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Prevalence of *Babesia* spp. and clinical characteristics of *Babesia vulpes* infections in North American dogs

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Abstract

Background: Babesiosis is an important cause of thrombocytopenia and hemolytic anemia in dogs. *Babesia vulpes*, reported in European dogs and North American foxes, rarely has been reported in domestic North American dogs. Newly optimized polymerase chain reaction (PCR) primers facilitate more sensitive amplification of *B. vulpes* DNA.

Objectives: To determine the prevalence of *Babesia sp.* infections in dogs being tested for Babesia infection, and to describe co-infections and clinicopathologic abnormalities in *B. vulpes* positive dogs.

Animals: Dog blood or tissue samples (n = 9367) submitted to a diagnostic laboratory between June 2015 and June 2018 were tested using an optimized *Babesia* PCR assay.

Methods: Comprehensive canine vector-borne disease diagnostic testing was performed on convenience samples.

Results: *Babesia* sp. DNA was amplified from 269/9367 (2.9%) North American dogs. *Babesia* sp. infections included *B. gibsoni* monoinfection (157; 1.7%), *B. vulpes* monoinfection (19; 0.20%), and *B. gibsoni* and *B. vulpes* coinfection (29; 0.31%). Forty-three of the 48 total *B. vulpes*-infected dogs were American Pit Bull Terrier-type breeds, of which 36 historically were involved with dog fights. Coinfections with *Mycoplasma*, *Dirofilaria immitis*, or *Wolbachia* and coexposures to *Bartonella*, *Ehrlichia*, and *Rickettsia* spp. were documented in *B. vulpes*-infected dogs. Clinicopathologic data in *B. vulpes*-infected dogs both with and without coinfections included anemia, thrombocytopenia, hyperglobulinemia, hypoalbuminemia, and proteinuria.

Conclusions and Clinical Importance: *Babesia vulpes* infection in domestic North American dogs is commonly found in conjunction with other coinfections, including *B. gibsoni* and hemotropic *Mycoplasma*. Similar to *B. gibsoni*, dog-to-dog transmission of *B. vulpes* may be a frequent mode of transmission.

KEYWORDS

B. vulpes, *Babesia*, hemolytic anemia, prevalence, tick, vector-borne disease

Abbreviations: CVBD, canine vector-borne disease; IFA, immunofluorescence assay; mtLSU, *Babesia* mitochondrial large subunit; PCR, polymerase chain reaction.

1 | INTRODUCTION

Babesia vulpes, a parasite of canids formerly known as *Babesia microti*-like *Theileria annae* and the *Babesia* "Spanish isolate,"[1-4] has been linked to clinical diseases in European dogs including hemolytic anemia, thrombocytopenia, azotemia, and death.[5-8] *Babesia vulpes* infections genetically indistinguishable from the *B. vulpes* infections in European dogs are common in both red and gray foxes in North Carolina and Canada.[9,10] However, *B. vulpes* has been reported only rarely in domestic dogs in the United States of America, making the clinical relevance in domesticated dogs in North America uncertain. *Babesia vulpes* is closely related to the pathogen *B. microti*, which infects humans. *Babesia vulpes* infects a wide range of vertebrate hosts, and based on genetic data and in some cases biologic behavior, it is only distantly related to *Babesia* sensu stricto species (which includes most other *Babesia* species that infect canids).[11-14]

The prevalence and role of *B. vulpes* as a cause of disease in dogs[14] in North America remain unknown. Because commonly utilized commercial polymerase chain reaction (PCR) assays to detect *Babesia* infections in dogs often are designed to amplify *Babesia* sensu stricto species (eg, *B. vogeli*, *B. canis*, *B. rossi*, *B. gibsoni*, and large unnamed *Babesia* spp.), they lack sensitivity for the amplification of *B. vulpes* DNA,[15] making the detection of this parasite less likely. Our goals were to (1) assess the prevalence of *Babesia* infection in blood and splenic specimens of dogs submitted to a North American diagnostic laboratory using a novel PCR assay designed to amplify *B. vulpes* DNA,[15] and (2) to describe coinfections and laboratory findings in North American *B. vulpes*-infected dogs. We hypothesized that *B. vulpes* would be present in North American dogs, and that infected dogs would have clinical signs and laboratory abnormalities consistent with babesiosis as caused by other *Babesia* spp.

