DETECTION OF BORRELIA AND EHRLICHIA IN RHIPICEPHALUS SANGUINEUS

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Rhipicephalus sanguineus, the brown dog tick, is endemic throughout the world wherever domestic dogs are present. It has been recently reported by some veterinarians in the city of Laredo, Texas, USA, that Lyme disease, the most common tick-borne disease in the Northern United States, is present in local domestic dogs. Fully engorged R. sanguineus ticks were collected and their DNA was purified. The ticks were screened to determine the prevalence of Borrelia, Rickettsia and Ehrlichia species. Sequences related to Borrelia burgdorferi in 9.8% (n=11/112), "Candidatus Borrelia lonestari" in 16.9% (n=19/112) and Ehrlichia canis in 12.5% (n=14/112) were detected by PCR. Sequencing has confirmed the presence of DNA from Ehrlichia canis and “Candidatus B. lonestari”, corroborating that Borrelia and Ehrlichia are present in domestic dogs in South Texas.

INTRODUCTION

Rhipicephalus sanguineus, known as the brown dog tick, is the most widely distributed tick in the world (13). This tick is a known vector of Ehrlichia canis (23), the causative agent of canine ehrlichiosis (1,31). The symptoms of chronic E. canis infection in domestic dogs may include thrombocytopenia, anemia, weight loss, bleeding, fever, inflammation of the eye, and anoxic hepatitis (35). Acute ehrlichiosis in domestic dogs may result in loss of appetite, lethargy, shortness of breath, bruises, joint pain and depression (25). R. sanguineus is also thought to be a vector of Rickettsia rickettsii (13,22), the cause of the Rocky Mountain spotted fever (14). Although R. sanguineus ticks typically prefer to feed on domestic dogs, they have been reported to parasitize humans as well (20,35). The presence of a disease agent in the domestic dog population can indicate that the disease could also be present in humans (29).

B. burgdorferi sensu stricto (39) has been identified as the sole etiologic agent of Lyme disease in North America (24,38). Lyme disease is the most common vector-borne illness in the Northern United States (8) and is considered an emerging infectious disease (32). The main vectors are Ixodes scapularis (17,18,37) and Ixodes pacificus (34). The agent has also been detected at a lower incidence in R. sanguineus (9,21,42) and Amblyomma inornatum ticks (30). However, the vector potential of these ticks has not yet been characterized. It has been previously reported that B. burgdorferi in domestic dogs may result in arthritis, similar to
humans suffering from Lyme disease (27). Symptoms in domestic dogs from the acute form of Lyme disease may include fever, swelling, pain, lameness, lymphadenopathy and malaise (12). Acute renal failure, myocarditis, cardiac arrhythmia, peripheral edema, neurological syndrome and arthritis have been described as clinical signs found in the chronic form of Lyme disease in domestic dogs (2). A Lyme disease–like illness has also been described in the southern United States since the 1980s (41). This condition is referred to as the Southern Tick Associated Rash Illness (STARI), and is thought to be caused by “Candidatus B. lonestari” (40) after being bitten by Amblyomma americanum (3). The symptoms of “Candidatus B. lonestari” infection in domestic dogs have not been determined.

A veterinarian in Laredo, Texas, USA has reported that Lyme disease is present in local domestic dogs (Dr. Sandra Leyendecker, personal communication). B. burgdorferi has been previously detected in coyotes in Webb County (7), Texas. However, to the extent of our knowledge it has not been detected in domestic dogs. House pets, including dogs, have an increased exposure to ticks, and can serve as sentinel organisms for some diseases that occur in humans (29). Given the limited information available on vector-borne diseases in South Texas counties, there is a need for tick and pathogen surveillance in the area. This surveillance can help define areas at high risk for transmission (26) of infection to mammals, including humans. Identifying areas at high risk of transmission can increase awareness, potentially leading to the implementation of better diagnosis and prevention methods. In addition, as Laredo is the largest land-based port of entry in the United States, there is the movement of a large number of people and animals to and from this city into the rest of United States (11). For example, truck drivers frequently travel to Laredo, TX with their domestic dogs to warehouses in the city. They may have to wait a day or two before leaving to their destination. The purpose of this study was to determine the prevalence of DNA from tick-borne disease agents in domestic dogs from Laredo, Texas, by investigating the prevalence of Borrelia, Ehrlichia and Rickettsia species in R. sanguineus ticks.

### MATERIALS AND METHODS

#### TICK COLLECTION AND IDENTIFICATION

Fully engorged adult R. sanguineus ticks were collected at multiple sites in Laredo, Texas. The ticks were collected from the walls of dog kennels, or from a CO$_2$ trap placed in the Laredo animal shelter. Ticks that were removed while grooming dogs were also collected from animal caregivers/owners. The researchers had no contact with any animal in the study. The ticks were counted and individually examined under the microscope to identify them to the species level using a published key (10).

