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Effects of Rickettsia amblyommatis Infection on the Vector Competence of Amblyomma americanum Ticks for Rickettsia rickettsia

Michael L. Levin[1], **Lauren B.M. Schumacher**[1], and **Alyssa Snellgrove**[1]

[1] Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, Georgia.

Abstract

Although *Dermacentor* spp. ticks are considered the primary vectors of *Rickettsia rickettsii* in the United States, other North American tick species are also capable of transmitting the agent, including the lone star tick—*Amblyomma americanum*. The lone star tick is an aggressive human-biting tick abundant in the South, Central, and Mid-Atlantic United States, which has been shown to be a competent vector of *R. rickettsii* in laboratory studies. However in nature, *A. americanum* frequently carry *Rickettsia amblyommatis*—another member of the spotted fever group—with the prevalence of infection reaching 84% in some populations. It has been postulated that the presence of an endosymbiotic *Rickettsia* in a significant proportion of a vector population would diminish or even block transmission of pathogenic *Rickettsia* in ticks from generation to generation due to transovarial interference. We measured the ability of *R. amblyommatis*-infected *A. americanum* to acquire *R. rickettsii* from an infected host with a bloodmeal, and transmit it transstadially, horizontally (to a susceptible host), and vertically to the next generation. Larvae from both the *R. amblyommatis-infected* and *R. amblyommatis*-free cohorts acquired *R. rickettsii* from infected guinea pigs, but the presence of the symbiont diminished the ability of coinfected engorged larvae to transmit *R. rickettsii* transstadially. Conversely, acquisition of *R. rickettsii* by cofeeding was unaffected in *R. amblyommatis-infected* nymphs and adults; prevalence of *R. rickettsii* in engorged adults reached 97% in both *R. amblyommatis-infected* and *R. amblyommatis-free* cohorts. In guinea pigs exposed to dually infected nymphs, *R. rickettsii* infection was milder than in those fed upon nymphs infected with *R. rickettsii* only. The frequency of transovarial transmission of *R. rickettsii* in the *R. amblyommatis*-infected cohort (31%) appeared lower than that in the *R. amblyommatis-free* cohort (48%), but the difference was not statistically significant. Larval progenies of dually infected *A. americanum* females transmitted *R. rickettsii* to naïve guinea pigs confirming viability of the pathogen. Thus, the vector competence of *A. americanum* for *R. rickettsii* was not significantly affected by *R. amblyommatis*.

Address correspondence to: Michael L. Levin, Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, MS G-13, Atlanta, GA 30333, mlevin@cdc.gov.

Author Disclosure Statement

No conflicting financial interests exist.

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Keywords

Amblyomma americanum; *Rickettsia rickettsii*; *Rickettsia amblyommatis*; vector competence; transovarial interference

Introduction

Rickettsia rickettsii, the causative agent of Rocky Mountain spotted fever, continues to cause severe illness and death in humans throughout North, Central, and South America, despite the availability of effective antibacterial therapy. In the United States and Canada, the American dog tick (*Dermacentor variabilis)* and the Rocky Mountain wood tick (*Dermacentor andersoni)* are known as major vectors of this pathogen (Burgdorfer 1975), while from Panama to Argentina various ticks of the genus *Amblyomma* have been implicated in both the natural maintenance of *R. rickettsii* and its transmission to humans (de Rodaniche 1953, Guedes et al. 2005, Paddock et al. 2008, Labruna 2009, Tarragona et al. 2016). Laboratory studies have demonstrated that among North American *Amblyomma* at least one species—*Amblyomma americanum*—can be a competent vector of *R. rickettsii* (Maver 1911, Parker et al. 1933, 1943, Levin et al. 2017).

A. americanum—the lone star tick—is an aggressive human-biting tick abundant in the South, Central, and Mid-Atlantic United States. It can acquire the pathogen from systemically infected hosts as well as from infected ticks of either the same or a different species. Interspecies transmission of *Rickettsia* through cofeeding allows new introductions of the pathogen into *A. americanum* populations as spillover from the primary vectors. Ticks acquiring *R. rickettsii* during feeding successfully retain the pathogen during the molting process from larval to nymphal and from nymphal to adult stages, and transmit it to susceptible hosts during subsequent feedings. Moreover, ~14–28% of infected *A. americanum* females transmitted *R. rickettsii* to their progeny (Levin et al. 2017). Considering the efficiency of horizontal transmission and the notorious tendency of *A. americanum* to cluster on a host in high numbers, the lone star tick may play a role in the transmission cycle of *R. rickettsii* in nature, notwithstanding a relatively low frequency of transovarial transmission (TOT).

In nature, however, *A. americanum* frequently carry another member of the spotted fever group *Rickettsia—Rickettsia amblyommatis* (previously *Ca. Rickettsi amblyommii)* (Karpathy et al. 2016)—with the prevalence of infection reaching 84% in some populations (Mixson et al. 2006, Killmaster et al. 2014). It had been postulated that presence of an endo-symbiotic *Rickettsia* in a significant proportion of a vector population will diminish or even block transmission of a pathogenic *Rickettsia* in ticks from generation to generation due to transovarial interference (Burgdorfer et al. 1981a, Burgdorfer 1988, Azad and Beard 1998, Macaluso et al. 2002, Goddard 2008). Thus, the high prevalence of *R. amblyommatis* infection in ticks may affect the ability of *A. americanum* to maintain and transmit *R. rickettsii* in nature.