2 | MATERIALS AND METHODS

2.1 | Samples and *Babesia* PCR testing

Ethylenediaminetetraacetic acid-anticoagulated whole blood or tissue specimens from dogs were submitted to a reference laboratory (Canine Vector-Borne Disease Diagnostic Panel, Vector Borne Disease Diagnostic Laboratory, North Carolina State University [NCSU VBDDL], Raleigh, North Carolina) from June 22, 2015, through June 4, 2018, for canine vector-borne disease (CVBD) diagnostic testing that included the use of a newly validated *Babesia* quantitative real-time PCR (qPCR).[15] Additional follow-up *Babesia* testing was performed retrospectively for *B. vulpes* PCR-positive dogs (*B. vulpes*+), for which all *Babesia* PCR primers, gene targets, and associated references are listed in Table 1.[3,10,16] A schematic of sample handling procedures and parasite identification protocols was created (Figure 1). Definitions for each *Babesia* species (*B. vogeli*, *B. coco*, *B. gibsoni*, and *B. vulpes*) were established based on PCR (Table 2). Briefly, PCR-positive samples for *Babesia* mitochondrial large subunit (mtLSU) DNA were speciated using at least 4 additional species-specific (*B. vogeli*, *B. coco*, *B. gibsoni*, and *B. vulpes*) 18S rRNA PCRs. Dogs with positive mtLSU PCRs but negative using the 4 18S rRNA species-specific PCRs were further screened using *B. canis* 18S rRNA primers, and if they are still negative, the mtLSU amplicon was submitted for sequencing to the NCSU VBDDL. For additional confirmation of *B. vulpes* infections, samples were subjected to an additional PCR that amplified a region of the *B. vulpes* β-tubulin gene. Amplicons were submitted for sequencing to GENEWIZ Inc (Research Triangle Park, North Carolina) and sequence alignments were made with GenBank sequences using a basic local alignment search tool and AlignX software (Vector NTI Suite 6.0, InforMax, Inc, Bethesda, Maryland).

TABLE 1 Oligonucleotide primers used in this study to amplify *Babesia* DNA

		F (5′-3′)		R (5′-3′)		
Organism	**Target**	**Primer name**	**Primer sequence**	**Primer name**	**Primer sequence**	**Ref**
Babesia genus	mtLSU	B-lsu-F	AGCAAAGTTCCCATTCCAGA	B-lsu-R2	TCTTAACCCAACTCACGTACCA	3
		BmicF	TTGCGATAGTAATAGATTTACTGC			
B. vogeli	18S rRNA	BCV-F	GTTCGAGTTTGCCATTCGTT	BAB722	ATGCCCCCAACCGTTCCTATTA	3
B. coco	18S rRNA	BCO-F	CCTTTTCTTTGCTTTGTCGC			3
B. vulpes	18S rRNA	Bmic18F	CTGCTTTATCATTAATTTCGCTTCCGAACG			3
B. gibsoni	18S rRNA	BGNC-F	ACTCGGCTACTTGCCTTGTC			3
B. canis	18S rRNA	BCC-F	TTGCGTTGACGGTTTGACC			3
B. vulpes	β-tubulin	BtubF	GATATGTACCAAGAGCCATTCTTATG	BtubR	TGTTACTCCACTCATAGCAGCAC	13
B. gibsoni	cox1	BG-cox1-F	CTTCAGCCAATAGCTTTCTGTTTG	BG-cox1-R	CCTGAGGCAAGTAAACCAAATAT	3
Babesia genus (bias toward sensu stricto)	18S rRNA	BcanisF	GCATTTAGCGATGGACCATTCAAG	Bcommon2R	TGCTTTCGCAGTAGTTCGTC	15
Babesia genus (bias toward sensu lato)	18S rRNA	Bcanis2F	GCCGGCGATGTATGATTCAAG	Bcommon2R	TGCTTTCGCAGTAGTTCGTC	15

