A Focus of Dogs and *Rickettsia massiliae*–Infected *Rhipicephalus sanguineus* in California

Emily Beeler, Kyle F. Abramowicz, Maria L. Zambrano, Michele M. Sturgeon, Nada Khalaf, Renjie Hu, Gregory A. Dasch, and Marina E. Eremeeva*

*Rickettsial Zoonoses Branch, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Veterinary Public Health and Rabies Control Program, Los Angeles County Department of Public Health, Los Angeles, California; VCA McClave Animal Hospital, Reseda, California; Vector-Borne Disease Section, Center for Infectious Diseases, California Department of Public Health, Ontario, California*

**Abstract.** A recurrent focus of *Rhipicephalus sanguineus* infestation was investigated in a suburban area of southern California after reports of suspected Rocky Mountain spotted fever in two dogs on the same property. Abundant quantities of *R. sanguineus* were collected on the property and repeatedly from each dog, and *Rickettsia massiliae* DNA was detected by polymerase chain reaction (PCR). Whole blood and serum samples from four dogs were tested by using PCR and microimmunofluorescent assay for antibodies against spotted fever group rickettsiae. Serum samples from all four dogs contained antibodies reactive with *R. massiliae*, *R. rhipicephali*, *R. rickettsii*, and 364D *Rickettsia* but no rickettsial DNA was detected by PCR of blood samples. Serum cross-absorption and Western blot assays implicated *R. massiliae* as the most likely spotted fever group rickettsiae responsible for seropositivity. To our knowledge, this is the first detection of *R. massiliae* in ticks in California.

**INTRODUCTION**

*Rickettsia massiliae* Mtu1 was first isolated in 1990 from a *Rhipicephalus turanicus* tick collected from a horse in Le Sambuc, Bouches-du Rhone, France. Its formal taxonomic species description appeared in 1993. Other isolates of *R. massiliae* were later obtained from *Rhipicephalus sanguineus* from Spain (Bar29), France (Mtu5), Greece (GS), and Arizona (AZT80), USA. Different genotypes of *R. massiliae* have been detected in several other ticks of the so-called *Rhipicephalus* spp. complex including Mtu5 genotype in *Rh. senegalensis*, and Mtu1 genotype in *Rh. sulcatus*, *Rh. lunulatus* and *Rh. mushamae* in central Africa, and Bar29 genotype in *Rh. sanguineus* and *Rh. turanicus* in Switzerland. The distribution of *R. massiliae* and its association with different *Rhipicephalus* spp. was subsequently extended to other Mediterranean and African countries, and to Argentina. The reported prevalence of *R. massiliae* in ticks has varied from 4.7% to 18%. However, the true prevalence and range of distribution of *R. massiliae* in *Rhipicephalus* ticks are unknown, especially in the New World. Whether the specific genetic types of *R. massiliae* have adapted preferentially to different tick vectors, animal hosts, and whether their potential to cause febrile illness varies are unknown.

The first confirmed human case of *R. massiliae* infection was reported in 2006. It was diagnosed by polymerase chain reaction (PCR) and sequence characterization of a 1984 cell culture isolate from a blood sample collected from a man hospitalized in Sicily with fever, maculopapular rash on the palms and soles, mild hepatomegaly, and an eschar. In 2008 in France, a second patient was diagnosed with similar clinical symptoms that included two eschars on the thigh and buttock, and bilateral chorioretinitis with acute blindness. The latest case was diagnosed in Spain by retrospective PCR testing of eschar tissue from a female patient from Buenos Aires, Argentina.

There may have been additional cases of human infection with *R. massiliae*. Children diagnosed with Mediterranean spotted fever in Catalonia, an area that is endemic for the Bar29 isolate of *R. massiliae*, were refractory to treatment with rifampin, tested as an alternative antibiotic when tetracycline is contraindicated. *R. massiliae* is resistant to rifampin in vitro, and *R. conorii* (the etiologic agent of Mediterranean spotted fever) is not resistant.

