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Rickettsial Infection in *Dermacentor variabilis* (Acari: Ixodidae) Inhibits Transovarial Transmission of a Second *Rickettsia*

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ABSTRACT This study examined the ability of ticks to maintain multiple species of spotted fever group rickettsiae via transovarial transmission. Using a capillary feeding method, previously established *Rickettsia montana*- and *Rickettsia rhipicephali*-infected cohorts of *Dermacentor variabilis* (Say) were exposed to *R. rhipicephali* and *R. montana*, respectively, in two reciprocal challenge experiments. Eggs collected from individual females, for two successive generations, of each cohort were assessed for rickettsial infection by polymerase chain reaction for each challenge experiment. Assessment of the eggs from challenged ticks identified that both *R. montana*- and *R. rhipicephali*-infected ticks were refractory to their respective challenge rickettsiae. The prechallenged infection rate for both F1 and F2 generations (100%) of the *R. montana*-infected cohort was resistant to transovarial transmission of the second rickettsia species, and only *R. montana* was detected in the eggs of F1 = (50%) and F2 = (74%) challenged females. The *R. rhipicephali*-infected cohort maintained a lower level of infection (20%) in the population and did not transovarially transmit the challenge species, however, detectable levels of infection were lost after the first generation. Second-generation ticks, no longer infected with *R. rhipicephali*, became susceptible to infection with *R. montana* and female ticks ($\approx 4\%$) were able to transmit *R. montana* to their progeny. The resistance of the ovaries to co-infection and apparent host-specific nature of infection suggests that rickettsial infection of tick ovaries may alter the molecular expression of the oocytes so as to preclude secondary infection with other rickettsiae.

KEY WORDS rickettsiae, capillary feeding, transovarial transmission

IXODID TICKS SERVE as reservoirs and vectors for a number of spotted fever group rickettsiae. Currently ten pathogenic species of the spotted fever group associated with ticks have been recognized, in addition to five spotted fever group rickettsiae of unknown pathogenicity (Parole and Raoult 2001). Ticks acquire these bacteria either during feeding on rickettsemic hosts or through transovarial transmission. Infection can occur in mammalian hosts when *Rickettsia*-infected ticks feed on susceptible animals, and uninfected ticks can acquire infection during feeding on rickettsemic hosts (Hayes and Burgdorfer 1989). Transovarial transmission serves as the primary mechanism for maintenance of rickettsiae of low or no pathogenicity (nonpathogenic to certain laboratory animals and/or tick vectors), and may serve a lesser role in the maintenance of pathogenic rickettsiae, as evidenced by the lethal effects of *Rickettsia rickettsii* on the tick (Burgdorfer and Brinton 1975, Niebylski et al. 1999).

The characterization of the tick-rickettsiae relationship by Burgdorfer et al. (1981) revealed that ticks infected with East side agent (identified as *Rickettsia peacockii*; Niebylski et al. 1997) were found to be

refractory for transovarial transmission with *R. rickettsii*. The mechanisms underlying this refractory attribute remain to be elucidated. However, it is suggested that cellular changes in transovarially infected tick ovaries associated with the primary infection were responsible for the interference or blocking of the secondary infection. This study on interspecific competition of rickettsiae in ticks has been reinforced by ecological observations that fewer cases of Rocky Mountain spotted fever (RMSF) have been reported in the eastern Bitterroot Valley where *Dermacentor andersoni* infected with *R. peacockii* were prevalent. In contrast to the East side, more human cases of RMSF were reported on the West side where *D. andersoni* ticks that had much lower *R. peacockii* infection rates occurred. The interference phenomenon was also tested under laboratory conditions in which blockage of transovarial transmission of *R. rickettsii* was observed in ticks infected with either *R. montana* or *R. rhipicephali* (Burgdorfer 1988). These studies corroborate findings from field studies indicating that *R. rickettsii* occurs with a lower frequency in *Dermacentor* ticks in contrast with nonpathogenic rickettsiae.