Therefore, we assessed the capabilities of *A. americanum* inherently infected with *R. amblyommatis* to (1) acquire secondary infection with *R. rickettsii* during a bloodmeal, (2)

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transmit it transstadially and to susceptible hosts, and (3) transmit both *Rickettsia* species transovarially to the next generation.

Materials and Methods

R. rickettsii isolate BSF-Di-6 used here was the same as in the previous study documenting the vector competence of *A. americanum* free of endosymbiotic rickettsiae (Levin et al. 2017). After its original isolation in 1961 from a spleen and liver homogenate taken from a Virginia opossum (*Didelphus virginiana)* trapped in Hanover County, VA (Bozeman et al. 1967), the isolate was propagated and stored in yolk sac culture for a total of five passages. Before the vector competence studies, the yolk sac culture was inoculated into guinea pigs, and spleen/liver homogenates were prepared as described before (Levin et al. 2017). Aliquots of homogenate containing 10^5 copies of *R. rickettsii* DNA per aliquot were stored in liquid nitrogen until used for infecting the study animals.

Two independent laboratory colonies of *A. americanum* ticks were developed and maintained at the CDC Medical Entomology Laboratory by feeding on pathogen-free naïve New Zealand white rabbits as previously described (Troughton and Levin 2007). A specific pathogen-free (SPF) colony and a *R. amblyommatis*-infected colony both originated from adult ticks collected from vegetation at a State Park 15 miles SW of Atlanta, Georgia. The colonies have been maintained independently in the laboratory for eight and two generations, respectively. Absence of either pathogenic or endosymbiotic *Rickettsia* spp. in the SPF colony has been assured in every generation by PCR-testing samples of larvae from progeny of every female and serological assessment of all rabbits used for tick feeding. In the *R. amblyommatis-infected* colony, only larval batches containing transovarially transmitted bacteria were retained and used for propagation. Both colonies were confirmed free of known tick-borne *Anaplasma, Borrelia, Ehrlichia* species and Heartland virus.

Pathogen-free and tick-naïve male guinea pigs 6–9 weeks old were used as model animals. For tick infestations, guinea pigs were fitted with feeding bags glued to their dorsum as described (Levin and Schumacher 2016). Throughout the study, guinea pigs were monitored daily for clinical signs of infection, including fever (defined as body temperature ≥ 39.5°C), scrotal edema, dermatitis, and edema of ears and footpads (Walker et al. 1977). Ear-skin biopsies for PCR were collected under anesthesia every 2–3 days using sterile 2 mm diameter ear punches (Kent Scientific Corporation, Torrington, CT). Animals were euthanized at 9–14 days after infection using barbiturate overdose (Beuthanasia®-D; Intervet International B.V.). Samples of liver, spleen, testis with epididymis, lung, and heart were collected at the time of euthanasia. Tissue samples were stored at −20°C until tested by PCR for the presence of *R. rickettsii* and *R. amblyommatis* DNA as described below.

For introduction of *R. rickettsii* into ticks, two groups of six guinea pigs were needle inoculated with 1 mL aliquots of liver/spleen homogenate prepared beforehand. The third group of guinea pigs remained pathogen free. On day 6 postinoculation, six of the *R. rickettsii-infected* guinea pigs were each infested with ~ 300 *A. americanum* larvae from the SPF colony for acquisition feeding. At the same time, the remaining six *R. rickettsii*-infected guinea pigs as well as six pathogen-free guinea pigs each received larval ticks from the *R.*

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amblyommatis-infected colony (Fig. 1A). Thus, we produced three cohorts of ticks infected with (1) *R. amblyommatis* only, (2) *R. amblyommatis+R. rickettsii,* and (3) *R. rickettsii* only. Samples of engorged larvae and freshly molted nymphs—25 per stage per guinea pig—were tested individually by PCR. Nymphal ticks from each of the 3 cohorts were then fed on 6 naïve guinea pigs—150 ticks per animal—to assess horizontal and transstadial transmission of the 2 rickettsial species (Fig. 1A). The guinea pigs were monitored for clinical signs of infection and by ear-skin biopsy as described above for 2 weeks, and euthanized on 14 days postinfestation (DPI) for necropsy and collection of internal tissue samples.

The remaining nymphs were fed upon New Zealand white rabbits, one rabbit per cohort of tick, for evaluation of vertical transmission (Fig. 1B). Thirty pairs (male+female) of the resulting adults from each cohort were again fed on naïve rabbits and allowed to oviposit. Once oviposition was completed, female ticks were individually tested by PCR for the presence of both *R. amblyommatis* and *R. rickettsii*, as were samples of their eggs (10 pools of 10 eggs from each individual clutch). Finally, larvae hatching from four individual egg clutches that tested positive for both rickettsial agents were again fed on naïve guinea pigs to verify the ability of coinfected larvae to transmit *R. rickettsii* to susceptible hosts. Approximately 600 larvae from each of the 4 batches were divided between 3 naïve guinea pigs (Fig. 1B). These guinea pigs were monitored for clinical signs as before, but euthanized and necropsied earlier—9 DPI.