The similar clinical manifestations, common vector within the area endemic for Mediterranean spotted fever, and the use of cross-reactive serologic assays for diagnosis of rickettsial diseases complicate understanding the true prevalence and distribution of *R. massiliae* and associated human or potentially canine infections. A limited canine serosurvey in northeastern Spain demonstrated that 8.6–25% (n = 93) of Catalonian dogs were exposed to *R. massiliae* Bar29 and 4–20% to *R. conorii* or another cross-reacting *Rickettsia*. A survey of *Rh. sanguineus*-infested dogs in Seville Province, Spain, showed that 18% were carrying ticks infected with *R. massiliae*. Because dogs are recognized sentinels for human rickettsial diseases, these findings may correlate with a higher prevalence of human cases caused by *R. massiliae* in areas where this agent is found.

We report the results of an investigation triggered by reports of sick dogs living on a property in Los Angeles County, California, with sustained infestation by *Rh. sanguineus*. We found that *R. massiliae* is present in California ticks and show the efficient long-term maintenance of *R. massiliae* in this naturally infected population of *Rh. sanguineus*. We also report serologic evidence for canine exposure to this *Rickettsia*, and demonstrate the lack of obvious negative effect of canine anti-rickettsial antibodies on tick feeding and circulation of *R. massiliae* in those ticks.

**MATERIALS AND METHODS**

**Study description.** In August 2007 and March 2008, the Los Angeles County Veterinary Public Health and Rabies Control Program (LACVPH) was notified about two ill dogs on the same property suspected of having Rocky Mountain spotted fever, a rare condition in the area. Both dogs were seropositive to spotted fever group rickettsiae (SFGR), as established by testing at the commercial laboratory, and had a heavy infestation with the brown dog tick *Rh. sanguineus*. The
LACVPH reviewed the clinical history, treatment outcome, and commercial laboratory testing results of these dogs. Whole blood treated with EDTA and serum were collected from both case dogs and from two housemate apparently healthy dogs and submitted to the Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention (CDC) (Atlanta, GA) for further testing. Ticks were also collected from the property and dogs and submitted to the CDC for testing. The owner was advised on tick control and prevention measures.

Preparation of antigens for electrophoresis and Western blotting. Rickettsia rickettsii strain Bitterroot, R. massiliae strain AZT80, R. rhipicephali strains 3-7-3-6 and CA871, and 364D Rickettsia were cultivated in African green monkey kidney cells (E6-Vero), harvested, and purified from the host cells using centrifugation through 25–45% (w/v) Renografin (Nycomed, Inc., Princeton, NJ) gradients as described. Purified rickettsiae were kept frozen in aliquots (10 mg of protein/mL) in sterile distilled water at −80°C until used for electrophoresis and Western blotting.

For electrophoresis, suspensions of purified rickettsiae were diluted in water to a concentration of 3 mg of protein/mL, and an equal volume of 2× Laemml sample solubilizing buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue dye) was added at room temperature.

Indirect microimmunofluorescence testing. Antigens consisted of the Rickettsia-infected nuclear fraction collected as a by-product of the rickettsial purification from infected cells; it was resuspended and frozen in SRM buffer (0.281 M sucrose, 5 mM potassium glutamate buffer, pH 7.0, supplemented with 1% bovine serum albumin and 1% normal goat serum) to the last visible endpoint or 1:2,048 dilution. For the detection of IgM, IgG was first spotted onto glass slides, air-dried, and fixed in acetone.

Serum samples were screened by indirect immunofluorescent antibody (IFA) assay at consecutive 2-fold serum dilutions starting at 1:32 in a dilution buffer (phosphate-buffered saline, pH 7.4, supplemented with 1% bovine serum albumin and 1% normal goat serum) to the last visible endpoint or a 1:2,048 dilution. For the detection of IgM, IgG was first absorbed with a Mini Rapi-Sep-M kit (Panbio Diagnostics, Columbia, MD) according to the manufacturer’s instructions. Fluorescein isothiocyanate–labeled goat anti-dog IgM (anti-μ chain) and anti-dog IgG (anti-γ chain) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were diluted 1:100 and 1:150, respectively. IgG and IgM titers ≥ 1:64 were considered positive.

