EMERGING PARASITIC INFECTIONS VECTORED BY BROWN DOG TICKS IN NORTH AMERICA

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Abstract:

The research conducted in this dissertation provides a better understanding of the prevalence and geographic distribution of emerging and zoonotic organisms transmitted by brown dog ticks throughout the United States. In Chapter 3, we tested skin biopsy samples from dogs in Oklahoma for evidence of *Cercopithifilaria* spp. infection by saline sedimentation and PCR and tested ticks collected from these animals by PCR. In this study, *Cercopithifilaria bainae* was detected in 6/250 (2.4%) dogs and 3 brown dog ticks, indicating that this parasite may be circulating in Oklahoma. Prior to the study outlined in Chapter 3, *C. bainae* had been detected in dogs and ticks in other parts of the world, with a single report in 2019 originating from the United States (Florida). In Chapter 4, we tested brown dog ticks collected from animals across the United States for molecular evidence of *Cercopithifilaria* spp. infection to better understand the geographic distribution of this emerging parasite on a national scale. Here, we identified *C. bainae* DNA in 80/1400 (5.7%) brown dog ticks collected from 55 different dogs across 11 states, suggesting a more widespread geographic distribution of the parasite within the United States than previously known. Lastly, in Chapter 5, we tested a cohort of the brown dog ticks that were tested in Chapter 4 for molecular evidence of infection with tick-borne pathogens including *Babesia* spp., *Ehrlichia canis*, and *Rickettsia* spp. While no *Babesia* spp. were detected, we did identify rickettsial agents in 24 ticks, including 1 *E. canis*, 3 *Rickettsia amblyommatis*, 11 *R. massiliae*, 3 *R. monacensis*, 5 *R. montanensis*, and 1 undefined *Rickettsia* species. The data from Chapter 5 documents *E. canis* in randomly sampled individual ticks and *R. monacensis* in brown dog ticks in the United States for the first time, and *R. massiliae* in new geographic regions. In summary, brown dog ticks across the United States may be harboring a variety of emerging and zoonotic infectious agents. Further research is needed to uncover the suite of organisms that brown dog ticks may harbor in the United States to inform national efforts aimed at protecting canine and human health.

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CHAPTER I

INTRODUCTION

Rhipicephalus sanguineus sensu lato, also known as brown dog ticks, are found throughout the world and can transmit a variety of tick-borne infections including *Acanthocheilonema dracunculoides*, *Anaplasma platys*, *Babesia* spp., *Cercopithifilaria* spp., *Ehrlichia* spp., *Hepatozoon canis*, and *Rickettsia* spp. (Yamane et al., 1993; Wikswo et al., 2007; Little, 2010; Dantas-Torres and Otranto, 2015; Baneth et al., 2018; Saleh et al., 2021). While these ticks primarily feed on dogs, they have been shown to occasionally parasitize humans. Brown dog ticks are unique in that they can live indoors, commonly establishing infestations in homes and kennels (Dantas-Torres and Otranto, 2015). There are two main populations of brown dog ticks in North America; the "tropical" lineage, also classified as *R. linnaei* by some groups, and the "temperate" lineage (Jones et al., 2017; Saleh et al., 2021; Šlapeta et al., 2021). These different lineages may have varying vectorial capacity in transmitting pathogens (Beall et al., 2012; Dantas-Torres and Otranto, 2015; Prakash et al., 2018).

Filarioids transmitted by brown dog ticks include *Cercopithifilaria* spp. Three species of *Cercopithifilaria* are known to infect canines, including *C. bainae*, *C. grassi*, and *Cercopithifilaria* sp. II; brown dog ticks are the only species that have

demonstrated vector competence in transmitting canine *Cercopithifilaria* spp. (Brianti et al., 2012; Solinas et al., 2014; Ionică et al., 2014; Ramos et al., 2016). Adults of *Cercopithifilaria* spp. are found in the subcutaneous tissues of canine hosts, while microfilariae reside in the dermis (Cortes et al., 2014). Due to the location of the parasite, a skin biopsy punch or skin snip is collected and a saline sedimentation and/or PCR is performed on the skin sample to detect microfilariae and/or DNA from the parasite, respectively (Otranto et al., 2011; Cortes et al., 2014; Andersson et al., 2017).

Cercopithifilaria bainae was first described from a dog in Brazil in 1984, and since then, has primarily been identified from dogs and brown dog ticks in Brazil and Europe, but has also been identified from dogs and/or brown dog ticks in Australia, China, India, Indonesia, Iran, Malaysia, South Africa, Spain, Taiwan, the Philippines, United States, and Vietnam (Gabrielle et al., 2014; Ionică et al., 2014; Latrofa et al., 2014; Otranto et al., 2015; Ramos et al., 2016; Boyd et al., 2019; Bezerra-Santos et al., 2022; Sazmand et al., 2022). Although infections with *C. bainae* are primarily nonpathogenic, clinical signs such as polyarthritis, cutaneous cysts, and erythematous, papular, and pruritic dermatitis have been reported (Gabrielli et al., 2014; Soares et al., 2020). The first case of *Cercopithifilaria* sp. in the United States was reported in 2019 from a 1-year-old dog residing in Florida with no travel history. The dog presented with reoccurring skin lesions on the head and was diagnosed with *C. bainae* infection, which was confirmed morphologically by observing microfilariae and molecularly by PCR (Boyd et al., 2019).

Another group of parasites brown dog ticks are known to transmit are protozoa, including *Babesia* spp. and *Hepatozoon canis*. These blood-borne apicomplexa are known to cause mild to severe disease in dogs globally (Allen et al., 2008; Dantas-Torres and Otranto,

2015; Baneth, 2018). In North America, *Babesia canis vogeli* is the only known *Babesia* spp. transmitted by brown dog ticks, but *B. gibsoni* is also suspected to use this vector, which has been shown in other parts of the world (Solano-Gallego et al., 2016; Baneth, 2018; Tuska-Szalay et al., 2021). *Babesia* spp. and *H. canis* tend to have low prevalence in dogs in North America (<3%), but this could be due to low parasitemia, making infections challenging to detect by blood smear examination and PCR (Jefferies et al., 2007; Allen et al., 2008; Bierkenheuer et al., 2020; Díaz-Sánchez et al., 2021).

Lastly, brown dog ticks in North America have been shown to vector a variety of rickettsial agents, including *Ehrlichia* spp. and spotted fever group *Rickettsia* spp. (SFGR) (Little, 2010; Dantas-Torres and Otranto, 2015). These ticks are the primary vectors for *E. canis* and may be secondary vectors for *E. chaffeensis* and *E. ewingii* in some regions (Yabsley et al., 2008; Ndip et al., 2010; Beall et al., 2012). *Ehrlichia canis* causes canine monocytic ehrlichosis (CME), which can cause severe disease and even fatality (Little, 2010). Brown dog ticks are vectors for a variety of SFGR; the two species of greatest concern due to their zoonotic potential, are *Rickettsia massiliae* and *R. rickettsii* (Wikswo et al., 2007; Yabsley et al., 2008; Fornadel et al., 2013). *Rickettsia massiliae* has only been identified in dogs and/or ticks in a few states, but national surveillance data are lacking (Eremeeva et al., 2006; Beeler et al., 2011; Fornadel et al., 2013). Brown dog ticks have recently been implicated as vectors for *R. rickettsii* in the southwestern United States, where the primary vectors in North America (*Dermacentor* spp.) are not typically found (Wikswo et al., 2007; Yaglom et al., 2018). While there has been a national increase in diagnosed RMSF cases over the past 20 years based on Centers for Disease Control data (CDC, 2021), this purported rise could instead be due to cross-reactivity of antibodies produced with other

SFGR infections that are also be detected by serologic testing; SFGR can be challenging to detect in the blood of patients by PCR, and therefore the identity of the true etiologic agent often remains unknown (Parola et al., 2013; CDC, 2021; Duncan et al., 2021).

Based on diagnostic limitations for detecting the above organisms transmitted by brown dog ticks, our understanding of the prevalence and geographic distribution of these agents in the United States is lacking. Testing brown dog ticks for molecular evidence of infection by PCR, however, may serve as an alternative approach to fill these knowledge gaps regarding the variety of known and novel infectious agents transmitted by brown dog ticks in the United States.

RESEARCH OBJECTIVES

1) Survey dogs in Oklahoma for *Cercopithifilaria* spp. through saline sedimentation and PCR of skin biopsies collected post-mortem.

2) Estimate the prevalence and geographic distribution of *C. bainae* infection in brown dog ticks collected from animals across the United States using PCR.

3) Survey brown dog ticks collected from animals across the United States for *Babesia* spp., *Ehrlichia canis*, and *Rickettsia* spp. by PCR to understand prevalence and geographic distribution in brown dog tick populations.

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CHAPTER II

REVIEW OF LITERATURE

INTRODUCTION TO BROWN DOG TICKS (*RHIPICEPHALUS SANGUINEUS* SENSU LATO)

Rhipicephalus sanguineus (Latreille, 1806) sensu lato (s.l.) are the most geographically widespread tick species complex and can be found worldwide. Members of this tick complex are unique in that they are adapted to live indoors, commonly infesting homes and kennels (Dantas-Torres, 2010). All motile stages (larvae, nymphs, and adults) preferentially feed on dogs, giving them the common name "brown dog tick". Although *R. sanguineus* s.l. prefer to feed on dogs, they have been found to also feed on cats and humans (Dantas-Torres, 2010; Saleh et al., 2021). Brown dog ticks can take on the behavior of an active host-seeker (hunter) or quester (ambush). These strategies increase the chance of finding a host, and transmitting any pathogens the tick may be harboring (Dantas-Torres, 2010). Globally, this group of ticks transmits bacteria, protozoa, and nematodes to canines, some of which are of zoonotic concern; these pathogens can be found in Table 1 (Dantas-Torres and Otranto, 2015).