#### DNA EXTRACTION

A total of 124 R. sanguineus ticks (55 males and 69 females) were used for DNA extraction using the E.Z.N.A. Mollusk DNA Isolation Kits (OMEGA Bio-tek, Norcross, GA, USA). A previously reported protocol was followed and modified as previously described (50). Briefly, the tick was homogenized in 300 µl lysis buffer.
Table 1. Primers and thermal cycler settings used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Specificity</th>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th>Cycles</th>
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<td>12S rRNA</td>
<td>85F</td>
<td>TTAAGCCTTTTCAGAGGAATTGTC</td>
<td></td>
<td>95°C, 30sec</td>
<td>45°C, 30sec</td>
<td>72°C, 1min</td>
<td>40</td>
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<tr>
<td></td>
<td>225R</td>
<td>TTTWWGCTGCACCTTGACTTAA</td>
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<tr>
<td>flaB</td>
<td>FlaLS</td>
<td>AACAGCTGAAGAGACCTTGGAAATG</td>
<td>Not Reported</td>
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<tr>
<td></td>
<td>FlaRS</td>
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</table>

The tick was crushed for 5 minutes using a sterile microtube and pestle. After adding proteinase K, the samples were incubated at 55°C for 3h. The sample purification was then completed following the manufacturer’s protocol.

POLYMERASE CHAIN REACTION (PCR)

The samples were screened using PCR for the tick 12S rRNA gene as previously described (30). Samples positive for tick rDNA (n=112) were subjected to PCR for amplification of *Borrelia*, “Candidatus B. lonestari”, *Ehrlichia* and *Rickettsia* bacteria species (43,44).
The PCR mixture was a 25 µL reaction volume containing 0.25 µL of GoTaq polymerase (Promega, Madison, WI), 1X GoTaq Buffer, 160 ng/µL bovine serum albumin, 1.0 mM MgCl₂, 200 µM of each dNTP, 2 pmol primers, and 5µL of template (1 µL for nested reactions). Amplifications were performed on a Bio-Rad MyCycler thermal cycler (Bio-Rad, Carlsbad, CA) (30). Different thermal cycler settings, as indicated in Table 1, were used for different primers due to different optimal annealing temperatures. An initial denaturation step (95°C) of 5 min. and a final extension step (72°C) of 5 min. were used.
Nested PCR procedures were performed for *Borrelia* and “*Candidatus B. lonestari*” using 1µl from the initial reaction as a template. Amplification of target sequences was performed in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Carlsbad, CA) with several denaturing, annealing, and extension times and temperatures (Table 1). For each PCR assay, 5µL of sterile distilled water was used instead of template DNA as a negative control. The positive controls, when used, were added using separate hoods and pipettors to reduce the risk of cross contamination.

**VISUALIZATION AND SEQUENCING OF PCR PRODUCTS**

Five microliters of each PCR reaction was subjected to gel electrophoresis, using 2% agarose gels stained with ethidium bromide in 0.5X TBE (45 mM Tris, 45 mM boric acid, 1 mM disodium ethylene diamine tetraacetic acid) with 0.00005% ethidium bromide. The gels were run at 100 V for 40min. After electrophoresis, the gels were examined under UV light. Positive samples were purified using SpinPrep PCR Clean-Up Kits (Novagen, La Jolla CA, USA) following the manufacturer’s protocols. Each purified PCR product was sent for Sanger sequencing at Eurofins (Alabama, USA) or MCLab (San Francisco, CA) using the primers used for PCR.

**NUCLEOTIDE SEQUENCE ACCESSION NUMBERS**

The DNA sequences were visualized using Finch TV, Geospiza, Inc software version 1.5.0 and compared to reported sequences in the NCBI GenBank using BLAST. The assigned GenBank accession numbers are: KR183798-KR183823.

**PHYLOGENETIC ANALYSIS**

Initial alignments for *Borrelia* and *Ehrlichia* genes were executed using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) (16) as performed by the European Bioinformatics Institute’s
Fig. 1. Baysian inference consensus tree inferred from flaB of Borrelia species. Node support is indicated by the posterior probabilities at the node. The name of the species is followed by the GenBank accession number. A25B-F5, A25B-M1, A25B-M2, A25B-M11, A25B-M16, A25B-M17, A25B-M18, A25B-M19, A32B-F2, A32C-F5, A35A-M3, A46BB-F1, A46BB-F3, A46BB-F4, B17Z09-F2, B17Z10-F1 and E49Z02-M1 represent amplicons from R. sanguineus and are underlined. The scale bar indicates the mean number of nucleotide substitutions per site.
Fig. 2. Bayesian inference consensus tree inferred from 16S rDNA of Ehrlichia species. Node support is indicated by the posterior probabilities at the node. The name of the species is followed by the GenBank accession number. A17S-M1, A17S-F1, A17S-F3, A17S-F5, A17S-F7, A25B-F16, A25B-F19 and A25B-F22 represent amplicons from R. sanguineus and are underlined. The scale bar indicates the mean number of nucleotide substitutions per site.
RESULTS