Table 1: Detection of persistent presence of spotted fever group rickettsiae in Rhipicephalus sanguineus, California*

<table>
<thead>
<tr>
<th>Date collected</th>
<th>Source of ticks</th>
<th>No. collected</th>
<th>Total PCR positive</th>
<th>Female (no. positive/no. tested)</th>
<th>Male (no. positive/no. tested)</th>
<th>No sequenced</th>
<th>Rickettsial prevalence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/24/2008 Dog B</td>
<td>15</td>
<td>12</td>
<td>9/12</td>
<td>3/3</td>
<td>5</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>4/3/2008 Property yard</td>
<td>50</td>
<td>32</td>
<td>2/2</td>
<td>30/48</td>
<td>4</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>4/13/2009 Dog A</td>
<td>23</td>
<td>0</td>
<td>0/11</td>
<td>0/12</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4/16/2009 Dog A</td>
<td>48</td>
<td>17</td>
<td>9/24</td>
<td>8/24</td>
<td>8</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>4/17/2009 Dog C</td>
<td>31</td>
<td>6</td>
<td>1/8</td>
<td>5/23</td>
<td>1</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>4/20/2009 Dog D</td>
<td>10</td>
<td>3</td>
<td>1/5</td>
<td>2/5</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

*PCR = polymerase chain reaction.
that there was no cross-contamination of reagents. All samples were tested as two replicates, and melting curves were analyzed for each amplicon by using an iCycler (Bio-Rad). For all PCR-positive ticks a 70–602 nucleotide fragment of ompA was amplified by semi-nested PCR, purified, and sequenced by using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s recommendations on an ABI 3130xl genetic analyzer. Sequencing reads were assembled by using Sequencher 4.8 (Gene Codes, Ann Arbor, MI). Homologous sequences were detected by using the National Center for Biotechnology Information (Bethesda, MD) Basic Local Alignment Sequence Tool search engine.

RESULTS

Description of canine cases and the study site. Dog A, a German shepherd, was brought to a local veterinarian on July 22, 2007, August 26, 2007, and April 13, 2009, with episodes of anorexia, lethargy, leukopenia, increased levels of liver enzymes, and heavy infestation of Rh. sanguineus. Seropositivity to SFGR was established by using R. rickettsii antigen and a commercial IFA (titer = 1:128) after the second episode of illness. Oral doxycycline was prescribed and resulted in complete recovery. Doxycycline administered after the third episode resulted in minimal improvement and the dog was humanely killed because of its continued poor health. Dog B, a Doberman pinscher mixed breed, had similar tick infestation and mild illness, accompanied by fever and IgG titers to R. rickettsii antigen (titer = 1:512) in March 2008. The dog owner used a pyrethrin-shampoo bath and topical fipronil for animal treatment and amitraz-impregnated collars for all dogs, and pyrethroid-based spray for environmental tick control in August 2007, and reported successful results until efforts ceased in 2008. Tick numbers then began to increase considerably again on the dogs by late spring of 2009.

Tick testing results. All ticks collected from dogs and from the property were morphologically identified as Rh. sanguineus; their 12S mitochondrial rDNA (HM014444) had the highest nucleotide sequence identity to the homologous gene of Rh. sanguineus from Oklahoma (AF081829) and the Mediterranean region (AF150020 and AF150018). Thirty-two of 50 questing ticks from property were morphologically identified as Rh. sanguineus. Three of the four dogs were infected with SFGR on each of the four dogs (Table 2). Antibody titers ≥ 1:64 to each of the SFGR antigens were identified in one or more serum samples from each dog evaluated. Dog A had a previous history of illness and coincident increased R. rickettsii IgG titers tested by a commercial laboratory in 2007. In 2009, dogs A, C, and D had IgG titers to R. massilae much higher than cross-reactive IgG titers to R. rhipicephali, R. rickettsii, and 364D Rickettsia. None of the blood samples obtained from the dogs on the day of their presentation to the veterinarian and necropsy samples of dog A, including brain, lung, heart, liver, spleen, mesenteric lymph node and adrenal gland, were PCR positive for SFGR. No useful etiologic observations were made after hematoxylin eosin staining and immunohistochemical staining for SFGR in postmortem tissues of dog A.

