, demonstrated maintenance of a disseminated TST infection in a 6 month post-eclosion adult female tick, and demonstrated TST to 36% of adult ticks assayed 6 months post-eclosion.

3.3 Infection of larvae and transstadial transmission to nymphs

To infect larval *I. scapularis* and test for TST to nymphs we used the immersion infection technique developed with *Borrelia burgdorferi* (Policastro and Schwan, 2003). Approximately 60% of 500 laboratory colony F1 larvae immersed and vortexed in medium containing Rmona658 survived. None of 10 larvae examined by EFM 24 hr later or 20 larvae examined a week later contained detectable Rmona658. PCR assays were not conducted due to probable surface contamination with Rmona658. Hamsters were infested with the remaining larvae and 111 engorged larvae were recovered of which 100 (90%) molted to nymphs. Rmona658 were not observed in 10 nymphs examined by EFM and tissue extracts were then prepared from 18 nymphs to screen for presence of Rmona658 by PCR analysis. All nymphal tissue extracts were PCR positive for the tick 16S mitochondrial rRNA gene (Fig. 3A, lanes 1 – 18), verifying presence of PCR competent DNA. None of the extracts yielded the predicted 660 bp product in first round PCR reactions using Rmona658-specific CAT gene primers (data not shown). However, 4 of the extracts yielded the predicted 355 bp product in PCR reactions using nested CAT gene primers (Fig. 3B, lanes 4, 6, 7 and 16). Southern blot analysis of first round CAT PCR reactions confirmed results of the nested PCR analysis and indicated presence of Rmona658 in a fifth nymph (Fig. 3C, lanes 4, 6, 7, 12 and 16). The results demonstrated TST of Rmona658 to 28% of the 18 nymphs analyzed.

3.4 Transovarial (TOT) and horizontal transmission tests

To test for TOT of Rmona658 in *I. scapularis*, we capillary fed 26 laboratory colony F1 adult female ticks with medium containing Rmona658 and maintained the 20 (77%) two-day PCF survivors for 1 month to allow dissemination of infection. Four ticks were sacrificed at 1 month PCF and examined by EFM. Fluorescent Rmona658 were observed in tracheal tissue of 1 tick and in healthy midgut and salivary gland tissues of all 4 ticks but not in ovarian tissues. A rabbit was infested with the remaining 16 capillary fed female ticks along with male ticks that had not been fed Rmona658. After 1 week, we recovered 2 partially and 2 completely engorged female ticks. One small and two large egg masses were obtained from three of the engorged ticks and EFM demonstrated abundant fluorescent Rmona658 in hemolymph and degenerate tissue of all 3 ticks and in intact tracheal tissue of 1 tick (data not shown). The tick that did not oviposit harbored a disseminated Rmona658 infection but we did not observe Rmona658 in intact ovarian tissue. However, Giemsa-stained ovarian tissue smears revealed that the ovaries were heavily infected with bacteria having morphology consistent with that of SFG rickettsiae (Fig. 2K). Six weeks after oviposition, larvae hatched from the two large egg masses.

Fluorescent Rmona658 were not observed in 16 dissected larvae from each egg mass. Both first round and nested PCR assays, using Rmona658-specific CAT and GFP primers and DNA extracts from 4 pools of 10 larvae from each egg mass (80 larvae total) were negative (data not shown).

In view of rickettsial reactivation by bloodmeal (Hayes and Burgdorfer, 1982) and our demonstration of Rmona658 TST from larvae to nymphs (above), we infested hamsters with the remaining larvae and recovered nymphs after 1 month. Fluorescent Rmona658 were not observed in 20 dissected nymphs from each egg mass but presence of rickettsial DNA in 11 of 12 single nymph tissue extracts was indicated by positive PCR assay results using the general citrate synthase gene-specific RpCS.877p and RpCS.1273r PCR primer pairs (data not shown). PCR assays using the pan-SFG rickettsia 190–602n and 190–70p primer pair specific for the *rompA* gene indicated presence of SFG rickettsial DNA in tissue extracts of 3 pools of 5 nymphs each from each egg mass (Fig. 5, lanes P1 to P6, 532 bp band) and in all tissue extracts of 10 individual nymphs from each egg mass (lanes N1 to N20). However, PCR assays of the same DNA extracts using first round followed by nested CAT and GFP primers specific for Rmona658 and Southern blot analyses of the nested PCR reactions were negative, demonstrating that the SFG rickettsiae present in the nymphs were not Rmona658. The results demonstrated disseminated Rmona658 infections in capillary fed adult female *I. scapularis* ticks, the presence of SFG rickettsiae in their progeny, and that Rmona658 had not undergone TOT.

