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Effects of homologous and heterologous immunization on the reservoir competence of domestic dogs for *Rickettsia conorii* (*israelensis*)

M.L. Levin[*], **G.E. Zemtsova**, **M. Montgomery**, and **L.F. Killmaster**

Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

Abstract

A number of spotted fever group (SFG) rickettsiae cause serious infections in humans. Several antigenically related rickettsial agents may coexist within the same geographical area, and humans or vertebrate hosts may be sequentially exposed to multiple SFG agents. We assessed whether exposure of a vertebrate reservoir to one SFG *Rickettsia* will affect the host's immune response to a related pathogen and the efficiency of transmission to uninfected ticks. Two pairs of dogs were each infected with either *Rickettsia massiliae* or *Rickettsia conorii israelensis*, and their immune response was monitored twice weekly by IFA. The four immunized dogs and a pair of naïve dogs were each challenged with *R. conorii israelensis*-infected *Rhipicephalus sanguineus* nymphs. Uninfected *Rh. sanguineus* larvae were acquisition-fed on the dogs on days 1, 7, and 14 post-challenge. These ticks were tested for the presence of rickettsial DNA after molting to the nymphal stage. The naive dogs became infected with *R. conorii israelensis* and were infectious to ticks for at least 3 weeks, whereas reservoir competence of dogs previously infected with either *R. massiliae* or *R. conorii* was significantly diminished. This opens an opportunity for decreasing the efficiency of transmission and propagation of pathogenic *Rickettsia* in natural foci by immunizing the primary hosts with closely related nonpathogenic SFG bacteria. However, neither homologous immunization nor cross-immunization significantly affected the efficiency of *R. conorii* transmission between cofeeding infected nymphs and uninfected larvae. At high densities of ticks, the efficiency of cofeeding transmission may be sufficient for yearly amplification and persistent circulation of a rickettsial pathogen in the vector population.

Keywords

Rickettsia conorii; Immunization; Reservoir competence; Horizontal transmission; *Rhipicephalus sanguineus*

Introduction

Spotted fever group (SFG) rickettsiae are closely related antigenically and exhibit strong cross-reaction in serological tests. While this group contains some highly virulent pathogens,

[*]Corresponding author at: Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, MS G-13, Atlanta, GA 30333, USA. Tel.: +1 404 639 3639; fax: +1 404 639 4436. MLevin@cdc.gov (M.L. Levin).

it also includes agents which may cause only mild or unapparent infections both in humans and animals. These agents exist in natural cycles involving arthropod vectors (mainly ticks) and their vertebrate hosts. The list of known rickettsial pathogens has tripled in the last 15–20 years and continues to grow. This extension is due to both discovery of new species and recognition of pathogenic potential in agents previously thought to be exclusive endosymbionts of ticks maintained in vector populations solely via vertical transmission (Walker et al., 2007).

Humans and animals recovering from spotted fever rickettsioses are often reported to develop a “solid immunity” that protects them from illness and death in case of reinfection (Lackman et al., 1965; Walker et al., 2007). In laboratory studies, immunizations with various pathogenic rickettsiae have been reported to protect experimental animals not only against challenges with the same (homologous) agent, but against other (heterologous) *Rickettsia* spp. as well. For example, guinea pigs immunized with either *R. rickettsii, R. conorii, R. rhipicephali*, or *R. montanensis* remained afebrile following heterologous challenges (Badger, 1933; Feng and Waner, 1980; Walker et al., 1984; Gage and Jerrells, 1992); similarly, sublethal infection with *R. conorii* and *Rickettsia australis* resulted in the absence of clinical manifestations of disease after challenge with a lethal dose of the heterologous agent (Feng and Walker, 2003).

Although antibody titers in dogs infected with *R. conorii israelensis* decline below the detectable threshold level within 8–10 months after infection, the reservoir competence of seropositive animals reinfected with the same agent appears to be lower than that in primary infection (Zemtsova et al., 2010; Levin et al., 2012). Based on the rationale that antibodies have limited access to obligately intracellular *Rickettsia*, it had been suggested that cell-mediated immunity is primarily responsible for curtailing a rickettsial infection, whereas humoral immunity to rickettsiae plays only a secondary role in vertebrate hosts (Jerrells, 1997). On the other hand, neutralization of bacteria inside ticks by antibodies from immune hosts has been suggested as one of the factors responsible for decreasing reservoir competence and preventing highly pathogenic rickettsiae like *R. sibirica* and *R. conorii* from infecting large proportions of vector populations (Grokhovskaya and Sidorov, 1966; Zemtsova et al., 2010).

As more than one species of tick-borne rickettsiae often exist within the same geographical area and may even be transmitted by the same vector species (Bitam et al., 2006; Carmichael and Fuerst, 2006, 2010; Eremeeva et al., 2006; Mediannikov et al., 2010; Moncayo et al., 2010; Medeiros et al., 2011; Spitalska et al., 2012), humans and reservoir animals can be exposed to multiple SFG agents circulating in the same or adjacent foci. If acquired immunity to one of these agents provides protection against others in immune animals, it may potentially reduce their reservoir competence for related rickettsial agents as well.