Previous studies examining the infection and maintenance of rickettsiae in ticks typically used either laboratory animals (Burgdorfer et al. 1981, Niebylski et al. 1999) or needle injection (Burgdorfer and Brinton 1975) to introduce the microorganism into the

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tick. Limitations to these approaches include the requirement to induce rickettsemia in the host animal to allow for tick infection and the potential to cause damage while injecting a tick. Recently, using a capillary feeding technique, we were able to introduce either *R. montana* or *R. rhipicephali* into previously uninfected female *Dermacentor variabilis* ticks and observe the transovarial transmission of rickettsiae imbibed by the fed females to their progeny (Macaluso et al. 2001). Using this method, it was possible to question the ability of *D. variabilis* to maintain multiple species of rickettsiae through transovarial transmission. This study was designed to test the hypothesis that a tick infected with one species of *Rickettsia* will be refractory to the maintenance of a secondary infection, via transovarial transmission.

Materials and Methods

Rickettsiae and Ticks. Frozen aliquots of both *R. montana*- and *R. rhipicephali*-infected Vero cells used in this study were maintained in our laboratory as previously described (Macaluso et al. 2001). *Rickettsia*-infected cohorts of *D. variabilis* were established in a previous study (Macaluso et al. 2001) and maintained separately at Old Dominion University to prevent cross-contamination. Adult ticks used for this study were maintained in a controlled environmental chamber at $27 \pm 1^\circ\text{C}$, 92% RH, and a 16:8 (light:dark) photoperiod. Adult ticks were fed on laboratory rabbits, *Oryctolagus cuniculus*, as described by Sonenshine (1993). All use of animals for this research was done in accordance with protocols approved by the Old Dominion University Institutional Animal Care and Use Committee. The approved protocols are on file in the Old Dominion University Animal Facility Office.

Rickettsial Challenge and Detection. *R. montana*- or *R. rhipicephali*-infected ticks (F1 and F2) were exposed to *Rickettsia*-infected Vero cells via capillary feeding. Female virgin ticks were forcibly detached from rabbits at day 4, weighed, and restrained ventral side up on double-sided sticky tape in the bottom of a large Petri dish. In an environmental chamber, each cohort of ticks was allowed to imbibe either *R. montana*-infected or *R. rhipicephali*-infected Vero cells (2.8×10^6 cells/ml) through a 10- μl microcapillary tube placed over their entire mouthparts. After overnight (≈ 14 h) capillary feeding, ticks were weighed and placed back on the rabbit hosts, using separate rabbits for each treatment. Uninfected *D. variabilis* males were added to the feeding capsules (ratio 1:2 = male:female) and the females were allowed to feed until repletion and naturally detach from the hosts. Replete ticks were weighed and held individually in labeled vials in an environmental control chamber until oviposition. Seven days postoviposition egg masses were weighed for a randomly selected subsample of ticks and comparisons between the treatments were made.

Detection of rickettsiae in ticks and tick eggs was determined by polymerase chain reaction (PCR), us-

ing primers designed from the *metK* genes of *R. montana* and *R. rhipicephali*, as previously described (Macaluso et al. 2001). Genomic DNA was extracted from adult ticks or subsamples from individual egg clutches (≈ 100 eggs) and used as the template for PCR. Amplicons were visualized on a 1% agarose gel stained with ethidium bromide.

Experimental Design. Challenge experiments were conducted in two separate trials, using adults of the F1 and F2 generations of tick cohorts for the first and second trials, respectively. For each trial, virgin female ticks ($n \approx 40$) from each cohort were allowed to feed for 4 days on the rabbit hosts before being removed. Subsamples of ticks ($n \approx 10$ –12) were assessed for prechallenge rickettsial infection rates, whereas the remaining ticks were used for capillary feeding challenge assays. Exposure to the second rickettsiae was done in a reciprocal fashion, *R. montana*-infected ticks were exposed to *R. rhipicephali*- and *R. rhipicephali*-infected ticks were exposed to *R. montana*. *Rickettsia*-infected ticks (F2) exposed to growth medium alone served as a control for assessing biological parameters of the ticks.