Based on biologic, genetic, and subtle morphologic variations, there are as many as 17 different species comprising four discreet operational taxonomic units (OTUs), or lineages, within the *R. sanguineus* s.l. complex found throughout the world (Dantas-Torres et al., 2018; Sanchez-Montes et al., 2021). Based on molecular analysis of mitochondrial 16S rDNA, there are thought to be two main clades; the "tropical" species, which are those related to ticks from Africa, and the "temperate" species, which are related to ticks from Europe (Dantas-Torres and Otranto, 2015). Cross-breeding experiments have been carried out between *R. sanguineus* s.l. from different geographical regions. A study conducted by Levin et al., 2012, compared laboratory-raised brown dog ticks from North America, the Mediterranean, and Africa. These cross-breeding experiments showed that different lineages within *R. sanguineus* s.l. are not able to produce viable offspring, suggesting they are different species, but still taxonomically grouped under the original type species name. Recently, a single male brown dog tick of the temperate lineage was described as neotype, and the temperate lineage was assigned the taxonomic classification of *Rhipicephalus sanguineus* sensu stricto (Nava et al., 2018). Based on a study conducted by Šlapeta et al. 2021, the "tropical" lineage has been categorized as a different species, *Rhipicephalus linnaei*. It has been suggested that different lineages of *R. sanguineus* s.l. are more likely to transmit certain pathogens, such as *R. sanguineus* s.l. in warmer areas primarily transmitting *Ehrlichia canis* and *Rhipicephalus* sp. I primarily transmitting *Hepatozoon canis* (Beall et al., 2012; Dantas-Torres and Otranto, 2015).

BROWN DOG TICKS IN NORTH AMERICA

In North America, there are currently two main populations of brown dog ticks: the "temperate" lineage and the "tropical" lineage (Jones et al., 2017; Saleh et al., 2021). Populations of the tropical lineage can be found in regions where the mean annual temperatures are ≥20°C while the temperate lineage are found where mean annual temperatures are ≤20°C. Occasionally, these populations may cross geographic regions, typically when attached to a dog that has a travel history (Jones et al., 2017). Although it was previously thought that dogs are not infested with multiple lineages, a recent study in Arizona found two dogs with infestations of tropical and temperate lineages (Jones et al., 2017; Brophy et al., 2022). In North America, brown dog ticks are known or suspected to transmit a variety of pathogens, including nematodes, protozoa, and rickettsial agents (Dantas-Torres and Otranto, 2015; Boyd et al., 2019; Saleh et al., 2021)

INFECTIONS TRANSMITTED BY BROWN DOG TICKS

Canine *Cercopithifilaria* spp.

Cercopithifilaria spp. are tick-borne nematodes belonging to the family Onchocercidae and superfamily Filarioidea (Ramos et al., 2016). Originally, it was thought that *Cercopithifilaria* spp. were most closely related to *Onchocerca* spp., but in 1980, the parasite was re-described from a group of feral baboons (*Cercopithifilaria kenyensis*) and classified as a new subgenus of *Dipetalonema* (Eberhard, 1980). In 1982, the differing morphological characteristics of *Cercopithifilaria* from *Dipetalonema*, including a smaller buccal cavity and lack of a posterior glandular part on the esophagus, raised *Cercopithifilaria*

to the genus level (Lefoulon et al., 2014). Different *Cercopithifilaria* spp. appear to utilize different species of ixodid tick vectors (Otranto, 2015).

Three known species of *Cercopithifilaria* parasitize domestic dogs: *Cercopithifilaria bainae*, *Cercopithifilaria grassi*, and *Cercopithifilaria* sp. II. When comparing mitochondrial DNA sequences (*cox1*) of canine *Cercopithifilaria* to other well-known canine filarioid species, there is 84% similarity to *Acanthocheilonema reconditum* (JF461456), 85% to *Dirofilaria immitis* (AJ271613), 87% to *Dirofilaria repens* (AJ271614), and 85% to *Onchocerca lupi* (HQ207644) (Casiraghi et al., 2001; Otranto et al., 2013a).

All three canine *Cercopithifilaria* spp. utilize brown dog ticks as vectors, and dogs can be co-infected with different *Cercopithifilaria* spp. These filarioid species can be differentiated by morphology and/or molecular analysis of the mitochondrial gene (Andersson et al., 2017; Maia et al., 2017). Reports of canine *Cercopithifilaria* are predominantly from canine and tick populations in Europe and South America (Otranto, 2015). Despite the broad distribution of brown dog ticks in the United States, there has been only one case report of *C. bainae* in a domestic dog from Florida (Boyd et al., 2019).

Third stage larvae (L_3) of *Cercopithifilaria* spp., are transmitted to canine hosts by blood-feeding of infected brown dog ticks (Otranto et al., 2012b). The L_3 disseminate and continue development to ultimately become sexually reproducing adults residing in the dermis. The offspring of *Cercopithifilaria* spp., called microfilariae, are not found circulating in blood, in contrast to other filarioidea including *A. dracunculoides*, *A. reconditum*, *D. immitis*, and *D. repens,* which can be detected by wet mounts or modified Knott's preparations of blood. This makes *Cercopithifilaria* spp. infections more challenging to diagnose. A skin biopsy or skin snip is required to detect the microfilariae in the dermis via

saline sedimentation, similar to the method used to detect *Onchocerca* spp. microfilariae (Magins et al., 2013; Bowman, 2014); more details regarding diagnostic techniques detecting canine *Cercopithifilaria* spp. in the dermis are provided below. As the adults of *Cercopithifilaria* are located in subcutaneous tissues of vertebrate hosts, they are also challenging to detect (Cortes et al., 2014). Although microfilariae reside in the dermis, they disseminate through lymphatic vessels, allowing them to be ingested by the tick vector when feeding (Bain et al., 2003). After ingestion, similar to other Filariodea in arthropod hosts, the microfilariae of *Cercopithifilaria* spp. develop to L_3.

Adults and microfilariae of *C. grassii* were first described as *Filaria grassii* from a dog in Italy in 1907 (Ionică et al, 2014). After the first documentation, *C. grassii* was not identified again until 1982–1983 from two brown dog ticks collected from dogs in Switzerland and northern Italy (Bain et al., 1982; Otranto, 2015) and from a brown dog tick in Pakistan (Latrofa et al., 2014). Since then, microfilariae of *C. grassi* have been isolated by skin sedimentation from dogs in Portugal, Spain, and Sicily (Ionică et al., 2014; Otranto et al., 2021). *Cercopithifilaria* sp. II adults have not been thoroughly described like the other canine *Cercopithifilaria* spp. (Otranto et al., 2013a). This species has microfilariae similar in length to those of *D. immitis* and *Acanthocheilonema* spp. (273–305 µm), but are approximately twice the width (12–15 µm). An adult of this species have not yet been described (Gabrielli et al., 2014). *Cercopithifilaria* sp. II has been reported from dogs in Italy and Spain by finding microfilariae in skin biopsies and confirming with molecular analysis (Otranto et al., 2013a; Solinas et al., 2014). A single case report of *Cercopithifilaria* sp. II infection in a red fox (*Vulpes vulpes*) in Portugal was confirmed by morphologically and molecularly identifying microfilariae isolated from a skin biopsy from the ear of the fox. This

suggests this species of *Cercopithifilaria* may also use wild canids as reservoir hosts (Maia et al., 2017).

Cercopithifilaria bainae was first described from a dog in Brazil in 1984 and was not reported in that country again until 2015 (Ramos et al., 2016). The first report of *C. bainae* in a dog outside Brazil, and only the second-ever report, occurred in 2010 in a dog from Sicily (Ionică et al., 2014). *Cercopithifilaria bainae* has been identified in dogs and/or ticks primarily in Mediterranean Europe, but researchers in other countries, including Australia, Brazil, China, India, Indonesia, Iran, Malaysia, South Africa, Spain, Taiwan, the Philippines, and Vietnam, have identified this parasite in canine and/or tick populations (Gabrielle et al., 2014; Latrofa et al., 2014 Otranto, 2015; Bezerra-Santos et al., 2022; Sazmand et al., 2022). The parasite had not been reported in the United States until the single case report from a dog in Florida (Boyd et al., 2019). A study conducted by Latrofa et al. 2014, testing and identifying *Cercopithifilaria* spp. in multiple brown dog ticks in various countries (Africa, Asia, and Europe), demonstrated the possibility of differing abilities of *R. sanguineus* lineages to carry transmit infections, so it is not surprising this parasite may be found world-wide.

The microfilariae of *C. bainae* are smaller than those of other species of *Cercopithifilaria* parasitizing dogs, with length of 170–197 μm and width of 6.1–9.4 μm. Female worms measure 14–19 mm while males are smaller, measuring 9–12 mm. A comparison of the size of microfilariae and adults of the different species of *Cercopithifilaria* in dogs can be found in Table 2. The adults of *C. baina*e appear to localize in the trunk and forelimbs of their host (Otranto et al., 2013b). The prepatent period seems to be less than six months, as microfilariae have been found in dogs approximately six months old (Ramos et

al., 2014). More information specifically regarding *C. bainae* is provided in the next three sub-sections of this literature review (*C. bainae* – Development and Transmission Patterns, Case Reports, and Understanding Prevalence in Dogs and Ticks).

When comparing *cox1* and *12S rRNA* sequences of the three known canine *Cercopithifilaria* spp., the sequence homology is 87–89% and 82–86%, respectively. Fourteen different canine *Cercopithifilaria* sp. haplotypes have been identified thus far based on *cox1*; these sequences were amplified from brown dog ticks infesting dogs and skin samples from dogs from different geographical areas in the Mediterranean Basin. This represents a significant level of genetic variation with 1.6% mean variability at the nucleotide level; this is higher than recorded for other sequences from onchocercids (*D. immitis* and *D. repens*), which are <1% disparate. The large genetic variation within *Cercopithifilaria* spp. is thought to be caused by a high mutation rate in the particular mitochondrial gene region, or due to inbreeding of the parasite within geographic areas or within hosts (Otranto et al., 2012a).