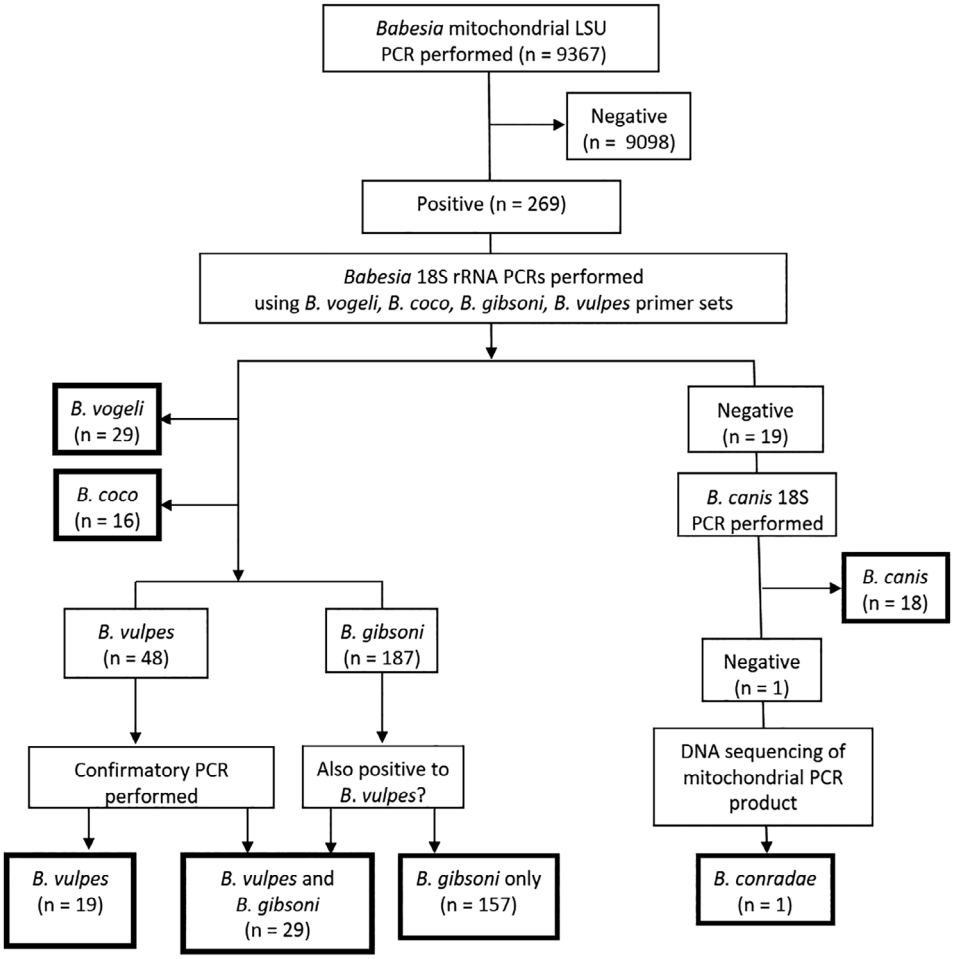

FIGURE 1 Schematic of experimental design

TABLE 2 *Babesia* species were defined by positive (+) or negative (–) reactions to polymerase chain reaction (PCR) amplification of broad screening primers (LSU mtDNA), species specific 18S rRNA, or additional PCRs as needed to define and confirm infection

		Specific PCR: 18S rRNA				Additional PCRs		
Species classification	*Babesia* LSU mtDNA	*B. vogeli*	*B. coco*	*B. vulpes*	*B. gibsoni*	*B. canis* 18S	*B. vulpes* B-tubulin	*Bgib Cox1*
B. vulpes only	+	–	–	+	–	NA	+	–
B. vulpes AND *B. gibsoni* coinfection	+	–	–	+	+	NA	+	+
B. gibsoni	+	–	–	–	+	NA	NA	NA
B. vogeli	+	+	–	–	–	NA	NA	NA
B. coco	+	–	+	–	–	NA	NA	NA
B. canis	+	–	–	–	–	+	NA	NA
B. conradae	*	–	–	–	–	–	NA	NA

Notes: NA indicates that the reaction was not performed. The asterisk indicates that the positive PCR product was sequenced to yield species determination.

2.2 | Coinfections for *B. vulpes* infected dogs

For all *B. vulpes*+ samples coinfected with *B. gibsoni*, a region of the *B. gibsoni* cox1 gene was amplified for further confirmation of the coinfection (Table 2). For 1 dog coinfected with *B. vulpes* and *B. gibsoni*, conventional PCR amplicons generated from a larger region of the *B. vulpes* 18S rRNA gene (using primers Bcanis2F and Bcommon2R) and *B. gibsoni* 18S rRNA gene (using primers BcanisF and Bcommon2R; Table 1) were cloned and sequenced to confirm the findings. Clones were constructed using the pGEM-T Easy Vector System Promega (Madison, Wisconsin) as recommended by the manufacturer and sequencing was performed as outlined above.