DNA extraction and PCR procedures were carried out in separate facilities to prevent contamination. DNA was extracted from tick and tissue samples using the Qiagen DNEasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA) according to manufacturer's protocols. Each sample in this study was submitted to two separate species-specific PCR tests. The presence of *R. rickettsii* DNA was detected by PCR using primers RRi6_F and RRi6_R, and FAM probe RRi6_P as described by Kato et al. (2013). The presence of *R. amblyommatis* DNA was detected using primers Ra477F and Ra618R, and FAM probe RA532Probe as described by Jiang et al. (2010). All tests were conducted in duplicate. Two negative (distilled water) and two positive (*R. rickettsii* or *R. amblyommatis* DNA) samples were included in each run. Samples demonstrating amplification in both duplicates before 40 cycles were considered positive. In instances (five total) when only one replicate was positive, samples were retested until congruent results were achieved.

Prevalence of rickettsial infection in cohorts of ticks and frequencies of TOT were compared by chi-squared and Fisher exact tests (two tailed) at the 95% confidence level.

Results

After feeding upon uninfected guinea pigs, 100% of ticks from the *R. amblyommatis*-infected colony were PCR positive for the symbiont when tested as either engorged larvae or freshly molted nymphs (Table 1). The same was observed in the cohort of *R. amblyommatis*-infected larvae fed upon *R. rickettsii*-inoculated guinea pigs; an apparent 2.7% decrease in the prevalence of *R. amblyommatis* among molted nymphs was not statistically significant. No *R. amblyommatis* DNA was detected in ticks originating from the SPF colony. Both

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uninfected and *R. amblyommatis-infected* larvae were able to acquire *R. rickettsii* from infected hosts (Table 1). In the *R. amblyommatis-free* cohort, the percentage of ticks with detectable *R. rickettsii* DNA increased from 8% to 24.7% between engorged larvae and molted nymphs—presumably due to proliferation of infection in individual ticks during molting. The prevalence of *R. rickettsii* infection in groups of nymphs from individual guinea pigs reached up to 52%. Conversely, no considerable increase in detectability of *R. rickettsii* took place in the *R. amblyommatis*-infected cohort, where only 8% of molted nymphs overall and up to 12% in groups from individual guinea pigs tested *R. rickettsii* positive. As a result, the prevalence of *R. rickettsii* in the dually infected cohort of nymphs was significantly lower ($p_{\text{chi-sq}} < 0.001$) than that in the *R. amblyommatis-free* cohort. Yet in the dually infected cohort, every nymph that tested positive for *R. rickettsii* also contained *R. amblyommatis.* None of the ticks in the "*R. amblyommatis*-only" cohort tested positive for *R. rickettsii* DNA; likewise, no *R. amblyommatis* DNA was present in ticks from the "*R. rickettsii-only*" cohort.

Guinea pigs infested with *A. americanum* nymphs from the cohort infected with *R. amblyommatis* only did not become febrile during the 2-week observation period (Table 2). One of them developed scrotal edema lasting for 2 days, and *R. amblyommatis* DNA was detected in ear-skin biopsies in three of the six animals on 1–3 sampling days. At the time of euthanasia (14 DPI), no gross abnormalities were observed in their internal organs, and rickettsial DNA was not detected in any of the tested tissues. All six guinea pigs fed upon by *A. americanum* nymphs from the *R. rickettsii*-only cohort developed fever reaching up to 41 °C, and five developed the characteristic scrotal edema lasting 2–5 days. *R. rickettsii* DNA was detected in ear-skin biopsies of all six guinea pigs on more than one occasion. During necropsy at 14 DPI, typical pathological signs of rickettsial infection were observed in all six animals, including discolored necrotic lesions of the liver (Fig. 2A), prominent congestion and erythema of the testes (Fig. 2C), and splenomegaly. Also, *R. rickettsii* DNA was detected in one or more internal tissue samples collected from five of the guinea pigs in this group (Table 2).

Among guinea pigs infested with nymphs from the dually infected cohort, five of six developed fever and three exhibited scrotal edema. *R. rickettsii* DNA was detected in ear-skin biopsies from three of the guinea pigs proving a disseminated rickettsial infection. In two guinea pigs, detection of *R. rickettsii* in skin biopsies coincided with scrotal edema. In addition, one guinea pig had a skin biopsy simultaneously positive for both *R. rickettsii* and *R. amblyommatis* (Table 2). No rickettsial DNA was detected in any internal tissues of these six guinea pigs collected at 14 DPI, and neither splenomegaly nor erythematous testes were evident during necropsy (Fig. 2D). However, discolored and depressed subcapsular lesions were present on the liver of four animals (Fig. 2B). These resembled the typical necrotic lesions described above (Fig. 2A) but already in the process of healing, which may suggest that rickettsial infection in guinea pigs exposed to dually infected ticks was milder compared with animals in the *R. rickettsii*-only group.