Similar to the diagnosis of *O. lupi*, *Cercopithifilaria* spp. infections in dogs are detected by observing microfilariae in saline sedimentations of skin biopsy punches or skin snips (Colella et al., 2018). Recovery is accomplished by placing the skin samples into a tube with saline solution, and allowing the samples to incubate at 30–37°C for a range of 10 minutes to 12 hours to allow microfilariae to migrate out of tissue. Once the skin samples have incubated, the tubes can be centrifuged to form a pellet. The supernatant is removed and the sediment is stained with 0.1% methylene blue. The stained sediment is examined on glass slides beneath a compound microscope; microfilariae present can be identified to species by length and width. Other features that aid in species identification between microfilariae of

filarioids include observing if the posterior end is filiform or non-filiform, and features of the cephalic hook (Otranto et al., 2011; Cortes et al., 2014).

Additionally, *Cercopithifilaria* spp. can be detected by extracting DNA from skin samples or sedimentations from skin samples and testing said extractions by PCR. Common gene targets include a 304 base pair region of the cytochrome oxidase subunit 1 mitochondrial gene (*cox*1) amplified with the primers Cbcox1F (5'-CGGGTCTTTGTTGTTTTTATTGC-3') and NTR (5'-ATAAGTACGAGTATCAATATC-3') and a 330 base pair region of the ribosomal *12S rRNA* gene amplified with the primers Fila12SF (5'-CGGGAGTAAAGTTTTGTTTAAACCG-3') and Fila12SR (5'-CATTGACGGATGGTTTGTACCAC-3') (Otranto et al., 2011; Latrofa et al., 2012).

When testing a skin biopsy sample by saline sedimentation, and then testing that same skin sample by PCR, microscopy seems to be more sensitive than PCR. This could be due to microfilariae migrating out during the skin sedimentation, leaving few to no *C. bainae* microfilariae (and therefore, little DNA) to detect. The opposite can be said about dogs that do not have microfilariae observed on skin sedimentation; the microfilariae may not migrate out of the skin sample during the incubation period, thus resulting in detectable *C. bainae* DNA for PCR (Otranto et al., 2012b; Solinas et al., 2014). Due to the contradicting results of testing skin snips by saline sedimentation or PCR, the best practice for diagnosing this parasite may be to perform both techniques to increase the likelihood of detecting infection.

Microfilariae of canine *Cercopithifilaria* spp., along with neutrophils, eosinophils, and lymphocytes, may be observed by histological examinations of skin samples, and appear to cause multifocal interstitial dermatitis (Otranto et al., 2013a). Histological examination of a skin biopsy sample from a dog in Florida presenting with non-healing wounds on the head

and medial canthi revealed an eosinophilic to lymphohistiocytic perivascular dermatitis with multiple microgranulomas. Microfilariae were observed within a thick, lightly eosinophilic cuticle and filled with basophilic nuclei measuring 1–2 μm. A modified Knott's test and skin sedimentation were performed, resulting in no microfilariae identified on the modified Knott's and microfilariae morphologically similar to *C. bainae* identified in the skin sedimentation. The microfilariae were molecularly confirmed as *C. bainae* by PCR (Boyd et al., 2019).

Cercopithifilaria bainae

Development and Transmission Patterns

Unlike *C. grassi* and *Cercopithifilaria* sp. II, which are speculated to use brown dog ticks as vectors based on molecular detection, *C. bainae* has been experimentally demonstrated to undergo development within brown dog ticks. In one study demonstrating *R. sanguineus* s.l. as the vector of *C. bainae*, nymphal brown dog ticks were fed until repletion on a microfilariae-positive dog. Replete nymphs were held in a humidity chamber and dissected at different time points through ecdysis. This study showed that nymphs molted to adults within approximately 30 days after taking a blood meal, and that emerged adults harbored third-stage larvae, indicating development and transstadial transmission of *C. bainae* in brown dog ticks (Brianti et al., 2012; Otranto et al., 2012b). A single adult brown dog tick may harbor as many as 1,469 developing larvae, suggesting the vector can tolerate infection with this parasite well (Otranto et al., 2012c).

An additional study demonstrated how nymphs played a key role in *C. bainae* transmission. In this study, ticks from the environment of a shelter where dogs commonly had tick infestations were collected. Engorged immature ticks were held under controlled

conditions until they molted, then dissected and checked for different stages of *Cercopithifilaria* (microfilaria, L_1, L_2, or L_3). The only tick stages that had developed L_3 of *C. bainae* were collected as unengorged adults or were engorged nymphs that were held through molt with larvae found in the emerged adults (Ramos et al., 2014). To support these findings, Santos et al., 2017 collected ticks from naturally infested dogs, dissecting apparently unengorged ticks immediately after collection, and holding engorged immatures through the molt in a controlled environment. This study detected *C. bainae* only in unengorged females, males, and engorged nymphs held through molt, with *C. bainae* larvae detected in the emerged adults.

Interestingly, to increase the possibility of *C. bainae* transmission, it is thought that the female worm may release more microfilariae in the seasons ticks are most active, which likely varies with geographic region. This has been demonstrated in Sicily and Apulia, where the highest average number of microfilariae were recovered from skin snips during the months that ticks are most active (June–September) (Otranto et al., 2012b). These behaviors are similar to that of other vector-borne filarioids. For example, *D. immitis* microfilariae reportedly are most numerous in circulation in infected dogs at night as that is when mosquitoes are more likely to take a blood meal. The abundance also changes seasonally, with microfilariae of *D. immitis* reported to be more abundant in the blood of dogs during July and August in temperate areas, when mosquitoes are more active and found in greater numbers (Hawking, 1967). Another factor that may increase transmission of *Cercopithifilaria* spp. is the feeding behavior of male brown dog ticks, which take multiple blood meals while seeking females to mate. During this feeding (and mating) period, male brown dog ticks move between canine hosts (Little et al., 2007; Otranto et al, 2012a). Further

promoting transmission of *C. bainae* to feeding brown dog ticks, *C. bainae* microfilariae are found in higher frequency and abundance in areas of where *R. sanguineus* s.l. prefer to attach and feed on dogs, including the head, ears, and neck regions (Otranto et al., 2012b; Saleh et al., 2019).

Case Reports

Although clinical manifestations of *Cercopithifilaria* spp. infection in dogs are thought to be uncommon, there have been a few case reports from Europe and in Brazil where infection with *Cercopithifilaria* spp. has caused clinical signs, such as a large mass, polyarthritis, and erythematous, papular, and pruritic dermatitis (Gabrielli et al., 2014; Soares et al., 2020). There are two case reports of dogs in Italy with clinical signs associated with *Cercopithifilaria* spp. infections. One dog had a history of tick infestation, and presented with lethargy, lameness, and joint pain with manipulation and was diagnosed with chronic polyarthritis. Upon collection of synovial fluid, active *C. bainae* microfilariae were observed, supporting the suspicion that microfilariae are not only present in the dermis, but are also distributed within the canine host via lymphatic vessels (Gabrielli et al., 2014). In survey of canine shelter dogs in Sicily, Italy, *A. reconditum* microfilariae were identified through blood collections from a mongrel dog. The dog was heavily infested with fleas and brown dog ticks, and during clinical examination, was noted to have a subcutaneous nodule on the right hind limb. Skin biopsy samples were processed via saline sedimentation and PCR, demonstrating co-infection of *A. reconditum* and *Cercopithifilaria* sp. microfilariae (Otranto et al., 2011). In Brazil, a 9-year-old mixed breed dog presented to a clinic with a large growth in the lumbosacral region, measuring approximately 15 cm. Fluid was collected from the mass and during the cytological examination, microfilariae were observed and

morphologically identified as *Cercopithifilaria* species. This diagnosis was later confirmed by PCR of the fluid collected and the parasite identified as *C. bainae*. After unsuccessful treatment with transdermal moxidectin, the tumor was removed by surgery (Soares et al., 2020).

The first case of a dog infected with *C. bainae* in North America was reported in 2019 from a dog in Florida. The 11-month-old mixed breed dog was referred to the University of Florida with a 1-month history of non-healing wounds on the head. Upon presentation, the dog had multiple plaques on the head and alopecia, erythema, and ulcerations around the eyes. Skin samples were collected using a 6 mm biopsy punch and histopathological examinations were performed on the tissues. Microfilariae were found, isolated, and confirmed to be *C. bainae* by morphology and PCR of the *18S rRNA* gene and mitochondrial *cox1* gene. The dog was treated with transdermal moxidectin (extra-label) every 2–3 weeks until the lesions completely cleared at 14 weeks post diagnosis (Boyd et al., 2019).

Understanding Prevalence in Dogs and Ticks

Most studies determining the prevalence of *C. bainae* in dogs have been conducted in Mediterranean Europe, where canine populations may have a prevalence as high as 21.6%, exceeding that of other filarioids, with the exception of *D. repens* (Otranto et al., 2012c; Otranto et al., 2013c). A small sample population of dogs (n=39) in Romania was tested for *Cercopithifilaria* spp. by skin sedimentation, and a single dog (2.6%) tested PCR positive for *C. bainae*. The latter study demonstrated that *Cercopithifilaria* is more widespread in dogs in Europe, and not limited to the Mediterranean region (Ionică et al., 2014). In the United

States, although there is the single case report in a dog from Florida, the prevalence of *C. bainae* infection in dogs is not known (Boyd et al., 2019).

A tick study conducted by Latrofa et al., 2014 surveyed *Rhipicephalus* spp. for *Cercopithifilaria* spp. within Africa, Asia, Australia, Europe, North America, and South America; of these regions, they identified *Cercopithifilaria* positive ticks in regions of all continents with the exception of North America. A study conducted in Romania surveyed *R. sanguineus* (temperate lineage) and *Dermacentor reticulatus* ticks for filarioid DNA and found a single female *D. reticulatus* positive for *C. bainae* DNA, while all *R. sanguineus* s.l. tested negative. This finding could demonstrate a new potential tick vector for *C. bainae* in the region, or could be the result of the tick recently feeding on a dog with *C. bainae* and ingesting microfilariae (Andersson et al., 2017). Although the single dog in Florida was likely infected with *C. bainae* by a brown dog tick, the dog did not have a history of tick infestations and ticks were not found on the dog at the time of examination (Boyd et al., 2019).