All *B. vulpes*+ dogs (n = 48) were tested using a CVBD PCR panel (NCSU VBDDL) that included testing for *Anaplasma* spp., *Bartonella* spp., *Ehrlichia* spp., hemotropic *Mycoplasma* spp., and *Rickettsia* spp.[15,17-21] *Babesia vulpes*+ dogs with corresponding serum samples (n = 22) also were tested using an indirect fluorescent antibody test (immunofluorescence assay [IFA]) for antibodies against *B. canis* (antigen slides contained both *B. canis* and *B. vogeli* that were isolated from dogs in the United States), *B. gibsoni*, *Bartonella henselae*, *B. koehlerae*, *B. vinsonii berkoffii*, *E. canis*, and *Rickettsia rickettsii* (spotted fever group rickettsia)[17] and using a SNAP 4DX Plus Test for *Anaplasma* spp., *Ehrlichia* spp., *Borrelia burgdorferi* antibodies and *Dirofilaria immitis* antigen.

2.3 | Data analysis

The prevalence of *Babesia* infections was determined in a population of dogs that had *Babesia* PCR testing requested in our diagnostic laboratory between June 22, 2015, and June 4, 2018. All equivocal results and subsequent results from dogs with repeat testing were removed before data analysis. Prevalence in this population refers to the total number of *Babesia* PCR-positive dogs divided by the total number of individual dogs tested. The submitting veterinarian or diagnostic laboratory was contacted for every *B. vulpes*+ dog to request supporting clinical data. If available, information compiled for *B. vulpes*+ dogs included age, breed, submission zip code, CVBD comprehensive panel results, and available clinicopathologic data consisting of a CBC, serum biochemistry panel, urinalysis, or some combination of these (Table 1). Because specimens from *B. vulpes*+ dogs were submitted to various diagnostic laboratories with different reference ranges, each available data point was scored as to whether it was below, within, or above the established reference range for the corresponding diagnostic laboratory.

3 | RESULTS

3.1 | Babesia prevalence

Canine whole blood (n = 9345) and tissue (n = 22) samples, representing individual dogs, were submitted to the NCSU VBDDL from June 22, 2015, until June 4, 2018, for CVBD diagnostic testing that included *Babesia* PCR. Of these, 269 (2.9%) dogs were *Babesia* PCR positive by mtLSU amplification. *Babesia gibsoni* (n = 186 dogs, 2.0% prevalence) was the most commonly identified *Babesia* spp. in dogs in these North America samples, followed by *B. vulpes* (n = 48 dogs, 0.51% prevalence), *B. vogeli* (n = 29 dogs, 0.31% prevalence), *B. canis* (n = 18 dogs, 0.19% prevalence), *B. coco* (n = 16 dogs, 0.17% prevalence), and *Babesia conradae* (n = 1 dog, 0.01% prevalence). Of the 48 *B. vulpes*+ dogs, 29 (60%) dogs were coinfected with *B. gibsoni*. Twenty-two *B. vulpes*+ dogs had both blood and serum samples submitted; of the remaining samples, 17 were submitted as blood only and 9 as splenic tissue samples. One hundred and fifty-seven *B. gibsoni* positive dogs were not coinfected with *B. vulpes*. No other *Babesia* coinfections were identified.

3.2 | *Babesia vulpes*-positive dogs

In addition to testing positive based on the *Babesia* mtLSU PCR, all 48 *B. vulpes*-infected dogs tested positive by *B. vulpes* 18S rRNA and *B. vulpes* β-tubulin species-specific PCRs (Table 2). All the *B. vulpes* β-tubulin amplicon sequences were identified as *B. vulpes*. High-quality sequences for 36 amplicons were aligned and compared to each other and *B. vulpes* reference sequences. One amplicon sequence was 100% (487/487) identical to *B. vulpes* isolate SN87-1 β-tubulin gene, originally identified in a fox from Cape Cod, Massachusetts (GenBank Accession # AY144707), and all other amplicons were 99% (485/487 bases) identical to SN87-1. Of the *B. vulpes* β-tubulin amplicons that were 99% identical, 2 different sequences were identified, differing by 2 bases, and were deposited in GenBank (Accession #s MK697353 and MK697354). Variables such as geographic location or coinfection status were not associated with a particular sequence.