R. conorii and *R. massiliae* provide an example of closely related SFG pathogens with overlapping geographical distribution, which are transmitted by the same vector species – *Rh. sanguineus* in particular (Brouqui et al., 2007). The *R. conorii* group includes causative agents of Mediterranean spotted fever, Astrakhan fever, Israeli spotted fever, and Indian tick typhus in the Mediterranean basin and Africa, southern Russia, Middle East, and India,

respectively (Zhu et al., 2005). *R. conorii* subspecies *israelensis*, the causative agent of Israeli tick typhus (ISTT), has been described in Cyprus, France, Israel, Italy, and Portugal with *Rh. sanguineus* being its main vector [reviewed by (Zemtsova et al., 2010)]. *R. massiliae*, originally isolated from *Rh. sanguineus* ticks collected in southern France (Beati and Raoult, 1993), has since been identified in several species of the genus *Rhipicephalus* in France, Greece, Italy, Portugal, Spain, Switzerland, Israel, north and central Africa, Argentina, and the United States (Bernasconi et al., 2002; Cicuttin et al., 2004; Bitam et al., 2006; Eremeeva et al., 2006; Fernandez-Soto et al., 2006; Brouqui et al., 2007; Marquez et al., 2008; Mura et al., 2008; Sarih et al., 2008; Labruna, 2009; Mediannikov et al., 2010; Milhano et al., 2010; Beeler et al., 2011; Harrus et al., 2011; Chochlakis et al., 2012; Khaldi et al., 2012).

Here, we assess effects of homologous and heterologous immune responses in a vertebrate host on the host's reservoir competence for *R. conorii israelensis* by comparing the efficiency of rickettsial transmission to *Rh. sanguineus* ticks feeding on naïve versus *R. conorii*- and *R. massiliae*-immunized dogs.

Materials and methods

Rickettsial isolates

R. conorii israelensis (strain T487) and *R. massiliae* (strain AZT80) were grown in Vero E6 cells at 32 °C in antibiotic-free minimal essential medium supplemented with 2% fetal calf serum and 2 mg/mL L-glutamine. Rickettsiae were purified by Renografin density gradient centrifugation as described (Paddock et al., 2006). Purified rickettsiae were stored in sucrose–phosphate–glutamate buffer (SPG: 218 mM sucrose, 3.76 mM potassium phosphate monobasic, 7.1 mM potassium phosphate dibasic, 4.9 mM potassium glutamate) with 5 mM $MgCl_2$, and 1% Renografin76 at −80 °C until used for inoculation.

Ticks and model animals

An uninfected laboratory colony of *Rh. sanguineus* from Oklahoma, USA, has been maintained in our laboratory by feeding all developmental stages on specific pathogen free New Zealand white rabbits as previously described (Troughton and Levin, 2007). *R. conorii*-infected ticks were produced by feeding larval ticks upon needle-inoculated dogs. Mixed-breed dogs were intravenously inoculated with 1×10^6 *R. conorii israelensis*, and *Rh. sanguineus* larvae were placed on each dog on the day of inoculation as described (Levin et al., 2012). Prevalence of rickettsiae in the infected cohort (11%) was evaluated by testing 25–50 freshly molted nymphs per dog. Between feedings, all ticks were kept in environmental incubators at 24 °C and 90% relative humidity.

Six 18–24-month-old purpose-bred mongrel (hound type) male dogs were used for all experiments in accordance with protocols approved by the CDC Institutional Animal Care and Use Committee. Dogs were housed indoors, in a climate-controlled animal facility that precluded an unintended exposure to any arthropod-borne agent including rickettsiae. The uninfected status in each dog was confirmed prior to inclusion into the study by PCR. Dogs were prescreened for antibodies (AB) against SFG rickettsiae by indirect

immunofluorescence assay (IFA), as described below, and all were non-reactive at the 1:16 dilution.

Immunization and challenge procedures

Two pairs of dogs were each infected with either *R. conorii israelensis* or *R. massiliae* live organisms via intravenous inoculations of 1×10^6 rickettsiae in 1 mL of phosphate-buffered saline solution. Development of antibodies against both agents was monitored by IFA twice weekly. After antibody titers against the immunizing pathogen peaked and then declined to approximately 1:512, 2 *R. conorii*-immunized dogs, 2 *R. massiliae*-immunized dogs, and a pair of naïve-control dogs were each challenged with *R. conorii israelensis*-infected *Rh. sanguineus* nymphs. Uninfected *Rh. sanguineus* larvae were placed on the dogs for acquisition feeding on days 1, 7, and 14 post challenge (Fig. 1).

Ticks were placed inside cotton feeding bags, glued to the dog's back, and allowed to feed to repletion, 4–5 days for nymphs and 3–4 days for larvae. The bags were checked twice daily for the duration of infestation. Engorged ticks were collected and kept at 22 °C and 95% relative humidity until they molted to the next stage. The challenging nymphs and acquisition-fed larval ticks were individually tested for the presence of rickettsial DNA after their eclosion – as freshly molted adults and nymphs, respectively. We tested all successfully molted adult ticks and at least 100 nymphs from each group of dogs per infestation.

The appetite, behavior, temperature, and level of activity of each dog were monitored daily throughout the study. Whole-blood (200 μL) and serum (500 μL) samples were collected aseptically from each dog twice weekly for approximately 10 months after the challenge and tested for the presence of rickettsial DNA and both anti-*R. conorii* and anti-*R. massiliae* antibodies as described below.

At 40 weeks after the first tick-borne challenge, the 2 control dogs were subjected to the second challenge with *R. conorii*-infected ticks followed by infestation with uninfected *Rh. sanguineus* larvae one day later. These dogs were monitored for additional 8 weeks by daily clinical observations and twice weekly by PCR and IFA tests.

PCR and serology

DNA extraction and PCR procedures were carried out in separate facilities. Qiagen DNEasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA) DNA was used to extract DNA from both ticks and blood samples in accordance with the manufacturer's protocols. The presence of rickettsial DNA was detected by real-time PCR using primers RR190-547F and RR190-701R which amplifies a 154-bp fragment of the *rOmpA* gene as described (Eremeeva et al., 2003). Samples were tested in duplicate with negative controls included in all extraction and PCR rounds. Water was used as a no-template negative control. PCR was interpreted as positive when both duplicate reactions had positive results.