Protozoa

Along with filariids, brown dog ticks transmit blood-borne apicomplexans (Apicomplexa: Hematozoa), including *Babesia* spp. and *Hepatozoon canis* (Dantas-Torres and Otranto, 2015). These protozoa infect dogs globally and can cause mild to severe disease (Allen et al., 2008; Baneth, 2018).

Babesia spp.

Babesia spp. are tick-borne protozoa found solely in erythrocytes. Several species infect dogs globally and may cause severe disease. The most common infections include *B. canis canis, B. c. vogeli*, *B. c. rossi*, and *B. gibsoni* (Beall et al., 2012; Baneth, 2018);

Babesia canis vogeli is found world-wide, while *B. c. canis* is primarily in Europe, *B. c. rossi* is typically limited to Africa, and *B. gibsoni* is found in Asia, Australia, Europe, and North America (Baneth, 2018; Birkenheuer et al., 2020). Of these species, *R. sanguineus* s.l. transmit *B. c. vogeli* and are thought to be a potential vector for *B. gibsoni* (Solano-Gallego et al., 2016). *Babesia canis canis* and *B. c. rossi* are vectored by *D. reticulatus* and *Haemaphysalis* spp., respectively. *Babesia gibsoni* is transmitted by *H. longicornis* in other areas of the world and is also spread by direct contact, typically during dog fights with an infected dog (Birkenheuer et al., 2005; Baneth, 2018).

A global study conducted by Bierkenheuer et al., 2020 examining positivity of *Babesia* spp. in clinically suspicious dogs found that North America had a prevalence of 1.5% of dogs testing PCR positive for at least one *Babesia* sp.; this prevalence was lower than all other countries tested, which included other parts of the Americas, Asia, Europe, and Australia. Due to the low parasitemia of *Babesia* spp. and the difficulty of detecting this parasite during subclinical and chronic infections, the prevalence of *Babesia* spp. could be higher than what is currently documented (Jefferies et al., 2007).

In North America, *B. c. vogeli, Babesia conradae, Babesia* sp. (Coco), and *B. gibsoni* all infect dogs (Köster et al., 2015; Birkenheuer et al., 2020). Of these species, *R. sanguineus* s.l. is known to vector *B. c. vogeli*, which is maintained in ticks trasstadially and transovarially (Yabsley et al., 2008; Chauvin et al., 2009; Lira-Amaya et al., 2017). Although the arthropod vector and other routes of transmission are not known for *Babesia* sp. (Coco) and *B. conradae*, ticks are suspected to serve as the vector (Köster et al., 2015). *Babesia gibsoni* is primarily transmitted in the United States among pit bull terriers or pit bull crosses by direct contact through biting and scratching leading to transmission of contaminated blood

from infected dogs to naïve dogs, which particularly occurs with fighting behavior, but infections may also occur transplacentally or through a tick vector (Birkenheuer et al., 2005; Tuska-Szalay et al., 2021).

Clinical disease in dogs infected with *Babesia* spp. can vary greatly. *Babesia canis vogeli* is usually characterized by mild symptoms including fever, lethargy, anemia, and vomiting (Lira-Amaya et al., 2017). Infections with *B. conradae* may cause anorexia, hemolytic anemia, splenomegaly, thrombocytopenia, and vomiting (Stayton et al., 2021). *Babesia* sp. (Coco) infections are typically identified in dogs with immunocompromised conditions and these dogs may present with anorexia and lethargy (Sikorski et al., 2010; Baneth, 2018). While infections with *B. gibsoni* can be subclinical in pit bull terriers, clinical signs such as lethargy, fever, hemolytic anemia, and splenomegaly can occur in other dog breeds (Baneth, 2018; Tuska-Szalay et al., 2021).

Hepatozoon canis

Rhipicephalus sanguineus s.l. is considered to be the primary vector for *Hepatozoon canis*, but *Rhipicephalus turanicus* has recently been demonstrated experimentally as a vector in some regions. Within *R. sanguineus* s.l., this protozoan can be transmitted transstadially, but has not been shown to transmit transovarially (Farkes et al., 2014). *Hepatozoon canis* infects canines and is geographically distributed across Europe, Asia, and the Americas (Allen et al., 2008). While data in the United States are limited, reports demonstrate infections in <3% of dogs, while researchers in other areas of the world have detected prevalence as high as 48% by PCR (Allen et al., 2008; Díaz-Sánchez et al., 2021). Infections in canines are considered subclinical, but clinical signs such as fever, lethargy, weight loss, and lymphadenomegaly have been reported. Clinical disease is often seen when canines have

co-infections with other pathogens such as *Babesia* spp. and *Ehrlichia* spp. (Allen et al., 2008; Pacifico et al., 2020).

Rickettsial agents

Members of Anaplasmataceae

Brown dog ticks transmit members of the family Anaplasmataceae including *Ehrlichia canis* and may be secondary vectors for *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in some regions (Yabsley et al., 2008; Ndip et al., 2010; Beall et al., 2012). Although brown dog ticks are also suspected to transmit *Anaplasma platys*, experimental transmission studies have failed to prove vector competence (Yabsley et al., 2008; Dantas-Torres and Otranto, 2015). Within the United States, *E. canis* infections in dogs are primarily detected in the southcentral and southwestern region, where *R. sanguineus* s.l. are commonly found (Little et al., 2021). While prevalence data of *E. canis* in ticks is lacking in North America, serologic prevalence of *E. canis* in dogs is <2% (Beall et al., 2012; Qurollo et al., 2014). *Ehrlichia canis* can be transmitted transstadially and intrastadially in ticks. Infection with *E. canis* causes canine monocytic ehrlichosis and has been shown to occasionally infect humans, categorizing it as zoonotic (Little, 2010; Almazán et al., 2016; Obaidat and Alshehabat, 2018). Infections with *E. canis* can cause severe disease in dogs, and may even be fatal. The severity of disease depends on factors such as prior health and immune response of individual dogs or specific breeds, co-infections with other tick-borne diseases, and amount of pathogen transmitted to the dog during tick feeding (i.e. infectious dose). Clinical signs include lethargy, anorexia, muscle pain, splenomegaly, lymphadenopathy, and bleeding diatheses (Little, 2010).

Spotted Fever Group *Rickettsia*

Rickettsia massiliae and *R. rickettsii*, species within the complex causing spotted fever group rickettsioses (SFGR), have been identified in brown dog ticks in North America (Wikswo et al., 2007; Yabsley et al., 2008; Fornadel et al., 2013). According to the CDC, the fatality rate of all SFGR is approximately 0.5% (CDC, 2021). In North America, *R. massiliae* has only been described from brown dog ticks collected in Arizona, California, Virginia, and northern Mexico, but prevalence data from other areas are lacking (Eremeeva et al., 2006; Beeler et al., 2011; Fornadel et al., 2013; López-Pérez et al., 2019).

Increased reports of *R. rickettsii*, the agent of Rocky Mountain spotted fever (RMSF), led to the recognition of the ability of brown dog ticks to vector this disease in North America. *Rickettsia rickettsii* infected *R. sanguineus* s.l. have been found in Arizona, California, and Northern Mexico (Wikswo et al., 2007; Yaglom et al., 2018). Brown dog ticks transmit *R. rickettsii* transstadially and transovarially (Piranda et al., 2011; Parola et al., 2013). Canines and humans develop similar clinical signs when infected, including fever, rash, fluid retention, and muscle pain (Yaglom et al., 2018). The national increase in RMSF cases in the United States over the past two decades could be caused by other SFGR that are mistaken for RMSF due to the lack of species-specificity of serologic assays commonly performed for diagnosis. To truly distinguish the *Rickettsia* spp. infections from one another, a more specific test such as PCR should be used (Parola et al., 2013; Drexler et al., 2016; Saito et al., 2019; Duncan et al., 2021). However, this may be problematic if blood is not collected during the acute phase of infection; approximately one-week post infection, the likelihood of detecting *Rickettsia* spp. in the blood decreases, as the serologic response increases (Robinson et al., 2019).

Co-infections

As brown dog ticks are known or suspected to vector a variety of organisms, it is not surprising that instances of co-infections in dogs occur. A study in Italy showed that all dogs infected with *C. bainae* (n=15) were also infected with *H. canis*, *A. platys*, and/or *B. c. vogeli*. While results of co-infections varied with season, this study indicated that the probability of *H. canis*, *A. platys*, and *B. vogeli* in *C. bainae* positive dogs was 78%, 22%, and 11%, respectively (Ramos et al., 2014).

While it can be challenging to survey dogs across a broad geographic range for multiple brown dog tick-vectored agents, testing brown dog ticks for molecular evidence of these agents can provide insight into where organisms are circulating regionally and how prevalent different infections are in sampled tick populations (Dantas-Torres et al., 2012; Bezerra-Santos et al., 2022). This approach may indirectly indicate risk of infection to dogs and/or humans with different organisms in given areas. One such study involved testing *Rhipicephalus* spp. from different geographical areas in Africa, Asia, Australia, Europe, and South America, for DNA of *A. platys*, *Cercopithifilaria* spp., and *H. canis* (Latrofa et al., 2014). Of the 204 ticks tested, 2.5% (5/204) tested positive for *A. platys*, and 7.4% (15/204) were positive for *H. canis*. From that sample set, 177 ticks were tested for *Cercopithifilaria* spp. and 9.6% (17/177) tested positive; with the exception of one *C. grassi*, all positives were characterized as *C. bainae*. Of the *C. bainae* positive ticks, four had evidence of co-infection with *A. platys* (n=1) or *H. canis* (n=3). These co-infection reports document that brown dog ticks serve as important canine disease vectors; their role in canine health in North America warrants further consideration.

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Table 1. List of bacteria, nematodes, and protozoa suspected or proven to be transmitted to dogs by brown dog ticks globally.