Of the 48 *B. vulpes*+ dogs, 36 samples were submitted from New York State. Other *B. vulpes*+ samples were submitted from Florida (3), Massachusetts (1), North Carolina (4), South Carolina (1), Texas (1), and Wisconsin (1). One sample was submitted from Calgary, Canada, but the dog recently had been relocated from Texas before sample acquisition. Thirty-six of the 48 *B. vulpes*+ dogs (75%) were managed by a humane organization after being seized as part of animal cruelty investigations or had a known history as a fighting or bait dog. Forty-four of 48 *B. vulpes*+ dogs (92%) were American Staffordshire or Pit Bull Terrier-type breeds. The remaining dog breeds included 1 each: Beagle, Dachshund, Schnauzer, Terrier, and an unknown breed. Sex distribution included 13 male intact, 4 male castrated, 12 female intact, 4 female spayed dogs, and 15 dogs of unknown status. Twenty of 48 dogs (42%) were estimated or known to be between 2 and 5 years old, 6 were <2 years old, 6 were >5 years old, and 16 were of unknown age.

3.3 | Coinfections

Thirty-eight (79%) *B. vulpes*+ dogs had evidence of infection with or exposure to other CVBD, determined by a CVBD comprehensive panel (Figure 2). Of the 48 *B. vulpes*+ dogs, 29 (60%) were coinfected with *B. gibsoni* based upon PCR testing. For 1 coinfected dog, a larger

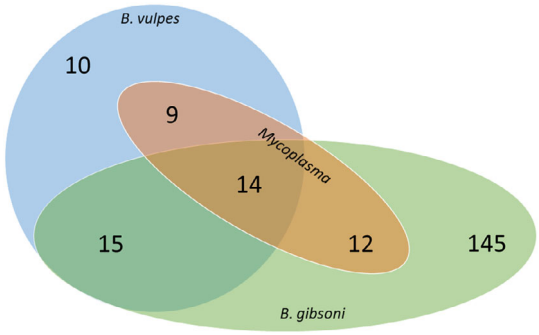

FIGURE 2 Venn diagram describing major coinfections found within all dogs positive for either *Babesia vulpes* (n = 48) or *Babesia gibsoni* (n = 186), for a total of 205 dogs infected with either piroplasm

portion of the 18S rRNA gene with the V4 hypervariable region from both *B. vulpes* and *B. gibsoni* was amplified and cloned. Sequence data obtained from the *B. vulpes* 18S rRNA clone, deposited in GenBank (Accession # MK693714), was 99% (602/605 bases) identical to several *B. vulpes* 18S rRNA sequences in GenBank, including the "Spanish isolate" strain amplified from a Pit Bull Terrier in Oklahoma (GenBank Accession # EU583387). Sequence data obtained from the *B. gibsoni* 18S rRNA clone, deposited in GenBank (Accession # MK694782), was 99% (568/569 bases) identical to *B. gibsoni* genotype Asia, including strains from Midwestern and Eastern United States (GenBank Accession #s AF205636, AF271081), respectively. Other *B. vulpes*+ CVBD coinfections included 23 (48%) with hemotropic *Mycoplasma* spp. (10 *M. haemocanis* and 13 *Candidatus* Mycoplasma haematoparvum) and 4 (8%) with *Wolbachia*. Within these coinfections, 14 were triply infected with *B. vulpes*, *B. gibsoni*, and a hemotropic *Mycoplasma* spp.

Of the 48 *B. vulpes*+ dogs, 22 (46%) had serum available for IFA and SNAP 4DX Plus serological testing. Six of 22 *B. vulpes*+ dogs (32%) tested by IFA had coexposures with either *Bartonella* (n = 3), *Rickettsia* (n = 1), or *Ehrlichia* (n = 1) spp. The *Ehrlichia* exposure also was detected by SNAP 4DX Plus. *Dirofilaria immitis* infections were detected in 4/22 (18%) dogs, and of those, 2 also were *Wolbachia* PCR positive. Of the 22 *B. vulpes*+ dogs tested by *Babesia* IFA, 14/22 (64%) were *B. canis* and *B. gibsoni* IFA seroreactive, 4/22 (18%) were only *B. canis* IFA seroreactive, and 4/22 (18%) were seronegative to both *B. canis* and *B. gibsoni*. Of the 14 dogs seroreactive to both *B. canis* and *B. gibsoni*, 12 (86%) were also *B. gibsoni* PCR positive. The 4 dogs seroreactive to *B. canis*, but not *B. gibsoni*, were infected with *B. vulpes*.