IFA was performed on dog sera to detect IgG(γ) antibodies against whole-cell *R. conorii israelensis* and *R. massiliae* antigens, using FITC-labeled goat anti-dog conjugate (Kirkegaard and Perry, Laboratories, Gaithersburg, MD), as previously described (Lennette et al., 1995). Each serum sample was tested and titrated using both antigens. Samples were

initially screened at 1/16 and 1/256 dilutions, and positive samples were titrated to endpoint in a two-fold dilution series. Serologic data are reported as the reciprocal of the last dilution showing positive fluorescence. IgG titers ≥1:16 were considered positive.

Statistical analysis

Prevalence of rickettsial infection in tick cohorts was compared using the Chi-square test. Differences were considered significant when the *P*-values were <0.05. Geometric means of the final IgG titers were calculated for both antigens, for each pair of dogs at every sampling time-point.

For comparison of reservoir competence between naïve and immunized dogs, the odds ratios for ticks to acquire rickettsial infection while feeding during the three-week period following dogs' exposure to infected ticks were analyzed using a logistic regression model with a random effect for individual animals. Main effects for immunization groups and the day post exposure, considered categorical, were included for evaluation; interaction between these effects was also evaluated. Models were fitted using maximum likelihood and compared using the likelihood ratio test. Significance was again fixed at 5%. Ninety-five percent confidence intervals (CI) for the pairwise log odds ratios were computed and exponentiated to the odds ratio scale, and adjustment for multiple comparisons to control was done using Dunnett's method. Computations were made such that odds ratios <1 compared to the control-unimmunized group indicated successful immunization. Analysis was done in R statistical software (R Core Team, 2012) using the lme4 package (Bates et al., 2011) and the multcomp package (Hothorn et al., 2008).

Results

Control – unimmunized dogs

Naïve dogs became febrile (39.5–39.7 °C) on the third day after placement of *R. conorii*-infected ticks, but the body temperature returned to normal 1–2 days later. Both dogs developed mild orchitis on the days 5–7 post infection (PI), which also resolved within 2–3 days. Otherwise, the dogs behaved normally and retained a healthy appetite. IgG antibodies reactive with both *R. conorii* and *R. massiliae* antigens appeared within 5–7 days after tick infestation. Antibody titers reactive to *R. conorii* antigen reached their peak on the days 15–22 PI (geometric mean 1:181). They quickly declined below the diagnostic threshold by day 42, and then continued to oscillate between negative and 1:32 for the following 8 months (Fig. 2A). Titers to *R. massiliae* reached their peak (geometric mean 1:128) on day 18 PI and remained above the threshold level of 1:16 until day 80 PI.

Nymphal *Rh. sanguineus* from the *Rickettsia*-infected cohort fed on these dogs were tested by PCR after they molted to the adult stage. Eight (21%) out of 39 tested ticks were PCR-positive for *R. conorii* (Table 1). Although this prevalence of infection was twice as high as in the same cohort before feeding, the difference did not reach the statistical significance ($P = 0.23$) due to the small numbers of adult ticks available for testing. The prevalence of infection in acquisition-fed ticks placed on unimmunized dogs on days 1, 7, and 14 after infestation with infected nymphs was 15%, 26%, and 7%, respectively (Table 2).

When these dogs were reinfested with *R. conorii*-infected nymphs for the second time (on day 280 PI), one of them became subfebrile (39.4 °C) on day 4 after placement of ticks and simultaneously developed mild orchitis, which resolved within 3 days. The other dog exhibited orchitis and subfebrile body temperature (39.3 °C) only on the 7th day after placement of infected ticks (day 287 of the study). Despite appearance of these clinical manifestations, titers of antibodies reactive with either *R. conorii* or *R. massiliae* antigens increased only slightly. The geometric mean titer to *R. conorii* rose to 1:45 within 10 days after the second challenge and fell below the diagnostic level in the following 10 days. Titer of cross-reaction with *R. massiliae* reached the 1:16 level only briefly on days 285–291 (Fig. 2A) and then again fell below the threshold. Both dogs supported transmission of *R. conorii* between cofeeding infected nymphs and uninfected larvae. The resulting prevalence of infection in acquisition-fed ticks (13.0 ± 3.4%) was the same as in those fed side-by-side with infected nymphs during the first challenge (Table 2).

Homologous immunization – dogs inoculated with R. conorii israelensis

The 2 dogs needle-inoculated with *R. conorii israelensis* exhibited one- or two-day-long fever on days 2–3 PI with rectal temperatures reaching 40.0–40.5 °C and became anorexic from day 4 to day 6 PI. At 17 days after inoculation, one of the 2 dogs developed orchitis, which resolved within a week. Dogs developed IgG antibodies reactive with both *R. conorii* and *R. massiliae* antigens within 7–9 days PI. Antibody titers to *R. conorii* antigen reached their peak (mean 1:1448) on day 21 PI and declined to 1:512 within 110 days PI. Titers of cross-reaction with *R. massiliae* antigen closely followed that of anti-*R. conorii* antibodies. The anti-*R. massiliae* antibody titers peaked on day 24 (mean 1:724) and slowly declined to 1:128 within 110 days PI. The mean titers of the cross-reaction always remained within one-to-two 2-fold dilutions below the titers of the anti-*R. conorii* antibodies. By the day of tick-borne challenge – day 0 – the mean antibody titers reactive with *R. conorii* and *R. massiliae* antigens were 1:512 and 1:218, respectively (Fig. 2B).