Pathogen	Geographic distribution	Zoonotic?	Reference
Acanthocheilonema dracunculoides	Africa, South America	No	Muñoz et al. (2020)
Anaplasma platys	World-wide	Yes	Little (2010); Dantas-Torres and Otranto (2015)
Babesia canis vogeli	Asia, Australia, Europe, North America, South America	No	Baneth (2018); Birkenheuer et al. (2020)
Babesia gibsoni	Asia, Australia, Europe, North America	No	Baneth et al. (2018)
Cercopithifilaria bainae	Asia, Australia, Europe, North America, South America	No	Brianti et al. (2012); Boyd et al. (2019); Bezerra-Santos et al. (2022)
Cercopithifilaria grassi	Asia, Europe	No	Bain et al. (1982); Bezerra-Santos et al. (2022)
Cercopithifilaria sp. II	Europe	No	Otranto et al. (2013a)
Coxiella burnetii	Africa, Asia, Australia, Europe, North America	Yes	Dantas-Torres et al. (2012)

Ehrlichia canis	World-wide	Yes	Bowman et al. (2009)
Ehrlichia chaffeensis	North America	Yes	Dantas-Torres et al. (2012)
Ehrlichia ewingii	North America	Yes	Harris et al. (2016)
Hepatozoon canis	Asia, Europe, North America, South America	No	Allen et al. (2008)
Leishmania infantum	Africa, Asia, Central America, Europe, North America, South America	Yes	Solano-Gallego et al. (2012); Schaut et al. (2015)
Mycoplasma haemocanis	Africa, Asia, Australia, Europe, North America, South America	No	Soto et al. (2017)
Rickettsia conorii	Africa, Asia, Europe	Yes	Parola et al. (2013)
Rickettsia massiliae	Africa, Asia, Central America, Europe, North America, South America	Yes	Parola et al. (2013)
Rickettsia rickettsii	Central America, North America, South America	Yes	Parola et al. (2013)
Rickettsia rhipicephali	Asia, Central America, North America, South America	No	Parola et al. (2013); Bermúdez and Troyo (2018)

Table 2. *Cercopithifilaria* spp. infecting dogs.

***Cercopithifilaria* spp.**	**Size of microfilariae**	**Size of adult worms**	**PPP**	**References**
C. bainae	170–197 µm x 6.1–9.4 µm	females 14–19 mm; males 9–12 mm	<6 months	Otranto et al. (2013a)
C. grassi	567–660 µm x 12.5–15.5 µm	females 17–21 mm; males 7–8 mm	UN*	Ionică et al. (2014)
Cercopithifilaria sp. ll	273–305 µm x 12–15 µm	UN*	UN*	Gabrielli et al. (2014)

*UN=unknown; PPP=Pre-patent period

CHAPTER III

DETECTION OF *CERCOPITHIFILARIA BAINAE* INFECTION IN SHELTER DOGS AND TICKS IN OKLAHOMA, USA[1]

Lineberry, M.W., Sundstrom, K.D., Little S.E., Stayton E.M., Allen K.E., 2020. Parasites & Vectors 13:216. Reprinted here with permission of publisher.

ABSTRACT

Background: *Cercopithifilaria bainae* is a filarioid nematode of dogs. Infection with the parasite was not reported in the USA until 2017, when a dog with skin lesions in Florida was diagnosed. Brown dog ticks, *Rhipicephalus sanguineus* (*sensu lato*), are the purported tick vectors, and are widespread in the USA. Therefore, *C. bainae* is likely present in additional states. Here, we tested dogs and ticks in Oklahoma for evidence of *C. bainae* infection.

Methods: Dermal punch biopsies were opportunistically collected from municipal shelter and client-owned dogs. Multiple skin samples collected from interscapular and head regions were tested by saline sedimentation to recover live microfilariae for morphometric identification and by PCR to amplify a 330 bp region of the filarioid *12S* rRNA gene. Also, ticks observed on surveyed dogs were collected, identified to species level, and tested for filarioid DNA.

Results: A total of 496 saline sedimentations were performed on 230 shelter and 20 client-owned dogs. *Cercopithifilaria bainae* infections were identified in 2.6% (6/230) of shelter dogs by morphometry of microfilariae in sedimentations and/or amplification of DNA from skin. DNA sequences amplified from PCR positive skin samples were 99–100% identical to *C. bainae* reported in Italy. All skin samples from client-owned dogs were negative for filarioid infection by saline sedimentation and PCR. A total of 112 ticks, comprised of four species, were collected. Two of 72 *R. sanguineus* (*s.l.*), both engorged females found attached to a *C. bainae* infected dog, harbored *C. bainae* DNA (99–100% identity). One attached *R. sanguineus* (*s.l.*) male on the same dog harbored filarioid DNA sequence which was difficult to interpret at numerous base-pair locations, but was closest in identity (~80%) to *C. bainae*.

Conclusions: The distribution of *C. bainae* is more widespread than previously known. To our knowledge, we document *C. bainae* infections in dogs and DNA in brown dog ticks in Oklahoma for the first time. As brown dog ticks are commonly found throughout the USA, veterinarians in this region should consider *C. bainae* infection as a differential diagnosis in canine patients with dermatitis or polyarthritis.

Keywords: *Cercopithifilaria bainae*, Dermal punch biopsy, Filarioid, Microfilariae, Mitochondrial *12S* rDNA, *Rhipicephalus sanguineus* sensu lato, Saline sedimentation, Third-stage larvae

BACKGROUND

Cercopithifilaria bainae is a tick-borne filarial nematode of dogs that was first described in Brazil in 1984 [1]. Adults of *C. bainae* parasitize the subcutaneous tissue of canine hosts, and microfilariae remain sequestered in the dermis, making detection of the parasite in infected dogs challenging [2]. *Cercopithifilaria bainae* is considered primarily non-pathogenic, but erythematous, papular and pruritic dermatitis, non-healing and ulcerative skin lesions, and subcutaneous nodules associated with infection have been reported [3–5]. One case of polyarthritis has also been documented [3].

Cercopithifilaria bainae infections in dogs have been documented predominantly in Mediterranean Europe and in Brazil, and DNA of the parasite has been reported in the suspected tick vector, *Rhipicephalus sanguineus* (*sensu lato*), collected in these areas. DNA of *C. bainae* has also been identified in *R. sanguineus* (*s.l.*) collected in other regions, including Australia, Malaysia, and South Africa [3, 4, 6, 7]. *Rhipicephalus sanguineus* (*s.l.*), commonly called brown dog ticks, are thought to be important natural vectors of *C. bainae* based on the development of third-stage larvae in adult ticks acquisition fed as nymphs on a naturally infected dog [8]. Although *C. bainae* has been molecularly detected in other ticks, including *Dermacentor reticulatus* and *Ixodes ricinus*, parasite development within these tick species has not been experimentally demonstrated [8, 9].

Despite the cosmopolitan distribution of brown dog ticks, *C. bainae* had not been documented in the USA until 2017. A dog from Florida with no travel history was presented with dermatitis, with plaques on the dorsal head, and alopecia, erythema, and ulceration of both medial canthi. Microfilariae isolated from skin biopsy samples via saline sedimentation were identified as *C. bainae* by PCR and microscopy [5].

Brown dog ticks are widespread in the USA, with all stages preferentially feeding on dogs, and it is likely that *C. bainae* is present in dogs in states in addition to Florida [10, 11]. To the authors' knowledge, however, no studies investigating geographic distribution of *C. bainae* in dogs in the USA have been conducted. Raising awareness of the emerging parasite in the USA will assist veterinarians in diagnosing infections, which will generate further information regarding clinical manifestations and pathology in infected dogs, and lead to investigations into treatment and prevention strategies. To determine if *C. bainae* is present in dogs in Oklahoma, multiple dermal punch biopsy samples were evaluated by saline sedimentation and PCR. Additionally, ticks observed on dogs were tested for filarioid DNA.

METHODS

Skin Biopsy Sample Collection

Skin biopsy samples were opportunistically collected from euthanatized dogs in Oklahoma, USA, over a 10-month period (January–October 2018). Shelter dogs were temporarily housed at animal control facilities prior to euthanasia following standard approved shelter protocols and client-owned dogs were submitted for necropsy at the Oklahoma Animal Disease Diagnostic Laboratory (Payne County, Stillwater, OK). When possible, sex and estimated age were documented. Travel histories were not available for the majority of animals, nor was information regarding prior treatment with parasiticides.

Multiple skin samples were collected from individual animals using sterile 6 mm biopsy punches within hours, but sometimes up to four days, after death. Carcasses were stored at 4°C until sample collection. Higher frequency of *C. bainae* microfilariae in interscapular and head regions has been previously described [12], and therefore these focal

regions were sampled; up to four interscapular and up to three head samples were collected from each animal. At times of skin biopsy sample collection, the skin was briefly examined for cutaneous nodules and other lesions.

Single interscapular biopsy samples were placed in microcentrifuge tubes containing phosphate buffered saline (PBS) and transported to the laboratory for storage at -20°C and later DNA extraction and molecular analyses. Additional biopsy samples were placed in PBS-filled, sterile 15 ml conical tubes and, upon transport to the laboratory, processed to recover microfilariae as described below. After processing, the majority of skin samples were stored at -20°C for subsequent DNA extraction and PCR.

Saline Sedimentation of Skin Biopsy Samples

To detect microfilariae in skin biopsy samples, up to three skin samples from individual dogs were placed in 15 ml conical tubes containing PBS and incubated for 1–3 hours at 37°C to allow live microfilariae to migrate out of the tissue [12]. The skin was removed and remaining PBS was centrifuged at 388 x g for 5 minutes to concentrate microfilariae. Supernatants were decanted and resulting pellets were stained with 0.1% methylene blue for microscopic examination.

Stained sediment was transferred to microscope slides and covered with 22 x 60 mm glass coverslips; all sedimentation material from each skin sample was scanned under 100X total magnification. When observed, microfilariae on slides were enumerated, and up to 10 microfilariae were measured using an ocular micrometer (length and width) under 400X total magnification. Microfilariae measurements were compared to those available in the literature identifying filarioid species including *Acanthocheilonema reconditum* (215–288 x 4.5–5.8 μm), *Cercopithifilaria bainae* (173.8–200 x 5.6–6.9 μm), and *Dirofilaria immitis* (280–325 x

5–7.5 μm) [13–15]. Microfilariae in sedimentations were gently washed from slides with PBS and stored at 4°C for DNA extraction within 48 hours for subsequent molecular identification.