Identification of rickettsial agents responsible for seroconversion by Western blotting and serum cross-absorption. Serum samples from all four dogs were tested by Western blotting with four SFGR antigens and showed strong reactivity with high molecular mass proteins ranging from 116 kD to 127 kD (Figure 1A). Samples also contained antibodies reacting with low molecular mass proteins and lipopolysaccharide type was identified (HM014444); amplicons sequenced were identical to each other and had 99% and 100% sequence similarity, respectively, to the homologous fragments of R. massilae AZT80 and Bar29. The rest of the 50 questing tick samples tested had relatively low copy numbers of the rickettsial DNA (cycle threshold > 36.9), which precluded amplification of the larger ompA fragment required for sequence identification of the SFGR. Twelve of 15 Rh. sanguineus collected from dog B in late March 2008 were PCR positive for SFGR DNA. Nucleotide sequence of the five amplicons analyzed had 100% sequence identity to the homologous ompA fragment of R. massilae Bar29.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Date of serum collection</th>
<th>Reciprocal IgG (IgM) IFA titer to antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8/26/2007</td>
<td>R. massilae AZT80: 256 (128)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R. rickettsi 364D: 1,024 (512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R. rickettsi 364D: 1,024 (512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R. rickettsi Butte: 1,024 (512)</td>
</tr>
<tr>
<td>B</td>
<td>3/24/2008</td>
<td>1,024 (512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td>C</td>
<td>4/17/2009</td>
<td>2,048 (128)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td>D</td>
<td>4/20/2009</td>
<td>2,048 (256)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (512)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,024 (2,048)</td>
</tr>
</tbody>
</table>

*IFA = indirect immunofluorescent antibody.
antigens. However, their detection and visualization were more variable and dependent on the serum working dilution tested. To evaluate the nature of the rickettsial agent responsible for seroconversion, serum absorption with each of four SFGR was performed and each of the depleted sera was tested side-by-side with diluent only–treated homologous serum samples at the same working dilution. Similar absorption results were obtained with serum from each of the four dogs, but are shown only for dog B serum. When absorption was performed with *R. massiliae* antigen (Figure 1B), it resulted in removal and absence of binding of homologous and heterologous SFGR antibodies from serum samples treated and negative results by Western blotting (Figure 1A). In contrast, when absorption was performed with *R. rhipicephali* (Figure 1C), 364D *Rickettsia* (Figure 1D), or *R. rickettsii* (Figure 1E) antigens, only cross-reactive antibodies to homologous antigens were removed, and reactivity of antibodies to *R. massiliae* was still detectable.

DISCUSSION

To our knowledge, this is the first report of *R. massiliae* in *Rh. sanguineus* from California. Our findings expand the known geographic range in North America for *R. massiliae*, which now includes southern California in addition to a reported focus in Arizona. Numerous ticks containing *R. massiliae* were present on this property during 2008–2009. Canine infection with *R. conorii* (the etiologic agent of Mediterranean spotted fever) shows clinical symptoms and serum and hematologic abnormalities similar to those of Rocky Mountain spotted fever. In both infections, rickettsiemia detectable between days 2 and 12 after inoculation develops in acutely ill dogs. This illness is followed by complete clearance and development of anti-rickettsial IgG; its persistence and titers depend on the number of inoculated rickettsiae. In contrast, dogs infected with *R. montanensis*, a SFGR of unknown pathogenicity, remain asymptomatic. However, such exposure is usually sufficient to elicit protective immune response to subsequent inoculation with *R. rickettsii*. Antibody responses in dogs infected with *R. rickettsii* show a similar pattern of reactivity to *R. rickettsii*, *R. montanensis*, *R. rhipicephali*, and *R. bellii*. However, treatment with tetracycline causes significant delays in serologic responses of infected dogs to heterologous rickettsial species.

Two of the four dogs had an illness compatible with mild-to-moderate canine rickettsioses caused by *R. rickettsii* and *R. conorii*. Although the two other dogs were never reported to be ill, their serologic evaluation confirmed strong seropositivity to SFGR. The repeated consistent detection of *R. massiliae* in engorged and questing *Rh. sanguineus* and serum cross-absorption Western blotting results implicate *R. massiliae* as the likely etiologic agent responsible for SFGR seropositivity detected in all four dogs. Because only whole blood samples from immune dogs were collected and tested, it is not surprising that PCR detection of rickettsial DNA was not observed. Therefore, subsequent episodes of febrile illness in dog A were probably not caused by repeated and persistent exposure to *R. massiliae*-infected ticks. Although canine susceptibility to *R. massiliae* has not been established experimentally or proven clinically, this human pathogen may also cause rickettsial infection in dogs. Furthermore, immune response to *R. massiliae* will probably prevent infection of dogs with other SFGR.