Nymphs from all three cohorts fed upon rabbits successfully developed into adult ticks. Prevalence of *R. amblyommatis* in both the singly and dually infected cohorts remained at almost 100% (Table 3). In contrast, the detectable prevalence of *R. rickettsii* in unfed adult

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ticks from the *R. amblyommatis*-free cohort (56%) was significantly higher than in those from the dually infected cohort (16%; $p_{\text{Fisher}} = 0.007$). Notably, *R. rickettsii* infection increased from nymphal to adult stage—8–16% and 24.7–56%—among *R. amblyommatis-infected* and *R. amblyommatis*-free cohorts, respectively—presumably as a result of cofeeding transmission within each cohort (Tables 1 and 3). After these adult ticks fed on naïve rabbits, presence of *R. rickettsii* DNA was detectable in 97 % of females from either *R. amblyommatis*-free or *R. amblyommatis*-infected cohorts (Table 3). Thus, the prevalence of *R. rickettsii* infection increased significantly ($p_{\text{Fisher}} < 0.001$) during adult feeding—as a result of cofeeding transmission—and became nearly universal regardless of the presence of *R. amblyommatis.*

All egg clutches produced by *R. amblyommatis*-infected female ticks contained *R. amblyommatis* DNA resulting in 100% frequency of TOT of these symbiotic bacteria. Within the infected clutches, 100% of tested egg pools were PCR positive in both singly and dually infected cohorts (Table 3). Hence, neither the frequency nor efficiency of TOT of *R. amblyommatis* appeared to be affected by the presence of *R. rickettsii.* Approximately half (48.1%) of egg clutches produced by *R. rickettsii*-infected female ticks contained *R. rickettsii* DNA, whereas only 8 (30.8%) of 26 dually infected ticks transmitted *R. rickettsii* transovarially. However, this difference was not statistically significant ($p_{\text{Fisher}} = 0.264$). Moreover, the efficiency of TOT of *R. rickettsia* widely overlapped between cohorts of singly and dually infected ticks (Table 3).

To confirm the viability of *R. rickettsii* transovarially transmitted by the dually infected *A. americanum* females, larval ticks from 4 of the dually infected egg clutches were placed on guinea pigs—3 guinea pigs per progeny, ~200 larvae per guinea pig. After placement of these ticks on animals, guinea pigs developed multiple clinical signs of rickettsial infection, including fever, scrotal edema, and desquamating dermatitis of ears and footpads (Table 4). Guinea pigs were euthanized on 9 DPI, and typical liver necrosis and/or splenomegaly were observed during necropsy in the majority of animals. Moreover, *R. rickettsii* DNA was detected in internal tissues collected from 7 of the 12 guinea pigs confirming a widely disseminated infection.

Discussion

We recently demonstrated that the lone star tick (*A. americanum*) is a competent vector of *R. rickettsii*, at least under laboratory conditions (Levin et al. 2017). Yet in nature, *A. americanum* frequently carry another member of the spotted fever group *Rickettsia—R. amblyommatis*—with the prevalence of infection reaching 84% in some populations. *R. amblyommatis* was originally described as being nonpathogenic for voles (*Microtus pennsylvanicus*), guinea pigs, and humans (Burgdorfer et al. 1981b, p. 599). However, recent studies have shown that repeated exposure to *R. amblyommatis* through *A. americanum* bites can lead to seroconversion in dogs (Barrett et al. 2014), and that at least some isolates of this symbiont may cause mild pathology in guinea pigs (Rivas et al. 2015). Furthermore, Billeter et al. (2007) reported detection of *R. amblyommatis* in a tick removed from a patient with a doxycycline-treatable skin rash (Billeter et al. 2007). In this study, we detected *R. amblyommatis* DNA in several ear-skin biopsies after placement of infected ticks on the

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dorsum of model animals, confirming that the agent is both transmissible through tick bites and capable of dissemination from the site of inoculation.

A. americanum very efficiently transmits *R. amblyommatis* transstadially and transovarially (Levin et al. 2017). In this study, 96–100% of ticks from the *R. amblyommatis-infected* colony and their progeny tested positive for this symbiont. Because it is generally assumed that a rickettsial endosymbiont can block transmission of pathogenic rickettsiae by their tick vectors, we needed to reassess the vector competence of *A. americanum* for *R. rickettsii* in the presence of transovarially transmitted *R. amblyommatis.*

A number of quantitative parameters governing survival (endurance) or *R. rickettsii* in the natural transmission cycle are currently unknown. These unidentified parameters include, among others, (1) the minimal dose of rickettsiae contained in an engorged tick needed for successful transstadial or TOT; (2) the amount of bacteria acquired by feeding ticks from animal hosts, and its relation to the survival or propagation of the pathogen during feeding, molting, or oviposition; (3) correlation between loads of rickettsiae in the tick body versus salivary glands or ovaries; and (4) the number of rickettsial cells injected by a tick into the host during feeding and its relation to the load of the pathogen in a flat tick. At present, even a minimal infectious dose of *R. rickettsii* resulting in illness in guinea pigs is unknown. In the absence of this information, any discussion regarding quantitative relationships between *R. amblyommatis* and *R. rickettsii* in ticks, or possible consequences of those quantitative balances on the long-term survival and transmission of the two *Rickettsia* spp. in nature, would only amount to an unsubstantiated speculation. Therefore, we focused this study exclusively on qualitative description of the phenomenon. Quantitative relationships between related rickettsial species within a tick should be addressed in the future.