Tick Collection and Processing

Animals were briefly examined (approximately 1–3 minutes) for ticks at the time of skin biopsy collection. When present, ticks were placed in 70% ethanol and stored at -20˚C. At the time of dissection, ticks were removed from ethanol and identified to species by microscopic examination and comparison with standard keys [16]. Identified ticks were then individually dissected and internal contents removed and digested in Proteinase K and lysis buffer solution at ambient temperature [17].

DNA Extraction Methods, PCR, and Sequence Analysis

Tick dissection, DNA extraction, PCR amplification, and amplicon purification were carried out in dedicated laboratory areas to prevent DNA contamination. Separate negative water controls were used for DNA extractions and for PCR. A sample containing DNA of *D. immitis* was used as a positive control.

Nucleic acid was extracted from approximately 30 mg sections of skin biopsy samples using the QIAamp® Fast DNA Tissue Kit (Qiagen, Valencia, CA, USA). Refrigerated microfilariae (washed with PBS from glass microscope slides) were extracted for DNA using the Illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA). After tissue digestion, individual tick samples were extracted for DNA using the QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). DNA extractions were carried out according to the manufacturer's instructions specific to each kit.

PCR amplifying a ~ 330-bp region of the filarioid *12S* rRNA mitochondrial gene was performed on DNA extractions from skin, microfilariae, and ticks using previously described primers Fila12SF and Fila12SR [2]. Individual reactions were carried out in a total volume of 25 µl containing 1X AmpliTaq Gold 360 (Applied Biosystems, Carlsbad, CA), 0.8 µM of each primer, and 2 µl of DNA. Thermocycler conditions were as follows: 94°C for 10 min, followed by 40 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 90 sec, and ending with a final extension step of 72°C for 7 min.

Additionally, PCR amplifying a 340–370 base pair region of the 12S rRNA mitochondrial gene was performed on *R. sanguineus* (*s.l.*) testing positive for *C. bainae,* using previously described primers 12SF and 12SR, to determine the genetic lineage (temperate or tropical) of the ticks as previously described [17, 18].

Standard gel electrophoresis in a 2% agarose matrix with GelRed® staining (Biotium, Fremont, CA) was used to detect amplicons. Correctly sized amplicons were purified either directly from the gel using the QIAquick® Gel Extraction Kit (Qiagen) or from PCR reactions using the QIAquick® PCR Purification Kit (Qiagen).

Purified amplicons were bi-directionally sequenced (Sanger method) by Eurofins Genomics (Louisville, KY) or the Oklahoma State University Molecular Core Facility (Stillwater, OK). Sequences from skin samples and ticks were compared to those available in the National Center for Biotechnology Information database (GenBank™) to determine filarioid species identity and *R. sanguineus* (*s.l.*) genetic lineage. Sequence alignments were constructed using ClustalW to determine percent similarities of Oklahoma filarioid *12S* rRNA mitochondrial gene sequences to each other and to additional filarioid sequences

previously contributed to the GenBank™ repository, as well as to determine *R. sanguineus* (*s.l.*) genetic lineage.

RESULTS

Dogs Surveyed

The sample set included 230 shelter dogs and 20 owned dogs. Shelter dogs consisted of 55.2% (127/230) males and 43.5% (100/230) females, with reported ages ranging from two months to 14 years ($\bar{x}$=2.4 years; 95% CI: 2.00–2.87). Sex was not recorded for three dogs and age was not reported for 106 dogs. One of the shelter animals was a coyote, but was included as part of the shelter cohort. Four shelter dogs tested positive for circulating heartworm antigen on a commercial patient-side diagnostic assay conducted by the shelter. A single shelter dog was noted to have alopecia and scabbing on the face and dorsum at the time of skin biopsy collection. Although sometimes difficult to ascertain in some animals due to overall poor condition, cutaneous lesions were not noted on other dogs. Owned dogs consisted of 10 males and 10 females, with reported ages ranging from 3 months to 12 years ($\bar{x}$=6.4 years; 95% CI: 4.14–8.73). Age was not reported in one dog. Necropsy revealed that two owned dogs were infected with adult *D. immitis*. No dermatological lesions were reported for any of the owned animals.

Microfilariae Recovered in Saline Sedimentations

A total of 496 saline sedimentations were performed on 230 shelter dogs and 20 owned dogs. Microfilariae were recovered from 8.7% (20/230) of shelter dogs. A total of eight microfilariae recovered from 1.3% (3/230) of dogs were consistent with *C. bainae* by morphometry, measuring 173–200 µm x 5.6–7.5 µm. Body regions where *C. bainae*

microfilariae were recovered and number of microfilariae recovered in individual biopsy samples are included in Table 1. *Demodex* sp. was recovered by saline sedimentation from the single shelter dog with obvious skin lesions (alopecia and scabbing). No microfilariae were recovered from skin biopsy samples collected from client-owned dogs.

Acanthocheilonema reconditum (215–288 x 4.5–5.8 μm) was identified in 1.3% (3/230) of dogs and *D. immitis* (280–325 x 5–7.5 μm) was identified in 5.2% (12/230) of dogs. One dog had a single microfilaria recovered from the interscapular region that desiccated on the slide, so an accurate measurement was not possible for species determination. This dog was later confirmed as having *D. immitis* by PCR of the sediment washed from the slide with PBS. One microfilaria (measuring 160 μm x 4.5 μm) recovered from a single shelter dog did not fall into known filarioid microfilariae size ranges.

On average, the numbers of microfilariae detected for *A. reconditum* and *D. immitis* in individual skin biopsy samples were higher when compared to *C. bainae*, which ranged in number from one to four. Of the dogs with *A. reconditum* or *D. immitis*, 93.3% (14/15) had detectable microfilariae in interscapular regions, ranging in number from one to 68, and 80% (12/15) had detectable microfilariae in head regions, ranging in number from one to 239.

DNA from microscopically identified *C. bainae* or *A. reconditum* microfilariae was not detectable in material rinsed from sedimentation slides. DNA of *D. immitis* microfilariae rinsed from slides was detected in 55% (11/20) of samples.

PCR of Skin Biopsy Samples

Skin samples from 228 shelter dogs and eight owned dogs were tested by PCR, all of which were also tested by saline sedimentation. A total of 9.6% (22/228) of shelter dogs were positive for filarioid DNA, with 2.2% (5/228) having DNA of *C. bainae* (Table 1); two of

these dogs were also positive for *C. bainae* microfilariae by microscopy. *Acanthocheilonema reconditum* and *D. immitis* DNA was also detected in 0.9% (2/228) and 6.6% (15/228) of dogs, respectively. When assessing dermal areas of skin biopsy collection, 8.3% (19/228) of dogs had detectable DNA in the interscapular region, including 1.8% (4/228) with *C. bainae*, 0.9% (2/228) with *A. reconditum,* and 5.7% (13/228) with *D. immitis* infections. In the head region, 4.8% (11/228) of dogs had detectable DNA, including 0.4% (1/228) with *C. bainae*, and 4.4% (10/228) with *D. immitis* infections. *Cercopithifilaria bainae* sequences obtained from shelter dogs were 99–100% homologous to each other and to *C. bainae* reported in Italy (accession number KF381408). *Cercopithifilaria bainae* sequences obtained from dogs in this study were submitted to GenBank™ (MN814265–MN814269). *Acanthocheilonema reconditum* and *D. immitis* sequences were 99–100% homologous to GenBank™ accessions JF461460 and MH051846, respectively. None of the samples from owned dogs had detectable filarioid DNA by PCR.

PCR of Dissected Ticks

A total of 112 ticks were collected from 17 dogs, including two dogs with *C. bainae*. A total of 110 ticks were collected from 16 shelter dogs (16/230, 7.0%) and were comprised of *Amblyomma americanum* (1 nymph, 10 males, 6 unengorged females and 8 engorged females), *Amblyomma maculatum* (5 males and 1 unengorged female), *Dermacentor variabilis* (4 males, 1 unengorged female and 3 engorged females), and *R. sanguineus* (*s.l.*) (47 males, 3 unengorged females and 22 engorged females). Two partially engorged *A. americanum* females were collected from one (1/20, 5.0%) client-owned dog.

Two shelter dogs with *C. bainae* microfilariae by sedimentation were noted to have *R. sanguineus* (*s.l.*) on them at the time of skin biopsy sample collection; three attached,

engorged females and two attached males were collected from one of these dogs. Two of the engorged *R. sanguineus* (*s.l.*) harbored DNA sequences that were 99% identical to each other and 99% homologous to *C. bainae* from Italy (KF381408); *C. bainae* sequences from the female *R. sanguineus* (*s.l.*) were 99–100% identical to *C. bainae* sequences amplified from skin of dogs in this study. One of the male *R. sanguineus* (*s.l.*) harbored sequence that was difficult to interpret at numerous base-pair locations due to heterozygous and mis-spaced peaks, suggesting co-infection with similar organisms, but was closest in identity (~80%) to *C. bainae* (KF381408). Attempts to clone amplicons from the male *R. sanguineus* (*s.l.*) into plasmid vectors to better elucidate nucleotide sequences of single gene fragments were unsuccessful. The *R. sanguineus* (*s.l.*) ticks which harbored *Cercopithifilaria* sp. sequences were identified as belonging to the temperate lineage. The *R. sanguineus* (*s.l.*) ticks which tested negative for *Cercopithifilaria* sp. DNA were not tested for genetic lineage. Both *A. americanum* collected from the client-owned dog were negative for filarioid DNA by PCR.

In addition to detection of *Cercopithifilaria* sp. DNA in ticks, DNA of *D. immitis* was detected in 13 ticks collected from six different dogs, including five *A. americanum* (two males, 1 unengorged female and 2 engorged females) and eight *R. sanguineus* (*s.l.*) (4 males and 4 partially to fully engorged females). Two dogs with detectable *D. immitis* DNA in infesting ticks were positive for *D. immitis* by skin sedimentation and/or PCR, two dogs did not have microfilariae or detectable filarioid DNA in skin, and two dogs with *D. immitis* positive ticks were positive for *C. bainae* microfilariae by skin sedimentations and/or PCR. No *A. reconditum* DNA was detected in any of the ticks tested.