Data Analysis. All values for biological parameters are reported as mean \pm SD. Comparisons were made between the mean weights of ticks before and directly after capillary feeding for each cohort, and the mean engorgement and mean egg mass weights. A mean percentage of egg mass to engorgement weight was also calculated for *Rickettsia*-challenged and control ticks in both cohorts. Data for all experiments were analyzed using PC-SAS version 8.0 (SAS Institute 1999). Differences in means were determined using independent sample *t*-tests, meaningfully paired sample *t*-tests, one-way analyses of variance, or two-way analyses of variance. Treatment means were considered statistically significant at the $\alpha = 0.05$ level.

Results and Discussion

Two cohorts, *R. montana*-infected and *R. rhipicephali*-infected, of *D. variabilis* were exposed to the reciprocal rickettsiae via capillary feeding. Biological parameters of female ticks including weight before and immediately after capillary feeding, engorgement weight, and egg mass weights 7 days postoviposition were assessed throughout the two trials (Table 1). Weight loss did occur during capillary feeding for each group during both trials. Statistical differences were not observed between the cohorts during either the F1 ($t = 0.88$; $df = 70$; $P = 0.384$) or F2 ($t = 0.79$; $df = 79$; $P = 0.487$) generations. In this study, the decrease in mean weight after capillary feeding was greater than that previously observed for uninfected *D. variabilis* exposed to *R. montana* (+2.4%), *R. rhipicephali* (–4.6%), or growth medium alone (–2.0%) for ≈ 16 h period (Macaluso et al. 2001). Although the biological significance of these differences remains undefined, it can likely be attributed to the fact that ticks imbibe fluid and salivate during feeding (Gregson 1960). In our hands, it has been determined that the amount of fluid taken up during capillary feeding is quite variable

Table 1. Mean \pm SD weight (mg) for biological parameters of *Dermacentor variabilis* used in capillary feeding challenge experiment

Trial	Group	Capillary feeding		Engorgement weight	Egg mass weight
		Prefed weight	Postfed weight		
I	<i>R. montana</i> -infected	28.1 \pm 9.4	26.9 \pm 8.4 ^a	571.8 \pm 139.7	203.5 \pm 30.1
	<i>R. rhipicephali</i> -infected	27.7 \pm 11.5	25.1 \pm 9.4	467.7 \pm 117.4	170.8 \pm 38.4
II	<i>R. montana</i> -infected	25.9 \pm 6.9	23.4 \pm 6.6 ^a	541.1 \pm 95.0	247.1 \pm 45.4
	<i>R. rhipicephali</i> -infected	32.4 \pm 8.5	29.5 \pm 8.1 ^a	475.5 \pm 158.5	199.5 \pm 95.4
Control	<i>R. montana</i> -infected	14.6 \pm 1.7	12.7 \pm 1.5 ^a	479.5 \pm 179.9	184.7 \pm 65.9
	<i>R. rhipicephali</i> -infected	15.4 \pm 3.4	14.2 \pm 3.3	390.9 \pm 118.4	154.6 \pm 70.7

^a Significant differences in weights after capillary feeding were observed for *R. montana*-infected tick cohorts exposed to rickettsiae (F1, $t = 2.09$; df = 35; $P = 0.044$; F2, $t = 9.11$; df = 40; $P < 0.0001$; Control, $t = 6.09$; df = 8; $P = 0.0003$), and the F2 of the *R. rhipicephali*-infected cohort ($t = 4.72$; df = 39; $P < 0.0001$).

in ticks, ranging from 0.06 μ l to 6.77 μ l, and measuring individual tick weights is not a precise indicator of fluid intake (Macaluso et al. 2001).