When challenged with *R. conorii*-infected ticks, these dogs did not become sick. Their body temperature was only slightly elevated, i.e. 39.0–39.5 °C at 7–19 days post-challenge, and only one of the 2 was noticeably depressed from day 11 to 19 post challenge. Surprisingly, there was no boost of antibody titers following exposure to infected ticks, rather titers continued declining gradually and fell below the threshold level within 9–10 months post challenge (Fig. 2B). Throughout this period, mean titers to *R. massiliae* remained approximately 4× lower than those to *R. conorii*.

Four (10%) out of 39 challenge ticks were found infected with *R. conorii* (Table 1). Their prevalence of infection was similar to that in the challenging nymphal cohort before feeding. The prevalence of infection in acquisition-fed ticks placed on dogs at 1, 7, and 14 days after challenge was 10.0%, 1.7%, and 0%, respectively (Table 2).

Heterologous immunization – dogs inoculated with R. massiliae

Dogs needle-inoculated with *R. massiliae* exhibited no clinical signs of infection in spite of the relatively high infectious dose. Body temperature in one of the dogs was elevated to 39.6 °C next day after the inoculation, but this resolved by the following morning. No

depression, anorexia, or orchitis were observed. Dogs developed IgG antibodies reactive with both *R. massiliae* and *R. conorii* antigens within 7–15 days PI. Titers to *R. massiliae* reached their peak (mean 1:4096) within 5 weeks PI and declined to 1:512 within 135 days PI. Titers of cross-reaction with *R. conorii* reached the peak titer of 1:1024 within 38 days PI and also began declining right after the peak. By the day of tick-borne challenge – day 0 – the mean antibody titers measured with *R. massiliae* and *R. conorii* antigens were 1:512 and 1:256, respectively (Fig. 2C).

Following an infestation with *R. conorii*-infected ticks, neither of the 2 dogs immunized with the heterologous agent developed obvious signs of infection. Their body temperature remained within the normal range of 38.0–39.3 °C throughout the observation period, and there was no noticeable depression, anorexia, or orchitis after the challenge. Both dogs responded to an infestation with *R. conorii*-infected ticks by only a slight increase in IgG titers. Titers to both *R. conorii* and *R. massiliae* oscillated for several weeks after the challenge, but in general continued their decline and fell below the threshold level within 230–260 days. Surprisingly, the *R. massiliae*-immunized dogs responded to the challenge with *R. conorii*-by producing more IgG reactive with the *R. massiliae* antigen, and titers to *R. massiliae* remained significantly higher than those to *R. conorii* antibodies for 3–4 months following infestation with *R. conorii*-infected ticks (Fig. 2C).

Rickettsial DNA was detected in 5% of the challenging ticks that fed to repletion upon *R. massiliae*-immunized dogs and successfully molted into adults (Table 1). This prevalence of infection was not significantly different from that in the challenging nymphal cohort before feeding ($P = 0.306$). Uninfected ticks placed on dogs at 1, 7, and 14 days after challenge, were able to acquire rickettsial infection at a prevalence of 13.6%, 5.0%, and 0%, respectively (Table 2).

Effect of immunization on reservoir competence

Based on the indices of pathogen acquisition by larval ticks (Table 2) and assuming that dogs are continuously infested with constant numbers of larvae, the overall prevalence of infection among ticks feeding on naïve, *R. conorii*-immunized, and *R. massiliae*-immunized dogs within the three-week period following the dogs' exposure to *R. conorii* was estimated as 12%, 3%, and 5%, respectively.

When odds ratios for ticks to acquire rickettsial infection were compared between naïve and immunized dogs using a logistic regression model with a random effect for individual animals, both the homologous and heterologous immunization resulted in a significant decrease in the prevalence of infection among acquisition-fed ticks in comparison to those feeding upon unimmunized dogs (Fig. 3). Odds ratios for ticks acquiring rickettsial infection from *R. conorii*-immunized and *R. massiliae*-immunized vs. unimmunized dogs were 0.22 (95% CI 0.10–0.52) and 0.37 (95% CI 0.16–0.80), respectively. Multiple comparisons-adjusted, pairwise 95% CIs for the odds ratios between *R. conorii*-immunized and *R. massiliae*-immunized overlapped, and the odds ratios in those 2 groups were not significantly different.

Discussion

A list of known tick-borne rickettsiae has tripled in number over the past 15–20 years and continues to grow. *Rickettsia* spp. belonging to the spotted fever group (SFG) include such highly virulent pathogens as *R. conorii, R. rickettsii*, and *R. sibirica* causing Mediterranean spotted fever, Rocky Mountain spotted fever, and Siberian tick typhus, respectively. The rickettsial SFG also includes obligate endosymbionts of ticks as well as agents causing only mild or unapparent infections in vertebrate hosts. Several of these antigenically related bacteria may coexist within the same geographical areas. Antibodies produced in response to infection with one SFG *Rickettsia* cross-react with antigens of the other species so extensively that in patients naturally infected with either *R. rickettsii* or *R. conorii* the serologic tests usually cannot identify the causative agents (Hechemy et al., 1989). Considering this strong cross-reactivity between SFG organisms in conventional serological tests, it would seem logical to expect that host immune responses may affect the natural cycles and the proliferation efficiency of these antigenically related agents.