3.4 | Clinical data

Clinicopathologic data were available and collected retrospectively for 14 *B. vulpes*+ dogs, of which 7 were coinfected with *B. gibsoni*, and 9 were coinfected with hemotropic *Mycoplasma* spp. (Table 1). Of dogs with available clinical data, 6 were triply infected with *B. vulpes*, *B. gibsoni*, and hemotropic *Mycoplasma*. Four dogs were PCR positive to *B. vulpes* only without evidence of *B. gibsoni* or *Mycoplasma* infection. Common hematologic abnormalities within the *B. vulpes*+ population, independent of coinfection status, included thrombocytopenia, anemia, hyperglobulinemia, hypoalbuminemia, and proteinuria (Table 1). Physical examination findings for 5 *B. vulpes*+ dogs were available and are included in the supplemental material (Table 1).

3.5 | Response to treatment

Treatment with atovaquone (13.5 mg/kg PO q8h) and azithromycin (10 mg/kg PO q24h) for 10 days[22,23] was recommended for all dogs as soon as the *B. vulpes* infection was diagnosed. Follow-up data were available for 9 dogs, all of which were clinically improved after treatment. In total, 6 of 9 *B. vulpes* infections were undetectable by PCR by 90 days after treatment. Four of these dogs were *B. vulpes*+ only (without concurrent *B. gibsoni* infection), and of these, 2 were cleared of *B. vulpes* based on PCR 90 days after treatment. Five treated dogs were coinfected with *B. vulpes* and *B. gibsoni*. Of these, 4 were cleared of *B. vulpes* and 3 were cleared of *B. gibsoni* after treatment. One dog initially coinfected with both *B. vulpes* and *B. gibsoni* was cleared of *B. vulpes* but not of *B. gibsoni* after treatment, and another dog remained positive for both piroplasms after treatment, but follow-up information was available only at 30 days posttreatment. Of the 2 splenectomized dogs included in the study group, 1 dog coinfected with *B. vulpes* and *B. gibsoni* was PCR negative after 60 days of treatment as mentioned above, and the other, infected with *B. vulpes* but not *B. gibsoni*, was not cleared of *B. vulpes*.

4 | DISCUSSION

Among the 9376 dogs tested by PCR for *Babesia*, we found an overall *Babesia* prevalence of 2.9%, with *B. vulpes* the second-most common *Babesia* spp. identified (overall prevalence 0.51%). Our findings indicate that the prevalence of *B. vulpes* is higher than expected in dogs from North America tested diagnostically. Based on sequence analysis of amplified regions of the 18 seconds rRNA and β-tubulin genes, the strain of *B. vulpes* identified in most of the dogs from our study is 99% identical to a *B. vulpes* strain detected in a Pit Bull in Oklahoma in 2009 and in North American red and gray foxes in 2010.[9,10,24] Detection of *B. vulpes* in our study most likely was a direct result of the use of novel PCR assays specifically designed to detect a broader range of *Babesia* spp., including *B. vulpes*, rather than a change in the epidemiology of this pathogen. Accurate identification of babesiosis can result in improved patient outcomes because specific treatments for *Babesia* typically are not employed empirically, and treatments recommended for large *Babesia* spp. differ from treatment regimens recommended for small *Babesia* spp. Results obtained before the routine use of PCR assays capable of detecting *B. vulpes* may have resulted in the use of treatments designed for alternate infectious or immune-mediated etiologies instead of *Babesia* spp.

The natural history of *B. vulpes* infections in dogs remains unknown. The primary routes of transmission for babesiosis in dogs around the world include tick vectors, direct dog-to-dog transmission, and inadvertent administration of infected donor blood. The tick vector or vectors for *B. vulpes* transmission have not been definitively identified in any region of the world. We found a high prevalence of *B. vulpes* in American Staffordshire and Pit Bull Terrier-type dogs that were rescued from alleged dog fighting operations. This epidemiological pattern of *B. vulpes* infection appears very similar to the epidemiology of *B. gibsoni* infections in American Staffordshire and Pit Bull Terrier-type dogs, in which primary routes of infection are believed to be direct dog-to-dog transmission by bite wounds and potentially transplacental transmission. These similarities make it plausible that *B. vulpes* also is being transmitted directly from dog to dog. The role of nonvectored transmission in both the United States and Europe, including vertical and dog-to-dog transmission, still requires clarification, as does the role of wild canids as reservoir species for tick acquisition of *B. vulpes* infection.