3.5 Horizontal Transmission Analysis

None of the animals used to blood feed Rmona658-infected ticks became visibly ill. Sera of 5 hamsters used to blood feed Rmona658-infected nymphs were tested by Western immunoblot analysis for evidence of infection with Rmona658. None of the sera reacted with protein extracts prepared from purified *R. monacensis* (data not shown). Heart, liver and spleen removed from the rabbit used to blood feed Rmona658-infected adult ticks were minced under sterile conditions and inoculated separately into tick ISE6 and monkey RF/6A cell cultures. The cultures were monitored for 6 weeks by phase contrast microscopy for rickettsiae, which did not appear. PCR assays, using Rmona658-specific CAT and GFP primers, of DNA extracts from each culture and from minced tissues used as inocula as well as rabbit blood further indicated that Rmona658 was not present (data not shown). The results suggested that Rmona658 did not establish infection in small mammal hosts as a result of horizontal transmission by *I. scapularis* nymphs and adults.

4. Discussion

Establishment of specific rickettsial infections in ticks by feeding on rickettsemic animals (Niebylski et al., 1999) is complicated by use of hosts with different rickettsial titers and variable immune status as well as potential antimicrobial activities derived from blood digestion products (Sonenshine et al., 2005). Intracelomic inoculation of rickettsial suspensions (Santos et al., 2002) is complicated by injury and the wound response that includes induction of the innate immune system (Nakajima, et al., 2005). Infection by capillary feeding (Macaluso et al., 2001) avoided such complications.

In contrast to the successful infection of *A. americanum* nymphs by intracelomic inoculation of *Rickettsia parkeri* and subsequent TST to adults (Goddard, 2003), our results suggested that capillary feeding of *R. monacensis* to *A. americanum* or to freshly molted nymphs did not result in establishment of infection. Macaluso and colleagues (2001) capillary fed *Rickettsia montanensis* and *Rickettsia rhipicephali* to *D. variabilis* and demonstrated disseminated infections, but capillary feeding of *R. monacensis* to *D. variabilis* did not result in disseminated infections. However, *I. scapularis*, supported disseminated infections and TST. The infection

characteristics of *Borrelia burgdorferi* in the same three ticks resemble those of *R. monacensis* and may be determined by differences in innate and blood meal-derived immune factors and presence or absence of appropriate receptor/ligand interactions between host and microbe (Soares et al., 2006).

Rickettsial interactions with tick hosts have been most thoroughly studied in *Dermacentor andersoni* infected with the virulent pathogen, *R. rickettsii*, and/or the nonpathogenic endosymbiont, *R. peacockii* (reviewed in Marchette, 1982; Hayes and Burgdorfer, 1989). *R. peacockii* is the most closely related of SFG rickettsiae to *R. rickettsii* (Baldridge et al., 2004), suggesting that subtle differences may underlie pathogenicity versus nonpathogenicity. In adult female *D. andersoni*, *R. peacockii* primarily infects ovaries and midgut posterior lobes but is not found in adult males. It infects salivary glands of some larvae, but not those of nymphs or adults and is thus primarily acquired and maintained by TOT. It may exclude ovarian infection by *R. rickettsii* (Burgdorfer et al., 1981; Niebylski et al., 1997). In contrast, *R. rickettsii* is acquired by blood feeding or TOT and can be widely distributed in tissues of both sexes. It is highly pathogenic to ticks, implying a major role for horizontal transmission in its maintenance and little or no significant contribution by TST (Burgdorfer et al., 1981; Hayes and Burgdorfer, 1982; Niebylski et al., 1999). Our analysis of *R. monacensis* infection dynamics in *I. scapularis* revealed characteristics that shared aspects of the endosymbiotic and pathogenic rickettsial infection paradigms described above.