The prevailing views on the maintenance and proliferation of tick-borne SFG rickettsial pathogens in nature are firmly rooted in conclusions made some 30–45 years ago. According to these, most vertebrate hosts, including dogs, are incapable of developing levels of rickettsemia sufficient for infecting ticks (Burgdorfer et al., 1966; Norment and Burgdorfer, 1984), and even when there is a sufficient level of rickettsemia, the pathogen is present in blood only for a few days (McDade and Newhouse, 1986). It had been also demonstrated that both vaccinated animals and those that recovered from an acute infection do not become sick when challenged with rickettsial pathogens that would otherwise cause illness or death in naïve animals (Badger, 1933; Keenan et al., 1977; Feng and Waner, 1980; Folds et al., 1983, Eisemann et al., 1984; Gage and Jerrells, 1992). In the absence of modern tools allowing recognition of unapparent and subclinical infections, the lack of illness or measurable pathological manifestations was interpreted as an absence of infection per se. Therefore, it was concluded that animals once infected with *Rickettsia* sp. become immune and are protected against reinfection for the rest of their life (Lackman et al., 1965). Thus, the opportunity for a tick to acquire infectious rickettsiae from a host seems to be limited to only a few days in the lifetime of an animal. As such, the role of vertebrate hosts in propagation of a rickettsial pathogen has been considered insignificant or nonexistent, and it is generally concluded that the persistence of SFG *Rickettsia* can be assured by vertical transmission in tick vectors alone (Burgdorfer and Varma, 1967).

Transovarial transmission of SFG rickettsiae should theoretically allow for a high prevalence in vector populations, yet, proportions of ticks caring highly pathogenic *Rickettsia* spp. (*R. conorii, R. rickettsii, R. sibirica*) are usually quite low in natural populations (Socolovschi et al., 2012). Reasons for this incongruence may stem from negative effects of pathogenic rickettsiae on the survival of their vectors (Niebylski et al., 1997; Levin et al., 2009; Socolovschi et al., 2009), anti-rickettsial immune responses in the susceptible vertebrate hosts (Keenan et al., 1977), competitive relationships between rickettsiae within vectors (Burgdorfer et al., 1981), or any combination of these processes.

As some pathogenic SFG *Rickettsia* can negatively affect survival and fecundity of their hosts (Niebylski et al., 1999; Levin et al., 2009; Socolovschi et al., 2009), it seems likely that these agents cannot be indefinitely maintained in nature solely by vertical transmission, and periodic amplification and horizontal dissemination of infection via vertebrate hosts appear necessary for continuous persistence of these pathogens within tick populations. We recently showed that domestic dogs are competent reservoirs for *R. conorii*, but immune responses to a natural tick-borne infection indeed can decrease the efficiency of horizontal transmission of the pathogen, though do not necessarily prevent it (Zemtsova et al., 2010; Levin et al., 2012). In spite of cross-reactivity between SFG rickettsiae in serological tests, immunization with *R. conorii* did not seem to preclude development of eschars in laboratory guinea pigs needle-challenged with several related SFG rickettsial agents (Bechah et al. 2012). However, guinea pigs are not natural hosts for either of the SFG pathogens used in that study, and the results may not be fully applicable to circulation of rickettsial agents in nature. Therefore, we considered it important to assess whether homologous or heterologous immune responses in natural hosts – dogs – may affect their reservoir competence for a pathogenic *Rickettsia*.

Immunity to obligate intracellular bacteria like *Rickettsia* typically involves both cell-mediated and humoral responses. Antibody is considered important in preventing reinfection with SFG rickettsiae by binding to the organisms and blocking attachment or penetration of the host cell (Jerrells, 1997). The presence of serum antibody was shown useful in predicting resistance to challenge with *R. rickettsii* using a guinea pig model (Folds et al., 1983). Therefore, we evaluated the use of IgG titers in dogs as an indication of total immune response and a measure of cross-reactivity between the agents.

As expected, antibodies generated in dogs to both *R. conorii* and *R. massiliae* were highly cross-reactive; antibody titers to homologous and heterologous antigens in immunized dogs differed by no more than 2 dilutions. This was similar to previous observations made in dogs and guinea pigs (Breitschwerdt et al., 1988; Gage and Jerrells, 1992). Unlike in studies where the rickettsial challenge was delivered by needle-inoculation, IgG titers in seropositive dogs were not boosted in response to the infectious challenge when it was delivered by *R. conorii*-infected ticks. This was observed in dogs which, at the time of challenge, had moderate titers of antirickettsial IgG (range 1:128–1:512) following previous exposure to either *R. conorii* or *R. massiliae*. In these animals, both homologous and heterologous titers did not increase after the challenge although they fluctuated for several days after the challenge, but otherwise continued to decline. It is noteworthy that in *R. massiliae*-immunized dogs, anti-*R. massiliae* titers remained higher than titers of anti-*R. conorii* antibodies for almost 3 months after the challenge with *R. conorii*-infected ticks, probably demonstrating the “original antigenic sin” effect (Francis, 1960).

Only in dogs originally infected via tick bite, where antibody titers fell below the diagnostic threshold by the time of the challenge, was a rise in antibody titer observed after the challenge with infected ticks, although the increase in titer was much less than the original response and short-lived. If a certain level of antigen presentation is needed to stimulate secondary immune response and boost antibody production, it follows that in dogs with high IgG titers at the time of infectious challenge, *R. conorii* was prevented from propagating at

the site of inoculation and disseminating from it in amounts sufficient to induce an antibody boost. Conversely, in dogs, whose antibody titers fell below the diagnostic threshold, the pathogen was permitted to propagate and consequently caused a temporary increase in IgG titers. It is noteworthy that the same 2 dogs developed orchitis after the second challenge indicating that the pathogen indeed spread from the site of tick bites on animals' backs, unlike in dogs with high antibody titers, which developed no clinical manifestations. Thus, titers of circulating IgG may be indicative of the immune system's preparedness to control colonization and propagation of *Rickettsia* in case of reinfection, although effects of cellular immunity were not measured in this study.