In addition to the capillary feeding weights, mean engorgement and egg mass weights of a subsample of *Rickettsia*-infected ticks used in the two challenge trials of this study were also recorded (Table 1). No differences were observed in mean engorgement weight between treatments or trials for either *Rickettsia*-infected cohort. Mean egg mass weights for the *R. montana*-infected cohorts were consistently larger, when compared with the *R. rhipicephali*-infected cohorts, for each trial and treatment (challenged or control). When examining these differences in egg mass weights, there was not a significant cohort-trial interaction ($F = 0.04$; df = 1,40; $P = 0.841$), or significant differences between the cohorts ($F = 2.68$; df = 1,40; $P = 0.109$) or between the trials ($F = 0.73$; df = 1,40; $P = 0.398$). Likewise, no significant differences ($t = 2.05$; df = 28; $P > 0.05$) in mean percentages of egg mass weights relative to engorgement weights were observed. However, the individual percentages of egg mass weights relative to engorgement weights varied greatly within both the *R. montana*-infected (20.0%–59.4%) and *R. rhipicephali*-infected (6.5%–63.1%) cohorts for both *Rickettsia*-challenged and control treatments.

A deleterious effect to tick populations with a persistent infection has been reported for both pathogenic and nonpathogenic rickettsiae. *D. andersoni* ticks chronically infected through twelve generations with a virulent strain of *R. rickettsii* (Sawtooth) began to illustrate higher mortality, decreased oviposition and egg hatches for their progeny during the fifth generation (Burgdorfer and Brinton 1975). A subsequent study confirmed the adverse effects of highly virulent *R. rickettsii* (Como-96 or Wachsmuth) infection on tick development/oviposition and reported decreased fecundity by ticks naturally infected with some nonpathogenic rickettsiae (*R. montana* and *R. rhipicephali*), but not all (*R. peacockii*) species examined (Niebylski et al. 1999). Previously, we found no difference in either engorgement weight or egg mass weight in the parental population of ticks initially infected with rickettsiae (Macaluso et al. 2001). The percentage of egg mass weight relative to engorgement weight was reported to be 38.4% and 32.4% for

adult ticks exposed to *R. montana* and *R. rhipicephali*, respectively, which was similar for that reported for uninfected ticks exposed to growth medium alone (36.7%).

To determine whether *Rickettsia*-infected cohorts were able to either transovarially transmit multiple species of *Rickettsia*, or whether the primary infection was capable of blocking transovarial transmission of the secondary exposure, eggs from the challenged ticks were assessed by PCR. Using the *metK* primers, amplification of a portion of the rickettsial gene resulted in amplicons of ≈ 350 and ≈ 250 bp for *R. montana* and *R. rhipicephali*, respectively. Using genomic DNA isolated from cultured *Rickettsia*-infected Vero cells as templates in the PCR, amplification of both fragments occurred in the same reaction, thus allowing for detection of dual infection. During each trial, partially fed adults or ≈ 100 eggs from both *Rickettsia*-infected cohorts were assessed for rickettsial infections. The results are presented in Table 2. The *R. montana*-infected cohort of ticks used in the challenge experiments maintained a 100% rate of infection

Table 2. Evidence of resistance of *Rickettsia*-infected ticks, *Dermacentor variabilis*, to co-infection with another rickettsial species as determined by PCR

Trial	Treatment/Tick	PCR positive for	
		<i>R. montana</i>	<i>R. rhipicephali</i>
I	Pre-reciprocal challenge ^a		
	<i>R. montana</i> -infected	10/10	0/10
	<i>R. rhipicephali</i> -infected	0/10	2/10
	Post-reciprocal challenge ^b		
II	<i>R. montana</i> -infected	16/32	0/32
	<i>R. rhipicephali</i> -infected	0/34	7/34
	Pre-reciprocal challenge		
	<i>R. montana</i> -infected	12/12	0/12
	<i>R. rhipicephali</i> -infected	0/10	0/10
	Post-reciprocal challenge		
	<i>R. montana</i> -infected	20/27	0/27
	<i>R. rhipicephali</i> -infected	1/24	0/24

^a Pre-reciprocal challenge infection rate was assessed for each *Rickettsia*-infected cohort. The values represent the number of female ticks that were positive for rickettsial infection over the total number of females examined.