Consistent with the endosymbiotic paradigm, *R. monacensis* infection of *I. scapularis* did not result in the obvious tissue damage and high mortality caused by the spotted fever pathogens *R. rickettsii* in *D. andersoni* (Niebylski et al., 1999) and *R. conorii* in *Rhipicephalus sanguineus* (Santos et al., 2002). However, infection and replication within midgut epithelial cells usually occurred throughout that tissue rather than being confined to its posterior lobes as in *R. peacockii* infections. In ticks harboring pathogenic rickettsiae, the salivary glands are among the most heavily infected tissues (Santos et al., 2002). In adult female ticks, but not in males, *R. monacensis* disseminated from the gut and infected salivary gland acini and ducts, although we have not demonstrated presence of *R. monacensis* within the duct lumen. Hamsters inoculated with *R. monacensis* raised a strong antibody response (Simser et al., 2001) but in this study we observed no evidence of infection in a rabbit or hamsters used to blood feed infected ticks or for an immune response by the hamsters. Recently, another SFG member not associated with disease, *R. massiliae* strain Bar29, was shown to infect the salivary glands of field collected *Rhipicephalus turanicus* (Matsumoto et al., 2005). Sera of eight spotted fever patients reacted to antigens of both *R. conorii* and *R. massiliae* (Cardenosa et al., 2003). Reactivity of polyclonal sera to heterologous rickettsial antigens is well documented and interferes with assessment of epidemiological situations.

Transstadial transmission is important in the maintenance of rickettsial endosymbionts in ticks. We demonstrated TST of *R. monacensis* from *I. scapularis* larvae to nymphs and from nymphs to adults of both sexes that was not accompanied by the high mortality and developmental abnormalities observed in TST of *R. conorii* in *Rh. sanguineus* (Santos et al., 2003). We did not obtain evidence for TOT of *R. monacensis* in *I. scapularis*. Rickettsial endosymbionts closely related to *R. monacensis* (Ishikura et al., 2002, 2003; Kurtti; unpublished) occur in *I. scapularis* populations throughout the United States (Benson et al., 2004). In *I. scapularis* collected in or near Minnesota, the endosymbionts were restricted to ovaries and underwent TOT to larvae and TST to females, but not males (Noda et al., 1997). Because endosymbionts occur at near 100% incidence in female ticks from the source population for our experiments (Kurtti et al., 2005), we speculate that ovarian superinfection resistance may explain our inability to demonstrate TOT of *R. monacensis* in *I. scapularis*. *R. monacensis* did not infect testicular tissues, consistent with absence of *I. scapularis* rickettsial endosymbionts in testicular

tissues (Noda et al., 1997) and in contrast to presence of *R. rickettsii* in *D. andersoni* and *R. helvetica* in *I. ricinus* testicular tissues (Hayes, et al., 1980).