In this study, all ticks were tested after molting to the next stage to insure that the only *Rickettsia* detected were those that could be successfully transmitted transstadially and not just remnants of DNA from killed bacteria ingested by ticks with the host blood. Consequently, the prevalence of *Rickettsia* in acquisition-fed ticks reflects the host's reservoir competence – an ability to transmit an infection to arthropod vectors. Infected nymphal *Rh. sanguineus* fed on dogs for up to 5 days. Cohorts of ticks used for acquisition feeding were placed on the dogs 7 and 14 days after nymphal infestation – several days following removal of all engorged nymphs. Direct (salivary) transmission between these ticks was not possible because infected nymphs and the 2 cohorts of uninfected larvae were never on the dogs at the same time. Thus, detection of *Rickettsia* in ticks, placed on dogs 7–14 days after the challenge, must be due to bacterial survival and persistence in the host animal, confirming that preexisting antibodies do not fully prevent reinfection of immunized dogs with either the same or a closely related *Rickettsia*. It also concurs with the observation that an existing immune response does not prevent development of eschars in intradermally inoculated guinea pigs (Bechah et al., 2012).

Still, dogs immunized with either *R. conorii* or *R. massiliae* displayed a significantly lower overall capacity for horizontal transmission of *R. conorii* after reinfection, compared to naïve dogs. While naïve dogs remained infectious for *Rh. sanguineus* larvae for at least 3 weeks after tick-borne challenge, the immunized dogs supported horizontal transmission of *R. conorii* only up to 2 weeks, implying a shortened persistence of the pathogen. As a result, the overall odds of *R. conorii* acquisition by larvae feeding on dogs within the three-week period after infection are decreased by approximately 66–80% on dogs immunized with *R. massiliae* and *R. conorii*, respectively. Effects of *R. massiliae* immunization on the reservoir competence of dogs for *R. conorii* were not statistically different from the effects of immunization with *R. conorii* itself.

Conversely, the efficiency of rickettsial dissemination between cofeeding ticks was not significantly affected by homologous or heterologous immunity; and transmission of *R. conorii* from infected nymphs to uninfected larvae feeding side-by-side on dogs previously exposed to either *R. conorii* or *R. massiliae* was as efficient as on naïve dogs. Approximately 10–15% of *Rh. sanguineus* larvae, feeding side-by-side with infected nymphs on either naïve dogs or dogs immunized with *R. conorii* or *R. massiliae*, successfully acquired *R. conorii* and maintained it through the molt. This is in agreement with previously published conclusions that cofeeding is an efficient route of rickettsial horizontal transmission and proliferation in tick populations (Zemtsova et al., 2010).

On the whole, our findings suggest that immune responses to rickettsial infection (whether cellular or humoral) do not fully prevent establishment of cutaneous reinfection, but can neutralize rickettsiae as they attempt to spread from the site of tick bite decreasing amounts of the pathogen to levels below the threshold sufficient for causing a boost in antibody production. Also, titers of the circulating IgG may be indicative of the immune system's preparedness to combat rickettsial proliferation and dissemination. By preventing proliferation and establishment of new foci of infection throughout the host organism, immunity shortens the period when a dog can be infectious to new ticks and thereby significantly reduces the reservoir competence of the host.

However, the preexisting immunity, apparently, does very little to control the transmission of *Rickettsia* at the site of attachment of infected ticks. It may be due to immune-modulating properties of tick saliva or other means, but uninfected larvae feeding side-by-side with infected ticks on immunized dogs are able to acquire *Rickettsia* with the same degree of success as on naïve dogs. Therefore in dogs continuously exposed to *Rickettsia*-infected ticks throughout the season, high efficiency of transmission between cofeeding ticks may negate effects of the preexisting immunity on dog reservoir competence.

In this study, the reduction in overall reservoir competence appears to be greater in dogs with homologous than with heterologous immunity, however, there was no statistically significant difference between the 2 groups. This suggests that the efficiency of transmission and propagation of pathogenic *Rickettsia* in natural foci may be reduced by immunizing the primary hosts (dogs) with closely related nonpathogenic SFG bacteria. However, effects of this decrease may not be sufficient to interrupt the transmission cycle in locations where ticks are sufficiently abundant and dogs are repeatedly infested with infected and uninfected ticks throughout the season. In these situations, transmission of the pathogen between cofeeding ticks can be adequate for uninterrupted circulation of the pathogen even if 100% of available dogs are seropositive for SFG *Rickettsia*. Therefore, any strategies aimed at controlling natural cycles of rickettsial transmission must include sustained efforts to decrease the abundance of tick vectors.