DISCUSSION

Cercopithifilaria bainae infections in dogs have predominantly been identified in Mediterranean Europe and Brazil, with detection in 10.5–13.9% and 1% of dogs surveyed, respectively; these are regions where researchers have been actively looking for the parasite [7, 19]. However, due to the cosmopolitan distribution of *R. sanguineus* (*s.l.*), the experimentally demonstrated tick vector, it is logical to deduce that *C. bainae* infections in dogs are similarly distributed, as are other infections transmitted by this tick group including *Anaplasma platys*, *Ehrlichia canis*, canine *Babesia* spp., and *Hepatozoon canis* [10, 20–22]. Here, we report *C. bainae* in dogs in Oklahoma for the first time, only the second documentation of the parasite in North America. The parasite was detected in 2.6% (6/230) of shelter dogs when PCR and sedimentation results are considered together. Although PCR and sedimentation results in the present study did not always agree, discrepant results between PCR and sedimentation assays have been documented previously in *C. bainae*-infected dogs [12]. It is also possible that the length of time between euthanasia and sample collection could have affected recovery of live microfilariae; the number of microfilariae recovered were lower than previously documented [12]. Longer durations of time may have led to parasite mortality and failure to migrate out of tissue. This may explain why some dogs in the study were positive for *C. bainae* by PCR of skin, but not by microscopy.

It is not surprising that filarioid infections were detected less commonly in client-owned dogs than shelter dogs. Owned dogs often receive more frequent veterinary care relative to shelter dogs, and therefore are more likely to be treated with compounds effective against helminths or ectoparasites [23, 24]. However, if approximately equal numbers of skin samples from pet dogs were tested, filarioid infections may have been detected in more

animals within the cohort. Not unexpectedly, we also detected *A. reconditum* and *D. immitis* infections in shelter dogs in this study; both parasites are well-documented in the USA [25, 26]. *Acanthocheilonema reconditum* infections were identified in 2.2% (5/230) of shelter dogs, and were more commonly detected by skin sedimentation of the head region or PCR of skin samples collected from the interscapular region. The prevalence of *A. reconditum* infection in dogs in Oklahoma has not been reported. *Dirofilaria immitis* infections were identified in 8.3% (19/230) of shelter dogs, and were more commonly detected by PCR of interscapular skin samples rather than detection of microfilariae by sedimentation. The overall heartworm prevalence observed in shelter dogs in this study was comparable to what has been previously reported in Oklahoma shelter dogs [27].

To the authors' knowledge, the present study is the first report of *Cercopithifilaria* sp. DNA in ticks in the USA, and suggests *R. sanguineus* (*s.l.*) may serve as vector in this region, as has been reported in other areas of the world [19]. All three of the PCR positive *R. sanguineus* (*s.l.*) were attached to one dog that was later determined to have *C. bainae* microfilariae; the female ticks were engorged, but it was not apparent for how long the male tick had been attached or if a blood meal was taken. The presence of *R. sanguineus* (*s.l.*) on a dog infected with *C. bainae* is noteworthy, and compels the authors to suspect that the parasite is cycling between this tick group and dogs in the USA. If *C. bainae* microfilariae had been ingested by immature *R. sanguineus* (*s.l.*) stages, they may have gone on to develop into infective third-stage larvae within ticks during ecdysis, as has been experimentally demonstrated in this tick group in other areas of the world [19].

Alternatively, the *Cercopithifilaria* sp. DNA amplified from the three ticks may have occurred following incidental ingestion of dermal microfilariae from the infected dog on

which they were found. This possibility is evidenced by the fact that DNA of *D. immitis* was detected in 20% (5/25) of the *A. americanum* and 11.1% (8/72) of the *R. sanguineus* (*s.l.*) tested. As *D. immitis* is adapted to mosquito intermediate hosts [13], it is extremely unlikely that developing larvae were present within ticks, but rather microfilariae were incidentally ingested in blood. Although previous studies have demonstrated molecular evidence of *C. bainae* in other tick species (*Dermacentor reticulatus* and *Ixodes ricinus*,), *R. sanguineus* (*s.l.*) is the only tick group which has been experimentally demonstrated to host developing stages of the parasite [8, 9]. In this study, *C. bainae,* was not detected in *A. americanum*, *A. maculatum*, or *D. variabilis*. However, if more specimens of each of these tick species were tested, then *C. bainae* DNA may have been detected, especially if ticks had recently fed on infected dogs.

CONCLUSION

Cercopithifilaria bainae infections in dogs in the USA are more widespread than previously thought. Here, we document infections in dogs and DNA of the parasite in engorged *R. sanguineus* (*s.l.*), the experimentally demonstrated tick vector, in Oklahoma for the first time. Due to the ubiquity of *R. sanguineus* (*s.l.*), practicing veterinarians should consider *C. bainae* infection as a differential etiology when diagnosing canine dermatitis and polyarthritis, especially for those animals with known histories of brown dog tick infestations.

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Table 1 *Cercopithifilaria bainae* infections in dogs in Oklahoma, USA

	Head Region		Interscapular Region	
Dog	Microfilaria	PCR	Microfilaria	PCR
60	-	-	-	+
85	1	-	-	-
88	-	-	-	+
105	-	+	-	-
112	1	-	4	+
220	-	-	2	+

CHAPTER IV

MOLECULAR DETECTION OF *CERCOPITHIFILARIA BAINAE* IN BROWN DOG TICKS COLLECTED FROM DOGS ACROSS THE UNITED STATES

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Lineberry, M.W., Sundstrom, K.D., Little S.E., Allen K.E., 2021. Veterinary Parasitology 299:109583. Reprinted here with permission of publisher.

ABSTRACT

Cercopithifilaria bainae is a filarioid nematode of dogs shown to use *Rhipicephalus sanguineus* sensu lato (s.l.), the brown dog tick, as the vector. Previously in the United States, *C. bainae* infections have been reported in a dog from Florida, and in dogs and ticks in Oklahoma, but data are lacking from other areas of the country. Here, we tested brown dog ticks from across the United States for *C. bainae* DNA to assess the geographic distribution of where this novel parasite may be cycling in ticks and dogs. Archival brown dog ticks were available for testing through the national tick survey *Show Us Your Ticks*. Ticks were morphologically identified, dissected, and tested by PCR to detect filarioid mitochondrial DNA. A total of 1,400 brown dog ticks were tested from 321 separate animals from 23 states, with 5.7% (80/1400) of the ticks testing positive for *C. bainae* DNA. At least one positive tick was detected in submissions from 9 states in addition to Florida and Oklahoma. *Cercopithifilaria bainae* DNA was detected in larval, nymphal, and adult stages of brown dog ticks and only in ticks removed from dogs. Of all dogs with brown dog ticks collected from them, 17.6% (55/312) were infested with at least one tick that harbored *C. bainae* DNA. Findings from this study demonstrate a wider geographic range of *C. bainae* than previously known, and that dogs are commonly infested with brown dog ticks with molecular evidence of infection.

Keywords: Brown dog tick, *Cercopithifilaria bainae*, Filarioid, Mitochondrial 12S rDNA, Mitochondrial *cox*1, *Rhipicephalus sanguineus* sensu lato

1. INTRODUCTION

Cercopithifilaria bainae is a filarioid nematode of dogs that was first described in Brazil in 1984 (Otranto et al., 2011). The adults reside in the subcutaneous tissues of dogs and female worms give rise to live microfilariae, which also live in the dermis but may disseminate away from the adults via lymphatic vessels (Otranto et al., 2012; Ramos et al., 2014a). Skin lesions in infected dogs, and more recently a 15 cm tumor in the lumbosacral region of a dog, are reported. Although commonly regarded as non-pathogenic, the variety and severity of clinical manifestations in dogs due to the parasitic infection remain poorly understood and may occasionally be attributed to other conditions (Soares et al., 2020a). Diagnosis of *C. bainae* infections can be challenging due to microfilariae residing in the skin; biopsy punch or skin snip with saline sedimentation is required to isolate and identify the microfilariae, or possibly detect DNA of *C. bainae* in skin (Otranto et al., 2012).

Transmission of *C. bainae* to dogs occurs through the blood-feeding of the tick vector, *Rhipicephalus sanguineus* sensu lato (s.l.), which is the most widespread tick species in the world (Saleh et al., 2021). All stages of *R. sanguineus* (s.l.), commonly called brown dog ticks, preferentially feed on dogs (Cortes et al., 2014; Soares et al., 2020b). Experimentally, after *R. sanguineus* (s.l.) ingest *C. bainae* microfilariae with a blood meal from infected dogs, it takes approximately 30 days for the ingested microfilariae to develop to the third-stage larvae (L3), the infective stage to canine hosts (Ramos et al., 2014a). The immature stages of *C. bainae* that have been microscopically documented in dissected nymphal and adult brown dog ticks are microfilariae and first-(L1), second-(L2), and third-(L3) stage larvae (Santos et al., 2017). While *C. bainae* has been molecularly detected in larval, nymphal, and adult

brown dog ticks, some surveys testing ticks collected from naturally infested dogs have shown that the majority of ticks harboring *C. bainae* DNA were unengorged adults (Latrofa et al., 2017; Santos et al., 2017).

Most surveys investigating *C. bainae* infections in dogs and ticks have been carried out in Brazil and Europe. Data in the United States are limited. Currently, *C. bainae* is reported in a dog from Florida and in dogs and brown dog ticks from Oklahoma, but other states have not been evaluated for presence of this parasite (Boyd et al., 2018; Lineberry et al., 2020). The logistical challenges of collecting skin samples from dogs on a national level and the invasiveness of the procedures (skin biopsy or snip) make this approach impractical. Thus, the aim of this study was to gain a better understanding of the geographic distribution of *C. bainae* infections in dogs in the United States by testing brown dog ticks collected from dogs across the region for molecular evidence of infection.