Results of this study suggest that, although *A. americanum* larvae infected with *R. amblyommatis* are able to acquire a second *Rickettsia* sp. during feeding on infected guinea pigs, the subsequent proliferation of *R. rickettsii* inside dually infected ticks may be reduced. This resulted in a significantly lower prevalence of *R. rickettsii* in the dually infected cohorts of nymphs compared with the cohorts infected only with *R. rickettsii*. These observations differ from results of an analogous experiment in the vector competence of *Rickettsia* peacockii-infected *D. andersoni,* where 100% of *R. peacockii*-infected larvae acquired *R. rickettsii* and transmitted it transstadially to resulting nymphs (Burgdorfer et al. 1981a). In contrast, Wright et al. (2015) observed that *R. amblyommatis-infected A. americanum* nymphs were significantly less efficient in acquisition of *Rickettsia parkeri* through cofeeding and its transstadial transmission, which is somewhat similar to our results.

Naïve guinea pigs infested with dually infected nymphal ticks developed *R. rickettsii* infection. Judging from the observation of healing subcapsular lesions on the liver of some animals on day 14 postinfection, rickettsial infection in guinea pigs exposed to dually infected ticks appeared milder than the typical illness caused by the feeding of ticks infected with *R. rickettsii* only. It is likely that moderation and curtailing of rickettsial infection may be explained by smaller doses of *R. rickettsii* delivered into the host by dually infected ticks. Alternatively, the noted mitigation of infection in guinea pigs fed upon by dually infected ticks may be due to an “interference phenomenon” in the model animals described by Price

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in 1953. In a large series of experiments, Price (1953) observed that 80–90% of guinea pigs intraperitoneally inoculated with a “lowly virulent strain” of *R. rickettsii* were protected against a simultaneous injection of a highly virulent strain of spotted fever exhibiting only mild signs of infection or not at all. Similarly, guinea pigs bitten by *R. amblyommatis*-infected ticks in our experiments may be to some degree protected against *R. rickettsii* transmitted simultaneously by the same ticks. Whether resulting from competition between the closely related rickettsiae inside the tick vector or the host, it is noteworthy that an exposure to dually infected *A. americanum* nymphs produced milder infections in these highly susceptible model animals.

After nymphal feeding, the prevalence of *R. rickettsii* increased approximately two times in both *R. amblyommatis*-free and *R. amblyommatis-infected* cohorts—from 25% and 8% in flat nymphs to 56% and 16% in adult ticks, respectively. This indicates that presence of *R. amblyommatis* did not affect the efficiency of *R. rickettsii* transmission between ticks through cofeeding route. Furthermore, the prevalence of *R. rickettsii* infection in both cohorts reached 97% after adults fed on new naïve rabbits.

Of 26 female ticks simultaneously infected with *R. rickettsii* and *R. amblyommatis,* 8 (30.8%) transmitted *R. rickettsii* to at least some larvae in their progenies. This frequency of TOT was somewhat lower than that among ticks infected with *R. rickettsii* only (48.1%), although the difference was not statistically significant. In this study, the frequency of TOT in both groups was somewhat higher than the 28% we observed for the same BSF-Di-6 isolate of *R. rickettsii* in our previous study (Levin et al. 2017). Within individual *R. rickettsii*-infected progenies of either *R. amblyommatis*-free or *R. amblyommatis-infected* females, between 1/10 and 10/10 larval pools (10 ticks/pool) contained *R. rickettsii* DNA, whereas in progenies of *R. amblyommatis*-infected females all larval pools contained the symbiont. The median number of *R. rickettsii*-positive larval pools in transovarially infected progenies of dually infected ticks (1.5 of 10) was lower than that in progenies infected only with *R. rickettsii*, indicating that the filial rate of infection was somewhat diminished. Still, the presence of *R. rickettsii* in any larvae produced by dually infected females indicates that the second *Rickettsia* species acquired at the larval stage of the parental generation is capable of successfully invading and establishing TOT in a tick population already universally occupied by a rickettsial symbiont.

Unfortunately, we were unsuccessful in attempts to detect *R. rickettsii* DNA in individual larvae even in progenies where 10 of 10 pools (10 larvae each) were PCR positive. Whether this lack of detection is due to low prevalence of infection, low amount of bacteria in a single larva (below the detection threshold), or both, available data do not allow a more precise assessment of the efficiency of TOT of *R. rickettsii* by individual *A. americanum* females. Yet, larval ticks from the coinfected progenies are obviously capable of transmitting *R. rickettsii* to vertebrate hosts as guinea pigs fed upon by these larvae developed typical clinical and pathological signs of disseminated rickettsial infection, and *R. rickettsii* DNA was detectable in multiple internal tissues at 9 DPI. Thus, results of this study unequivocally demonstrate that the pre-existing infection with *R. amblyommatis* in *A. americanum* does not block the TOT of *R. rickettsii,* although the filial rates of *R. rickettsii* may be diminished.