PCR DETECTION OF BORRELIA AND EHRLICHI A

Of the 112 positive samples for tick rDNA, 44 ticks (18 males and 26 females) were positive for B. burgdorferi-like species, “Candidatus B. lonestari” or Ehrlichia bacteria species (Table 2). “Candidatus B. lonestari”, the most commonly detected tick-borne pathogen, was detected in 16.9% (n=19, 16.3% of males (8/49) and 17.5% (11/63) of females) of all ticks. B. burgdorferi-like species were detected in 9.8% (n=11, 12.2% (6/49) of males and 7.9% (5/63) of females) of all ticks. E. canis was detected in 12.5% (n=14, 8.2% (4/49) of males and 15.9% (10/63) of females) of all samples. Two male ticks (4.1%, n=2 of 49) were positive for both E. canis and B. burgdorferi-like species, indicating a 1.8% co-infection rate (2 of 112) of all ticks. One male and one female tick (male: 2.0%, n=1 of 49; female: 1.6%, n=1 of 63) were positive for both “Candidatus B. lonestari” and B. burgdorferi-like species, indicating a 1.8% co-infection rate (2 of 112) of all ticks. No tick was positive for the spotted fever group rickettsial rompA gene.

PHYLOGENETIC ANALYSIS

Samples with strong bands by gel electrophoresis were prepared for sequencing. Sequencing confirmed the PCR results. A Bayesian inference tree including these sequences and other published sequences are shown in Figs. 1 and 2. Phylogenetic analysis showed that “Candidatus B. lonestari” amplicons were in the same clade as known sequences of “Candidatus B. lonestari” and B. burgdorferi-like species amplicons clustered with B. burgdorferi species complex (Fig. 1). “Candidatus B. lonestari” sequences were all identical except A25B-F5, which was polymorphic at one position. Three of the B. burgdorferi-like species sequences (B17Z10-F1, A25B-M1 and E49Z02-M1) were different at one position. Ehrlichia amplicons were in the same clade as known sequences of E. canis (Fig. 2).

DISCUSSION

The ticks used for this study were fully engorged. Thus, the detection of pathogen DNA either represents the most recent blood meal or potentially a prior infection of the tick. We detected the presence of B. burgdorferi-like species, “Candidatus B. lonestari” and E. canis DNA in R. sanguineus ticks from Laredo, Texas. Our study does not address the issue of vector competency of R. sanguineus ticks in regard to B. burgdorferi-like species and “Candidatus B. lonestari”.

The detection of pathogens in canines can indicate a potential risk for infection of humans (28,29). We have detected B. burgdorferi-like species DNA in 9.8% of ticks collected from the local animal shelter, as well as ticks submitted by pet caregivers. The main vector for B. burgdorferi in the Northeastern United States is I. scapularis (17,18,37). This tick is present throughout much of Texas and Northern Mexico. B. burgdorferi was previously detected in 45% of tested I. scapularis ticks (19). However, in Webb County TX, no I. scapularis were identified in the combined collection of over 70,000 ticks (5). B. burgdorferi-like
species have been previously reported in A. inornatum from Webb County (50) and A. mixtum from Northeastern Mexico (21). We also detected “Candidatus B. lonestari”, which is thought to be the cause of STARI (40), in 16.9% of ticks. Cohen et al. (1990) reported a 5.5% seroprevalence for Borrelia in domestic dogs from Texas. However, of the dogs in their study 0 of 5 dogs that came from Webb County were seropositive (9). Likewise, Bowman et al. (2009) reported B. burgdorferi in Central and Northern Texas, but did not have any results for South Texas. This is the first report of Borrelia from R. sanguineus ticks and domestic dogs in Webb County, Texas.

R. sanguineus is the only known vector of E. canis (23), and is widely distributed throughout the United States (6). We detected E. canis in 12.5% of ticks. However, all of the ticks that were positive for E. canis were collected from the Laredo animal shelter. Many pet owners acquire their pet from the animal shelter. These dogs are at a high risk for acquiring E. canis at this location. However, domestic dogs in the city of Laredo appear to be at a low risk for acquiring E. canis. The previously reported seroprevalence for E. canis (2.0%) was higher in Texas than in much of the United States (4). We did not detect E. chaffeensis and E. ewingii in R. sanguineus, even though they are present in the area (30). We also did not detect any spotted fever group Rickettsia. This would suggest that any spotted fever group Rickettsia are either absent from the area or were present at a very low prevalence.

Further research on R. sanguineus distribution and the prevalence of B. burgdorferi-like species, “Candidatus B. lonestari” and Ehrlichia species in South Texas is needed. Further research will help elucidate if R. sanguineus is a vector of a B. burgdorferi-like species. This additional research would allow for better and more accurate diagnosis of tick-borne illnesses, ultimately leading to better treatment and health care for domestic dogs and humans. This research supports the observation of Dr. Sandra Leyendecker and suggests that domestic dogs should be screened for Lyme disease if they present with appropriate symptoms.

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REFERENCES