^b Post-reciprocal challenge infection rate (transovarial transmission rate) was assessed for each *Rickettsia*-infected cohort. The values represent the number of egg clutches from individual female ticks that were positive for rickettsial infection over the total number of egg clutches examined.

throughout both the F1 and F2 generations. In contrast, in the *R. rhipicephali*-infected cohort, infection of ticks was detected in only 20% of the ticks for the F1 generation and was undetectable in the adult ticks of the F2 generation. Rickettsial challenge infection of ticks of the F1 generation resulted in only the original *Rickettsia* species being detected in the progeny for each cohort, at a 50% and 20.6% level of infection for *R. montana* and *R. rhipicephali*, respectively. Similar results were observed for the second trial (F2) for *R. montana*-infected ticks exposed to *R. rhipicephali*. Eggs from 74% of the adult ticks from this cohort were positive for *R. montana*, although *R. rhipicephali* was not detected in the eggs from any of the ticks assessed. However, after the rickettsial challenge, when eggs from the ticks of the F2 generation of the *R. rhipicephali*-infected cohort were tested for rickettsial infection, only one positive was identified (4.2%), and it was *R. montana*.

Because this study was designed to examine the ability of a cohort of *Rickettsia*-infected ticks to block transmission of a second species of *Rickettsia*, we did not use uninfected ticks to assess infectivity of the challenge rickettsiae. However, the rickettsiae used in this study were aliquots of frozen stocks that we routinely used successfully for propagation and Vero cell and tick infection.

These data indicate that ticks are incapable of maintaining two different species of *Rickettsia* via transovarial transmission. The understanding of the underlying mechanism of interference resulting in blockage of transovarial transmission of a second *Rickettsia* species in ticks may explain the observed infection of ticks in nature (Burgdorfer et al. 1981). It is interesting that *D. andersoni* infected with *R. montana* and/or *R. rhipicephali* could acquire secondary infection with *R. rickettsii* but are unable to maintain both infections via transovarial transmission (Burgdorfer 1988). We have also shown that interference occurs in this study, however, this is the first report in which the competition between two nonpathogenic species results in exclusion of the other species. These data also support the observation that ticks collected from various geographic regions are found not to be infected with more than one spotted fever group rickettsiae (Azad and Beard 1998).

Another interesting finding was that *D. variabilis* could maintain high levels of *R. montana* infection via transovarial transmission through at least two generations. However, these same ticks were unable to sustain infection with *R. rhipicephali* for more than one generation. Consequently, the loss of a detectable level of rickettsial infection in the *R. rhipicephali*-infected cohort during successive generations allowed for individuals of this population to become experimentally infected with *R. montana*. Although detected in *D. variabilis* and *D. andersoni* in nature (Bell et al. 1963), it is possible that *Dermacentor* ticks are not optimal hosts for *R. rhipicephali*, a species originally isolated from the brown dog tick *Rhipicephalus sanguineus* (Hayes and Burgdorfer 1979).

Vertical transmission of microorganisms resulting in benign associations is favored evolutionarily (Werren 1997). Laboratory studies and field observations support this concept for the relationship between nonpathogenic rickettsiae and ticks. Based on available data, virulent *R. rickettsii* maintenance has been shown to exert negative fitness to infected *D. andersoni* filial progenies. However, maintenance of *R. montana* via transovarial transmission in our hands had no effect on the reproductive fitness of *D. variabilis*. These studies may explain, at least in part, the reason for the low prevalence of *R. rickettsii* in its vector ticks in nature, compared with that observed for nonpathogenic *R. montana* (Schrieffer and Azad 1994). Taken together, we can speculate that competition between rickettsiae for establishment in tick reservoirs favors a single, nonpathogenic rickettsial infection. The data in this study demonstrate that the single infection in ticks is maintained irrespective of the pathogenicity of the challenging species, indicating that changes in tick tissues associated with primary infection prevent secondary infection. The mechanisms of infection and the changes within the tick postinfection are the focus of ongoing studies in our laboratory.

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