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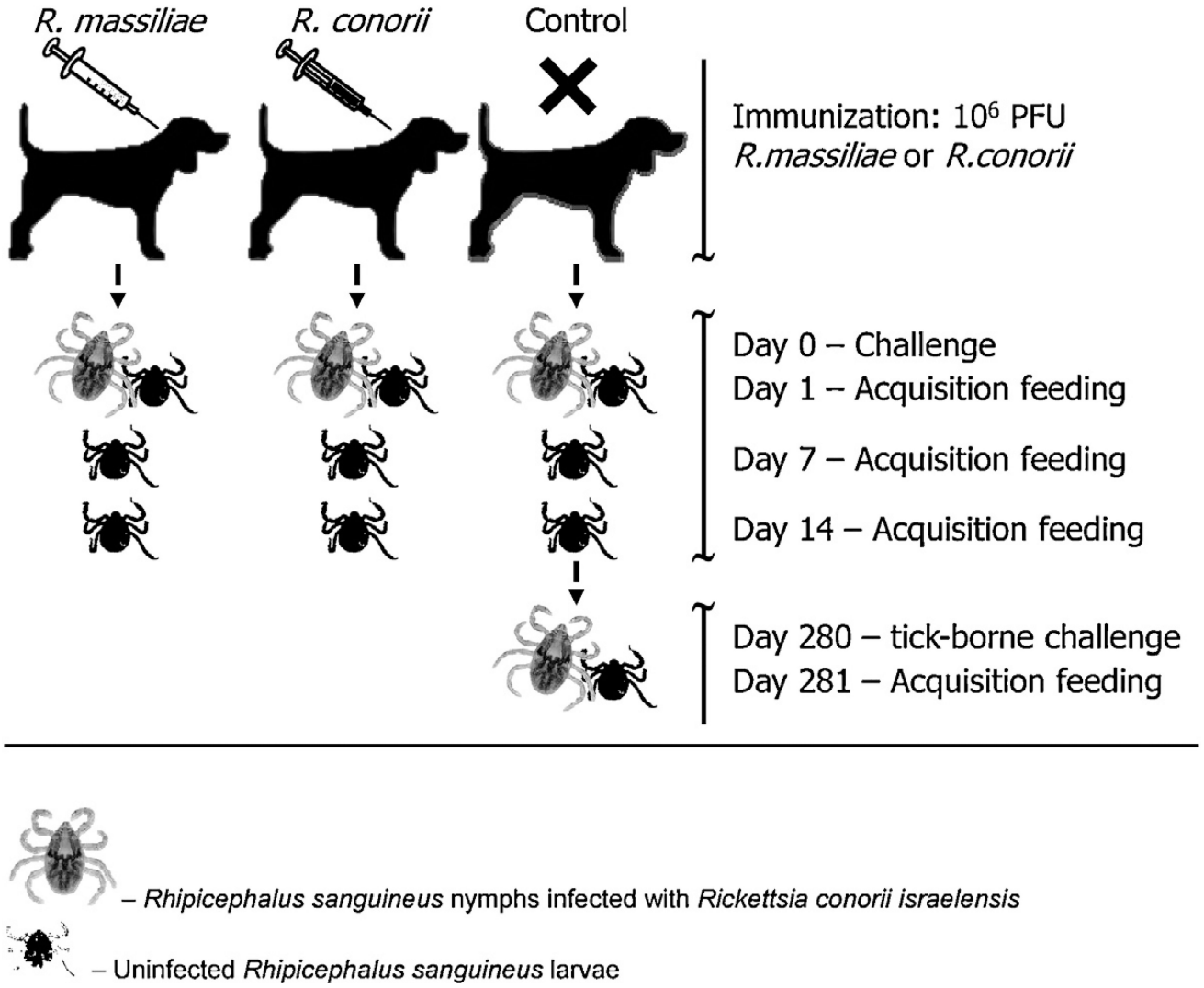

Fig. 1.
Flowchart of the experiment.

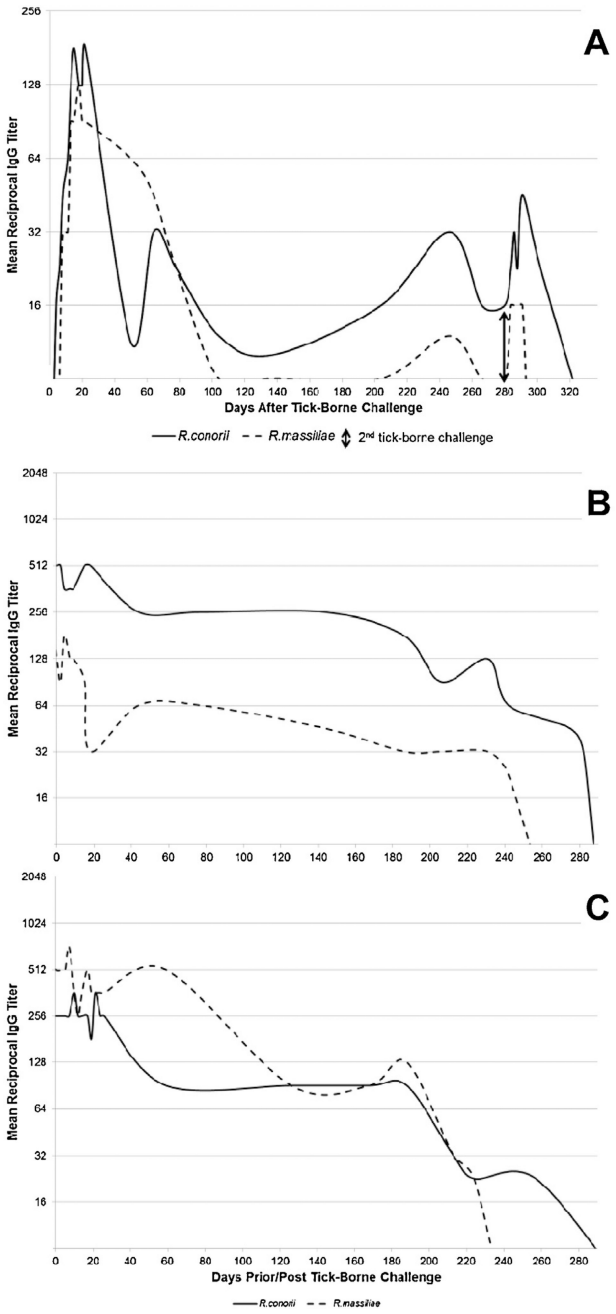

Fig. 2.