Natural maintenance of SFGR depends in part on the efficiency of transovarial and transstadial transmission in their tick vectors. However, this vector–agent interaction appears to vary significantly among different rickettsial species and ticks. Our observations demonstrated inefficient and damaging vertical transmission of *R. rickettsii* in *Rh. sanguineus* from eastern Arizona. In contrast, *R. massiliae* Mt1 shows efficient transovarial and transstadial transmission in *Rh. turanicus*, excretion of *R. massiliae* in saliva and feces of infected
ticks, and transmission through co-feeding to Rh. sanguineus. High rates of R. massiliae infections were demonstrated in Rh. sanguineus collected from dogs in the current study (19–80% prevalence in different collections), in questing Arizona ticks from one household used for isolation of R. massiliae AZT80 (25% of 20). Because infection with the agent was not detected in any of 62 Rh. sanguineus from two other sites in California, it appears that high prevalence rates of R. massiliae infections in ticks may arise either from clonal expansion from a single infected female tick or by efficient co-feeding transmission of this agent between ticks in a restricted focus.

Dogs evaluated during this study had high titers of IgG antibody reactive with R. massiliae. Similarly, 80% of 152 dogs infested with R. massiliae-infected Rh. sanguineus in Buenos Aires had antibodies reactive with R. conorii. However, a high percentage of ticks removed from seroconverted dogs were PCR positive in both studies, which suggested that antibodies against SFGR in the dogs did not affect significantly either transovarial and transtadial transmission or co-feeding acquisition of R. massiliae. How general this finding may be or which route is impacted more severely by the presence of antibodies in dogs is being evaluated in controlled laboratory studies.

Rhipicephalus sanguineus is found throughout the United States. Although it is commonly stated that this tick does not bite humans in this country, there are multiple reports of human exposures to Rh. sanguineus. Such human encounters may become more frequent when severe infestations occur. High temperatures are thought to increase the risk of humans being bitten. In temperate climates, Rh. sanguineus may establish persistent infestations in the environment and also can penetrate inside houses.

Common steps for prevention of most Ixodid tick exposures, which include avoiding tick-infested areas and wearing protective clothing, are not sufficient for addressing nidi- colous Rh. sanguineus infestations. Such infestations may also be expensive to eliminate and may recur (as in this report) after partial attempts at eradication. Routine canine inspections and use of shampoos and tick treatments are effective approaches, but persisting infestations require environmental control measures, including the services of professional exterminators. Households with fewer resources may be less able to control such infestations. Homeless persons surrounded by dogs are especially vulnerable to bites from Rh. sanguineus. Physicians and veterinarians are encouraged to maintain skills on basic tick identification, to differentiate R. sanguineus ticks from other Ixodid ticks, and to counsel patients and dog owners on tick control. Physicians in California and elsewhere should consider R. massiliae as a potential cause of illness in their patients, especially in those exposed to dogs and Rh. sanguineus.

Received June 22, 2010. Accepted for publication November 1, 2010.

Acknowledgments: We thank Gail Van Gordon and RobynSpano for facilitating this collaboration and for help with specimen storage, Karen Ehnert and Alexandra Swanson for reviewing the manuscript, Sarah Janis and Christopher Tsai for assistance with collection of specimens, Shari Lydy for membrane protein solubilization protocol, Christopher D. Paddock for performing immunohistochemical testing, and Peggy Barr and Linda Kidd for helping set up local surveillance for spotted fever group rickettsiae in dogs. We also thank the owner of the dogs for interest in this study and permission to perform testing.

Disclaimer: The findings and conclusions are those of the authors and do not necessarily reflect the views of the U.S. Department of Health and Human Services.

Authors’ addresses: Emily Beeler, Veterinary Public Health and Rabies Control Program, Los Angeles County Department of Public Health, Los Angeles, CA, E-mail: ebeeler@ph.lacounty.gov. Kyle F. Abramowicz, Maria L. Zambrano, Michele M. Sturgeon, Gregory A. Dasch, and Marina E. Ereomeeva, Rickettsial Zoonoses Branch, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, E-mails: kabra@cdc.gov, mzanbrano@cdc.gov, msturgeon@cdc.gov, gdasch@cdc.gov, and meremeeva@cdc.gov. Nada Khalaf, VCA McClave Animal Hospital, Reseda, CA, E-mail: nada.khalaf@vca-hospitals.com. Renjie Hu, Vector-Borne Disease Section, Center for Infectious Diseases, California Department of Public Health, Ontario, CA, E-mail: renjie.hu@cdph.ca.gov.

REFERENCES