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The outcomes of our study contradict the oft-repeated paradigm that only the primary rickettsial species invades tick ovaries and excludes a second species from being transmitted transovarially (e.g., Azad and Beard 1998, Goddard 2008). In their seminal experiments that eventually gave rise to the above generalization, Burgdorfer et al. (1981a) reported lower than expected frequency and efficiency of *R. rickettsii* TOT in the presence of another endosymbiotic *Rickettsia.* When the authors introduced *R. rickettsii* into *R. peacockii*-infected *D. andersoni* by feeding *R. peacockii*-infected larvae upon needle-inoculated guinea pigs, only 9 (45%) of 20 resulting *D. andersoni* females transmitted *R. rickettsii* to some or most eggs. From this, Burgdorfer et al. concluded that the preexisting *R. peacockii* infection in *D. andersoni* prevented ticks from transmitting *R. rickettsii* to their progeny transovarially. Unfortunately, this pilot study did not include a control group of ticks infected only with *R. rickettsii*, which would be essential for comparison of TOT frequency between singly and dually infected ticks. Elsewhere, Burgdorfer et al. reported “that all infected female ticks tested passed rickettsiae via eggs to almost 100% of their offspring” (Burgdorfer 1963). This, however, differed from earlier reports by Ricketts and Price that TOT may be achieved by up to 50% *D. andersoni* or up to 30% of *D. variabilis,* and that any “brood of an infected female may include many uninfected larvae” (Ricketts 1909, Price 1954a, 1954b). As the efficiency of TOT depends primarily on the degree of rickettsial infection in tick ovaries, the noted incongruities are likely due to differences between strains of *R. rickettsii* or species of ticks or both (Burgdorfer and Brinton 1975, Burgdorfer 1988). Differences in detection technology may also account for the mentioned dissimilarities as Ricketts and Burgdorfer visualized bacteria using either light or fluorescent microscopy, whereas Price cultured live rickettsiae from eggs and larvae in chick embryos.

It is worth reiterating that in our recently published experiments, the frequency of *R. rickettsii* TOT by *A. americanum* infected with the BSF-Di-6 isolate was 28%, whereas in this study 48% females from the same colony infected with the same rickettsial isolate (control group) transmitted the pathogen to their progenies. This clearly indicates that in addition to differences caused by agent isolates and vector species, TOT may be affected by other, yet unaccounted for, factors, which can fluctuate between even successive experiments. It also underlines the paramount necessity for the proper, simultaneous, parallel controls in any such study. In the absence of appropriate controls in the 1981 study by Burgdorfer et al., it is difficult to ascertain not only the significance of differences in the frequency of *R. rickettsii* TOT strictly attributable to *R. peacockii* infection but even the direction of the change. No follow-up studies corroborating (or disproving) the hypothesis of rickettsial interference (“Eastside hypothesis”) were published by Burgdorfer et al. Yet, this hypothesis was accepted by the scientific community as a proven fact and after numerous reiterations in scientific papers, reviews, and textbooks it became one of the best known axioms of rickettsiology (Azad and Beard 1998, Goddard 2008).

As far as we know, the only study carefully assessing transovarial interference between two species of spotted fever group (SFG) rickettsiae was published by Macaluso et al. (2002) where the authors examined the ability of *D. variabilis* to maintain more than one species of endosymbiotic rickettsiae through TOT. In their study, adult ticks from the previously established *Rickettsia montanensis-infected* and *Rickettsia rhipicephali*-infected colonies were reciprocally exposed to *R. rhipicephali* and *R. montanensis* through capillary feeding.

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Assessment of the eggs from challenged ticks showed that both *R. montanensis*- and *R. rhipicephali-infected* ticks were refractory to their respective challenge rickettsiae even though only portion of individual eggs (between 20% and 74%) contained the "pre-established" *Rickettsia* species. This led authors to a conclusion that rickettsial infection of tick ovaries may alter the molecular expression of the oocytes precluding secondary infection with other rickettsiae. These purported changes in the molecular expression of the oocytes are yet to be elucidated, and it is not known whether and to what extent similar changes take place between other SFG rickettsiae and other tick species. It is apparent, however, that no absolute exclusion took place either in *D. andersoni* ticks infected with *R. peacockii,* where authors documented "mild" dual infections in some of the examined individual eggs (Burgdorfer et al. 1981a), or in *A. americanum* of this study, where a number of tick progenies contained both the "pre-established" rickettsial endosymbiont and *R. rickettsii.* Likewise, Goethert and Telford (2005) observed simultaneous transovarial passage of two closely related *Francisella* species within the same egg batch of *D. variabilis,* thus providing another evidence against the generality of transovarial interference between intracellular tick-borne agents.

Overall, the vector competence of *A. americanum* for *R. rickettsii* does not appear significantly affected by *R. amblyommatis,* although rickettsial infection engendered by the dually infected ticks in model animals was milder. In this study, a decrease in the pathogen acquisition by larvae and transstadial transmission to nymphal stage was the only significant effect on the vector competence of *A. americanum* for *R. rickettsii* attributable to the pre-existing *R. amblyommatis* infection in ticks. This decrease was negated during subsequent nymphal and adult feeding. Contrary to the hypothesis of transovarial interference, there was no significant difference in the frequency of vertical transmission of *R. rickettsii* between *A. americanum* infected with and free of *R. amblyommatis.* While the "interference phenomenon" hypothesis predicts that an invasion of a symbiotic species would gradually push the pathogenic species out of circulation, our study shows that *R. rickettsii* can successfully invade and establish a foothold in a tick population already universally occupied by a rickettsial symbiont. This demonstrates that processes and mechanisms of interspecific competition between closely related rickettsiae are neither the same across various rickettsiae and vector species nor unidirectional. As Hechemy et al. (2009) have pointed out, "The relationship between the rickettsias and their vectors and the transovarial interference of rickettsial species in the vectors remains a promising area of research that has not been elucidated."