The presence of a tick vector that is capable of transmitting *B. vulpes* to domestic dogs in North America cannot be excluded.

Ixodes hexagonus, the hedgehog tick, has been posited as the primary vector in Spain,[25] but *B. vulpes* has been identified in regions without *I. hexagonus*,[9] and this tick is not believed to be endemic in North America. *Ixodes scapularis* is known to transmit *B. microti* to rodents and humans, and may serve as a vector for *B. vulpes* organisms. *Babesia vulpes* has been identified recently within unfed *Dermacentor reticulatus* in Austria,[26] suggesting that other ixodid tick species may transmit these piroplasms.

Our study found a high proportion of dogs coinfected with both *B. vulpes* and *B. gibsoni*. Efforts to amplify multiple species-specific PCR gene targets were required to confirm that these were true coinfections rather than mis-amplification of *B. gibsoni* DNA because of lack of PCR primer specificity (Table 2). Fighting dogs are known to be at higher risk for coinfections, including both *B. gibsoni* and hemotropic mycoplasmas.[27] Based on known or suspected vectors, the remaining coinfections and coexposures suggest that some of these dogs were exposed to arthropod vectors including ticks, fleas, or mosquitos.

Babesia spp. frequently are reported in coinfections with other vector-borne disease agents or as mixed *Babesia* infections.[28-31] In humans, *B. microti* and *B. burgdorferi* have a synergistic relationship including cotransmission and complex immune modulation.[32,33] It is unclear whether or not *B. vulpes* and *B. gibsoni* also have such a relationship or if they are simply being cotransmitted in a high-risk population. Controlled laboratory transmission studies most likely will be required to determine whether complex immunological interactions are more common in coinfected dogs, as compared to dogs infected only with *B. vulpes* or *B. gibsoni*.

Unfortunately, only limited clinical data were available for dogs in our study, and interpretation was confounded by coinfections. Although limited information was available, we observed that dogs infected with *B. vulpes*, with or without concurrent *B. gibsoni* infection, exhibited a range of laboratory abnormalities similar to those previously reported with other *Babesia* spp. infections,[6,22] including regenerative anemia, thrombocytopenia, hyperglobulinemia, hypoalbuminemia, and proteinuria. Azotemia, although previously reported as a common complication for dogs infected with *B. vulpes* in Europe,[6] was not common in the dogs in our study.

Complete clinical data were not available for all dogs including physical examination findings, response to treatment, duration of clinicopathologic abnormalities, or long-term survival rates. In addition, the data were limited to samples submitted to the NCSU VBDDL, which may have created a selection bias toward dogs exhibiting clinicopathologic abnormalities found in association with CVBD and a geographic bias toward the Eastern Atlantic United States, because most samples were submitted from this region.[34] Regardless of the limitations of our study, *B. vulpes* infects dogs in the United States and causes disease manifestations that are similar to those of other *Babesia* spp. infections.

In conclusion, we found that optimized primers could identify *B. vulpes* infection in dogs that might otherwise have remained undiagnosed. Of 205 dogs diagnosed with either of the 2 piroplasms, 77% had *B. gibsoni* without *B. vulpes* coinfection, 9% had *B. vulpes* without *B. gibsoni* coinfection, and 15% were coinfected with both piroplasms. Whether or not *B. vulpes* has been present as a coinfection in dogs with *B. gibsoni* infections in the United States or other parts of the world remains unknown. If *B. vulpes* infections were present but undetected in dogs with *B. gibsoni*, this coinfection could have affected interpretation of previous reports of dogs with *B. gibsoni* infection. Although clincopathologic data for *B. vulpes*+ dogs was inconsistently available, typical clinical features of babesiosis were reported, and some dogs responded to treatment with atovaquone and azithromycin. *Babesia vulpes* should be considered as a differential diagnosis in dogs showing clinical signs consistent with babesiosis, especially in dogs with *B. gibsoni* infections or in those with extensive cutaneous scarring or in those known to have been involved in dog fighting.

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CONFLICT OF INTEREST DECLARATION

Nanelle R. Barash received a Boehringer-Ingelheim Resident-Postdoctoral Scholar Sponsorship.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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