Ultrastructural TEM studies have led to reports of “rickettsia-like organisms” (RLOs) within transverse sectioned tracheae in Ixodid ticks (Tarasevich and Rehacek, 1972; Rehacek et al., 1976; Sutakova and Rehacek, 1989) and insects (Huger, 1964; Mitsuhashi and Kono, 1975; Adams and Azad, 1990). In longitudinally sectioned Siphonapteran tracheae, RLOs, later suggested to be *Rickettsia felis* (Bouyer et al., 2001), were observed in epithelial cells that surround and secrete the chitinous tracheae (Ito and Vinson, 1980). We have shown that *R. monacensis* infected the tracheal system in *I. scapularis* and disseminated from the midgut of female ticks to the salivary glands. Hayes and Burgdorfer (1989) proposed that structural changes induced by blood meal digestion allow rickettsiae to penetrate the gut basal lamina and enter the hemocoel to infect hemocytes that disseminate the infection to other tissues. Our demonstration of *R. monacensis* infection of the tick tracheal system suggests that it might serve as a dissemination pathway for rickettsiae. That hypothesis derives support from the demonstrations that nuclear polyhedrosis viruses disseminate from the gut of larval Lepidoptera via the tracheal system (Engelhard et al., 1994; Barret et al., 1998; Rahman and Gopinathan, 2004) and that arboviruses penetrate the mosquito gut basal lamina to disseminate via tissue conduits involving tracheae and visceral muscle (Romoser et al., 2004; Scholle et al., 2004). The epithelial cells that surround and secrete tracheae are metabolically active and envelope and penetrate tissues with high oxygen demand (Wigglesworth, 1972), a characteristic of the well-tracheated tick midgut (Fielden et al., 1999). The tracheal system ramifies to all internal tissues, thus offering rickettsiae a protected intracellular environment for dissemination to other tissues. Tracheal tissue might also serve as an infection reservoir during tick morphogenesis. Tick larvae are atracheate. As they prepare to molt, future tracheal epidermal cells enlarge and fuse into multinucleate cells (Balashov, 1972) in which rickettsiae could remain in an intracellular environment during tissue reorganization and then invade other tissues after development of the nymphal tracheal system. At the final molt, the tracheal system remains largely intact in contrast to almost completely regenerated tissues such as the salivary glands (Balashov, 1972). The tracheal system might thus serve as both a rickettsial dissemination pathway and infection reservoir during the tick life cycle.

Infection of *I. scapularis* with *R. monacensis* expressing GFP establishes a new model system for exploration of rickettsial tissue tropisms and infection dynamics.

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Figure 1.

Epifluorescence imaging of GFP transformant *Rickettsia monacensis* (Rmona658) in *Amblyomma americanum*, *Dermacentor variabilis* and *Ixodes scapularis* adult female ticks. Scale bar at lower left in each image indicates 10 μ m and small arrows indicate Rmona658. (A) One week PCF *Amblyomma* gut tissue. (B) Three week PCF *Amblyomma* gut tissue. (C) One week PCF *Dermacentor* gut tissue. (D) Four week PCF *Dermacentor* gut tissue. Large arrow indicates dense cluster of Rmona658. (E) One week PCF *Ixodes* gut tissue. (F) Two week PCF *Ixodes* gut tissue imaged in red fluorescence channel with Rmona658 imaged in green channel. Arrowheads indicate trachea associated with gut. Large arrow indicates dense cluster of Rmona658 associated with trachea. (G and H) Visible light and epifluorescence

images, respectively, of two week PCF *Ixodes* gut. Arrowheads indicate dark areas of dense hematin content. (I and J) Epifluorescence and visible light images, respectively, of two week PCF *Ixodes* salivary gland. Large arrows indicate distal edges of salivary gland acini. (K) *Ixodes* trachea with branch point at right side of image. Large arrow indicates cluster of doublet cell Rmona658. Arrowheads indicate taenidia. (L) *Ixodes* tracheoles (arrowheads) imaged in red fluorescence channel and associated with single and doublet cell (large arrow) forms of Rmona658 imaged in green fluorescence channel.

Figure 2.

Imaging of GFP fluorescent Rmona658 in *I. scapularis* ticks infected as nymphs by capillary feeding. Scale bars at lower left in each image indicate 10 μ m and small arrows indicate Rmona658. (A) One week PCF nymph gut tissue. Large arrow indicates chain of multiple Rmona658 cells. (B) Adult female salivary gland 4 days post-eclosion. Large arrow indicates distal edge of acini and arrowhead indicates salivary gland duct. (C) Adult female salivary gland duct 4 days post-eclosion. Arrowhead indicates junction of main and lateral salivary ducts. (D and E) Fluorescence and visible light images, respectively, of heavily infected adult female salivary gland duct 4 days post-eclosion. Large arrow indicates dense cluster of Rmona658. (F) Heavily infected complex of trachea and gut tissue from 4 day post-eclosion adult female. Large arrow indicates dense mass of Rmona658. Arrowhead indicates hematin granule. (G) Adult male gut tissue at 6 months post-eclosion. Arrowhead indicates hematin granule. (H) Adult female gut tissue imaged in red fluorescence channel at 6 months post-eclosion. Large arrow indicates dense cluster of Rmona658 imaged in green channel.