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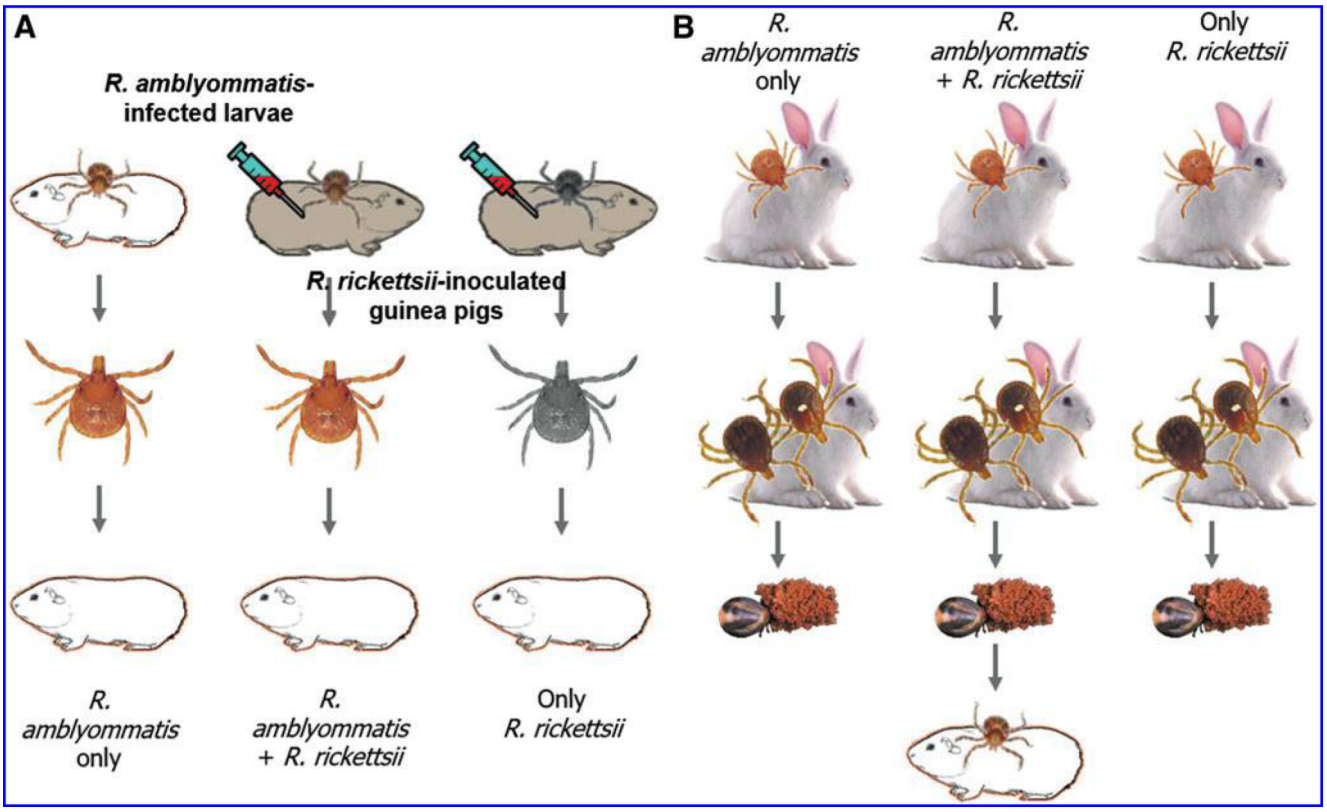

FIG. 1.

Flowchart of the study: (**A**) introduction of *Rickettsia rickettsii* into *Rickettsia amblyommatis-infected* and uninfected ticks, and evaluation of the tick-to-host transmission of the two *Rickettsia* spp.; (**B**) assessment of transovarial transmission of *R. rickettsii* and *R. amblyommatis* by singly and dually infected ticks.