Geometric mean titers of antibodies reactive with the *R. conorii* and *R. massiliae* whole-cell antigens in unimmunized and immunized dogs following infestation with *R. conorii*-infected ticks. (A) Unimmunized dogs; (B) dogs previously immunized with *R. conorii*; (C) dogs previously immunized with *R. massiliae*.

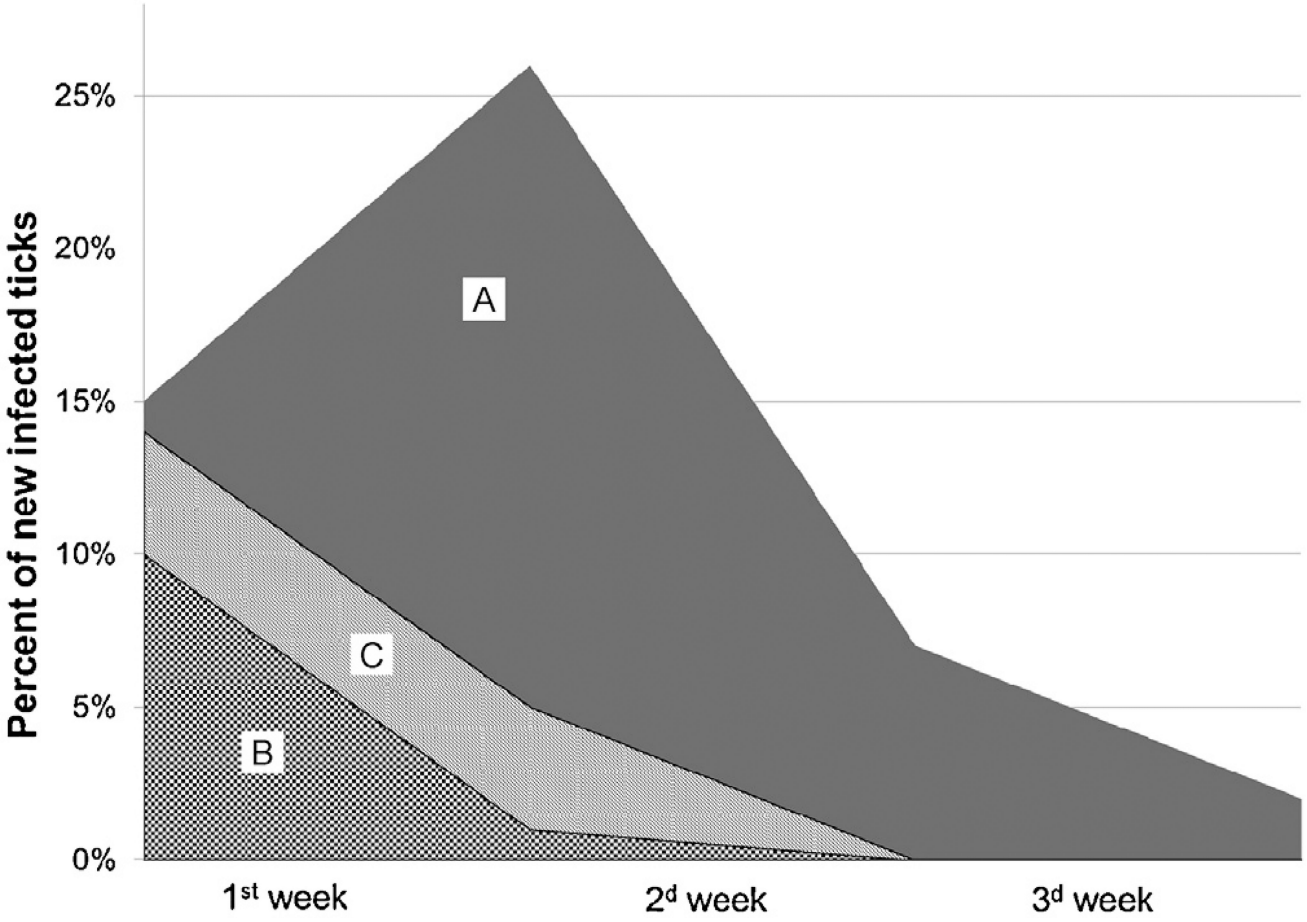

Fig. 3.

Comparative reservoir competence of naive and immunized dogs following an exposure to *R. conorii*-infected ticks: rates of the pathogen acquisition by ticks feeding upon (A) unimmunized dogs; (B) dogs previously immunized with *R. conorii*; (C) dogs previously immunized with *R. massiliae*.

Table 1

Prevalence of *Rickettsia conorii israelensis* in the challenging ticks prior to and after feeding on immunized and unimmunized (control) dogs.

Tested prior to feeding as nymphs			Tested after feeding as adults			
Tested	**Positive**	**%**	**Dogs**	**Tested**	**Positive**	**%**
45	5	11%	Unimmunized	39	8	21%
			R. conorii-immunized	39	4	10%
			R. massiliae-immunized	40	2	5%

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Table 2

Prevalence of *Rickettsia conorii israelensis* in nymphal *Rhipicephalus sanguineus* acquisition-fed as larvae on immunized and unimmunized (control) dogs.

Dogs	Day of larval infestation post-challenge (% ± SE)		
	1	7	14
Unimmunized	15.0 ± 3.6%	26.0 ± 4.4%[a]	7.0 ± 2.6%[a]
R. conorii-immunized	10.0 ± 2.6%	1.7 ± 1.2%	0.0 ± 0.0%
R. massiliae-immunized	13.6 ± 3.4%	5.0 ± 2.2%	0.0 ± 0.0%

SE, standard error.

[a]Statistically significant difference between immunized and unimmunized dogs.