Arrowhead indicates branching trachea associated with gut. (I) Adult female trunk and lateral tracheae imaged in red fluorescence channel at 6 months post-eclosion with Rmona658 imaged in green channel. (J) Heavily infected adult female trachea imaged in red fluorescence channel at 6 months post-eclosion. Large arrow indicates dense cluster of Rmona658 imaged in green channel. Arrowhead indicates taenidia. Infected tracheoles appear above scale bar. (K) Giemsa-stained adult ovarian tissue smear infected with rickettsia-like bacteria at 6 months post-eclosion.

Figure 3.

PCR and Southern blot detection of Rmona658 transstadial transmission from *I. scapularis* nymphs to adults. (A) PCR amplification of a 460 bp tick mitochondrial 16S rRNA gene product from individual adult male (lanes 1 – 7) and female (lanes 8 – 14) tick tissue extracts. (B) Nested PCR amplification of an Rmona658-specific 400 bp GFP gene product from the same tick extracts as in A. (C) Southern blot detection of a 720 bp GFP gene 1st round PCR product from the same tick extracts as in A and B. Lanes L = 100 bp ladder with 500 bp band indicated at left. Lanes –C = no template and uninfected tick control extract in panel A and panels B and C, respectively. Lanes +C = plasmid template positive control.

Figure 4. PCR and Southern blot detection of Rmona658 transstadial transmission from *I. scapularis* larvae to nymphs. (A) PCR amplification of a 460 bp tick mitochondrial 16S rRNA gene product from individual nymphal tick tissue extracts (lanes 1 – 18). (B) Nested PCR amplification of an Rmona658-specific 355 bp CAT gene product from the same tissue extracts. (C) Southern blot detection of a 660 bp CAT gene 1st round PCR product from the same tick extracts as in A and B. Lanes L, -C and +C as in Fig. 3.

Figure 5. PCR detection of SFG rickettsiae in nymphal progeny of Rmona658-fed adult female *I. scapularis*. Upper and lower panels: amplification of a 532bp SFG rickettsial *ompA* gene product from DNA extracts of 6 pools of 5 nymphs each (lanes P1 to P6) and from 20 individual nymphs (lanes N1 to N20). Lanes L, -C and +C as in Fig. 3.

Table 1

PCR and sequencing primers.

Primer Acronym	Sequence (5' to 3')
190-70p	ATGGCGAATATTTCTCAAAA
190-602n	AGTGCAGCATTCGCTCCCCCT
RpCS.877p	GGGGACCTGCTCACGGCGG
RpCS.1273r	CATAACCAGTGTAAGCTG
CATF	CCCCCATATGGAGAAAAAATCACTGGATATACC
CATRC	AACCGCTTAGCCCCGCCCGCCTGCCACTCATC
nCATF	TCCAATGGCATCGTAAAGAAC
nCATR	CCAGGTTTTACCGTAACACGC
GFPuvF	GTCGACTTAGAGGATCCCCGGGTACCGGTAGAACATATGAG TAAAGGAG
GFPuvRC	GGGGTCTAGACGCTTAGCCTTATTGTAGAGCTCATCCATGCCATG
nGFPuvF	TTCTGTCAGTGGAGAGGGTGAAGGTGATGC
nGFPuvRC	CCATTCTTTTGTGCTGCCGTG
16S+1	CTGCTCAATGATTTTTTAAATTGCTGTGG
16S-1	CCGGTCTGAACTCAGATCAAGT