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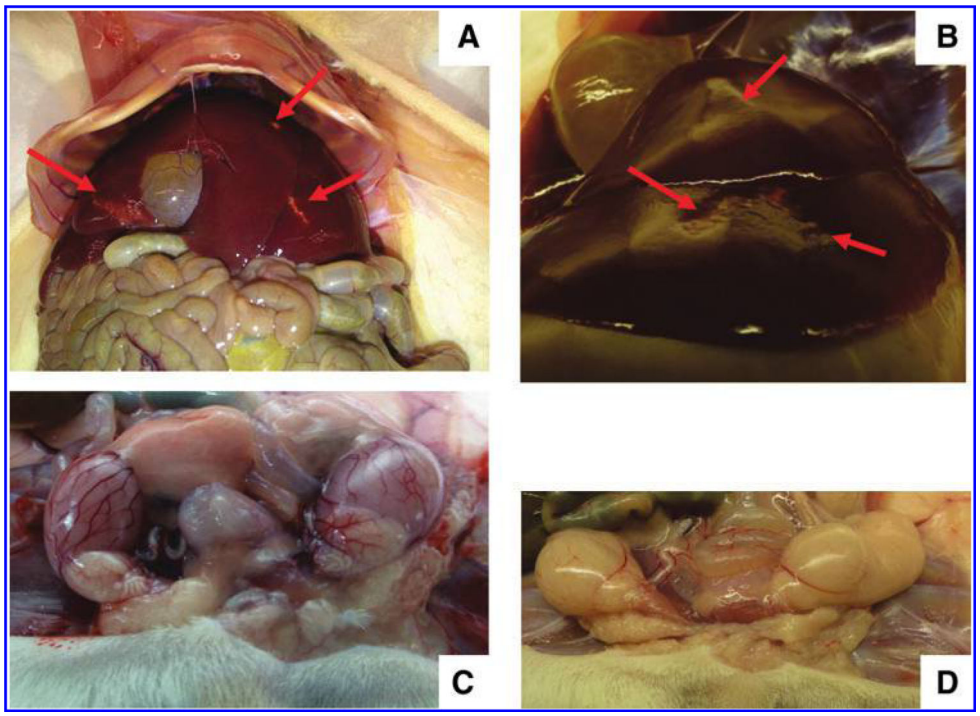

FIG. 2.

Pathological signs of rickettsial infection in guinea pigs fed upon by *Amblyomma americanum* nymphs infected with *R. rickettsii* only **(A, C),** and coinfected with *R. rickettsii* and *R. amblyommatis* **(B,D)** as observed on day 14 postinfestation: **(A)** discolored necrotic lesions of the liver (*arrows*), **(B)** discolored and depressed subcapsular lesions of the liver (*arrows*), **(C)** prominent congestion and erythema of the testes; and **(D)** unaffected testes.

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Table 1.

Acquisition of *Rickettsia rickettsii* by *Amblyomma americanum* Larvae Transovarially Infected with *Rickettsia amblyommatis* Versus Uninfected

		R. amblyommatis[a] (%)		R. rickettsii[a] (%)	
A. americanum *larvae*	***Guinea pigs***	***Engorged larvae***	***Molted nymphs***	***Engorged larvae***	***Molted nymphs***
Infected with *R. amblyommatis*	Naïve	100	100	0	0
	Inoculated with *R. rickettsii*	100	97.3 (92–100)	6.7 (4–12)	8.0 (4–12)
Uninfected	Inoculated with *R. rickettsii*	0	0	8.0 (4–12)	24.7 (12–52)

[a]The overall prevalence of infection (N = 150) and range of infections in cohorts of ticks fed on individual guinea pigs (n = 25).

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Table 2.

Signs of Infection in Guinea Pigs Fed Upon by *Amblyomma americanum* Nymphs Infected with *Rickettsia amblyommatis* Only, *Rickettsia rickettsii* Only, and Coinfected with Both Agents

Nymphs infected with	Fever >39.5° C	Scrotal edema	Ear-skin PCR	Internal tissues PCR
R. amblyommatis only	0/6	1/6	3/6	0/6
R. amblyommatis and *R. rickettsii*	5/6	3/6	1/6 3/6	0/6 0/6
R. rickettsii only	6/6	5/6	6/6	5/6

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Table 3.

Rickettsia rickettsii and *Rickettsia amblyommatis DNA* in Adult *Amblyomma americanum* and Their Progeny

Cohorts infected with	Unfed female ticks[a]	Postoviposition female ticks[a]	Infected egg clutches[a]	Median positive egg pools in positive clutches (range)
R. amblyommatis only	24/25 (96 ± 6)	30/30 (100)	29/29 (100)	10/10
R. amblyommatis and *R. rickettsii*	24/25 (96 ± 6)	30/30 (100)	26/26 (100)	10/10
	4/25 (16 ± 15)	29/30 (97 ± 7)	8/26 (31 ± 18)	1.5/10 (1/10–10/10)
R. rickettsii only	14/25 (56 ± 20)	28/29 (97 ± 7)	13/27 (48 ± 19)	2.5/10 (1/10–10/10)

[a]Positive/tested (% ±95% confidence interval).

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Table 4.

Signs of *Rickettsia rickettsii* Infection in Guinea Pigs Fed upon by *Amblyomma americanum* Larvae Transovarially Infected with Both *Rickettsia amblyommatis* and *Rickettsia rickettsii*

ID of progenies	*Fever >39.5° C*	*Scrotal edema*	*Dermatitis*	*Liver necrosis*	*Splenomegaly (>0.15% body weight)*	**R. rickettsii** *DNA in internal organs—9 DPI (positive organs)*
C	1/3	2/3	1/3	0/3	3/3	2/3 (Testes: 2, bladder: 2)
JJ	3/3	0/3	1/3	1/3	2/3	1/3 (Liver: 1)
DD	2/3	2/3	1/3	2/3	2/3	2/3 (Liver: 2, Testes: 2)
D	1/3	2/3	1/3	1/3	2/3	2/3 (Testes: 2, Liver: 1)
Total	7/12	6/12	4/12	4/12	9/12	7/12

DPI, days postinfestation.