2. MATERIALS AND METHODS

2.1. Ticks

Ticks were acquired through the ongoing national tick survey (showusyourticks.org) conducted by researchers at the Oklahoma State University's College of Veterinary Medicine (OSU-CVM). In the study, enrolled veterinary practices and shelters from across the United States send in ticks collected from animals (Saleh et al., 2019). Ticks were identified as *R. sanguineus* sensu lato using entomological keys (Strickland et al., 1976; Dubie et al., 2017), preserved in 70% ethanol at -20°C, and at the time of dissection for DNA extraction, the engorgement status (unengorged or engorged) was recorded. Along with the tick specimens, information from the animals that the ticks were collected from was provided by enrollees;

these data included reported age, breed, and sex, in addition to other information gathered as previously described (Saleh et al., 2019).

Due to the high numbers of brown dog ticks occasionally collected and submitted from individual animals, a maximum of 20 ticks was tested from each animal. This allowed for all ticks and stages from most submissions to be tested, while capping the number of ticks tested from occasional submissions with high numbers of ticks removed/submitted from a single animal. Although the majority of the ticks submitted were adults, immature stages were also tested when available.

2.2. DNA Extraction and PCR

To prevent DNA contamination, tick dissections, DNA extractions, PCR amplifications, and amplicon purifications were performed in dedicated laboratory areas. To detect any contamination, no template water controls were included with all DNA extractions and PCR. Ticks were dissected and the internal contents were removed and placed in a Proteinase K and lysis buffer mixture. Extractions were then carried out based on the Illustra™ genomicPrep Mini Spin Kit protocol (GE Healthcare, Piscataway, NJ, USA).

To detect *C. bainae* mitochondrial DNA, PCR was carried out using primers Fila12SF/Fila12SR and Cbcox1F/NTR to amplify a 330-bp region of the filarioid 12S rRNA gene and a 304-bp fragment of the cytochrome c oxidase subunit 1 gene (*cox*1), respectively (Otranto et al., 2011). A positive extract control containing *D. immitis* or *C. bainae* DNA was used to ensure the PCR methods were carried out properly. To determine the genetic lineage of the *R. sanguineus* (s.l.) (tropical or temperate) of ticks testing positive for *C. bainae* DNA, a separate PCR amplifying a 340–370-bp region of the 12S rRNA gene was also performed on DNA extracts (Jones et al., 2017). All PCR reaction and thermocycler conditions were

carried out as previously described (Otranto et al., 2011; Jones et al., 2017; Lineberry et al., 2020).

PCR amplicons were run horizontally in a 2% agarose gel stained with GelRed® (Biotium, Fremont, CA, USA). Any positive amplicons of the correct base-pair size were purified with the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA). These samples were then bi-directionally sequenced at the Oklahoma State University Molecular Core Facility (Stillwater, OK) using the Sanger method. Annotated sequences were compared to those sequences available in the National Center for Biotechnology Information database (GenBank^{TM}) to determine filarioid species amplified and identify *R. sanguineus* (s.l.) genetic lineages that harbored *C. bainae* DNA.

2.3. Statistical analyses

Chi-square (χ^2) analysis (with Yate's correction in two-by-two comparisons) or Fisher's Exact Test was used to compare proportions of *C. bainae* positive ticks by state. Additionally, proportions of *C. bainae* positive ticks were compared according to tick life stage (larva, nymph, female or male) and engorgement status (unengorged or engorged). Demographic data of animals from which ticks were collected were also compared including if client-owned or residing in shelters, ages, breed description, and sex. A paired t-test was performed to compare the mean ages of dogs with *C. bainae* positive ticks to those without positive ticks. The significance level was set at $P < 0.05$ for all analyses. Statistical analyses were performed using GraphPad QuickCalcs.

3. RESULTS

3.1. Ticks tested

The brown dog ticks tested as part of the current study were collected from animals during March 2018–March 2020, with ticks being collected in all months of the year. A total of 1,400 *R. sanguineus* (s.l.) were tested from 321 separate submissions to the OSU-CVM national tick survey from animals in 23 states. Survey enrollees in states submitting the greatest numbers of ticks (Arizona, California, Oklahoma, and Texas) were from multiple locations that were geographically widespread within those states. All states with ticks available for testing and numbers tested as part of this study were as follows: Arkansas (n=26), Arizona (n=116), California (n=166), Colorado (n=38), Florida (n=63), Hawaii (n=7), Idaho (n=6), Indiana (n=3), Kentucky (n=3), Michigan (n=2), Minnesota (n=2), North Carolina (n=2), New Mexico (n=70), Nevada (n=3), New York (n=7), Ohio (n=1), Oklahoma (n=175), Oregon (n=1), South Carolina (n=26), Tennessee (n=11), Texas (n=659), Utah (n=6), and Wisconsin (n=7). There were no brown dog ticks submitted from the remaining 27 states at the time of our study.

The stages of ticks tested were made up of 605 (43.2%; CI 40.6–45.8) females, 530 (37.9%; CI 35.4–40.4) males, 231 (16.5%; CI 14.7–18.5) nymphs, and 34 (2.4%; CI 1.7–3.4) larvae. At the time of dissection, engorgement status was noted for females and immatures and the results for the proportions of engorged stages were as follows: 382/605 (63.1%; CI 59.2–66.9) females, 206/231 (89.2%; CI 84.5–92.6) nymphs, and 33/34 (97.1%; CI 83.8–100) larvae.

3.2. PCR of ticks

Of the 1400 *R. sanguineus* (s.l.) tested, 80 (5.7%; CI 4.6–7.1) were positive for *C. bainae* DNA by *12S* PCR. The breakdown of the states with *C. bainae* positive ticks can be found in Table 1; tick stages and number of animals (submissions) from which positive ticks were collected are also indicated. Of the 23 states with ticks available for testing, 11 (47.8%) had at least one *C. bainae* positive tick. Positive ticks were submitted from Arkansas, Arizona, California, Colorado, Florida, Kentucky, New Mexico, Oklahoma, Texas, Wisconsin, and Utah. California and Oklahoma had significantly fewer PCR positive brown dog ticks than the other states with PCR positive ticks ($P < 0.001$). Positive ticks included 43/605 (7.1%; CI 5.3–9.5) females, 27/530 (5.1%; CI 3.5–7.3) males, 7/231 (3.0%; CI 1.4–6.2) nymphs, and 3/34 (8.8%; CI 2.3–23.7) larvae (Table 1). When comparing PCR results of the different stages and sexes of *R. sanguineus* (s.l.), there were significantly more positive female ticks than nymphs ($P = 0.0326$). There was not a significant difference in positive ticks that were visibly engorged (immatures or females) versus not at the time of extraction.

The 80 ticks testing positive for *C. bainae* DNA by *12S* PCR harbored sequences that were 97–100% homologous to GenBank accessions KX156956 and KF381408 originating from Brazil and Italy, respectively. These sequences were 95–100% similar to those collected previously from ticks and dogs in Oklahoma (Lineberry et al., 2020). The same 80 ticks testing positive by *12S* PCR were then tested by *cox*1 PCR to obtain further phylogenetic information about the *C. bainae* detected. Of these ticks, 52 (65%; CI 54.0–74.6) were PCR positive by *cox*1 PCR. The *cox*1 sequences of *C. bainae* were

95–100% similar to accessions MF479726, JQ305156, and JQ305157, originating from the Mediterranean.

Brown dog ticks harboring *C. bainae* DNA were tested to determine genetic lineage. Lineage was able to be determined for ticks collected from 51/55 dogs with *C. bainae* positive ticks. This included 44 (86.3%) dogs infested with the temperate lineage and 7 (13.7%) dogs infested with the tropical lineage.

3.3. Animals with ticks tested (i.e. submissions)

The total ticks tested for *C. bainae* as part of this study were pulled from 321 separate submissions of brown dog ticks identified through the national tick survey (Saleh et al., 2019). Each submission was from an individual animal (i.e. 321 animals had 1–20 ticks tested). On average, 4.36 (CI 3.79–4.93) ticks were tested from each submission. Animals from which brown dog ticks were collected included 312 dogs, 7 cats, and 2 other or not recorded. All dogs and cats were primarily patients visiting veterinary clinics (n=235 animals; 73.7%) versus housed in shelters (n=84 animals; 26.3%). The average age of dogs in this population was 3.2 years (CI 2.81–3.58 years) and the average age of cats was 0.9 years (CI 0–2.55 years). The sexes of dogs from which ticks were collected were evenly split, with 151 (49.8%) males and 152 (50.2%) females. All AKC breed groups were represented in the dog population. Six (85.7%) male cats and 1 (14.2%) female cat had brown dog ticks removed from them and tested as part of this study.

3.4. Animals with PCR positive ticks

Brown dog ticks testing positive for *C. bainae* DNA originated from 55 separate submissions; all of these PCR positive ticks were collected and submitted from dogs (Table 1). Thus, 17.6% (55/312; CI 13.8–22.3) of dogs from which all brown dog ticks were tested

as part of this study had at least one tick that was PCR positive for *C. bainae* DNA. The number of PCR positive ticks collected from individual dogs ranged from 1–6 ticks. There was not a statistical difference when comparing the mean ages of the dogs with *C. bainae* positive ticks to those with *C. bainae* negative ticks. The number of ticks tested from dogs in different age brackets were as follows: <1 year (n=375), 1–2 years (n=311), 2–5 years (n=315), and 5+ years (n=226). When comparing age brackets, dogs in the range of 2–5 years had significantly more PCR positive ticks than dogs less than 1 year or greater than 5 years of age ($P \leq 0.0046$). There was no significant difference in proportions of PCR positive ticks by dog breed description (specified or mixed) or sex.

4. DISCUSSION

Surveys of *C. bainae* infections in brown dog ticks and dogs have primarily been carried out in Europe and Brazil (Ramos et al., 2014b; Ramos et al., 2016). Although a case report of *C. bainae* infection in a dog from Florida and a survey of dogs and ticks in Oklahoma have shown this emerging parasite is present in the United States, we do not yet know how geographically widespread or prevalent infections are (Boyd et al., 2018; Lineberry et al., 2020). Here, we conducted the first national survey looking for *C. bainae* DNA in brown dog ticks to gain a better understanding of where the emerging parasitic infection may be enzootic in dogs. In this study, we tested ticks from 23 states and detected *C. bainae* DNA in 9 states in addition to Florida and Oklahoma – states that span the East to West Coast. With molecular evidence of *C. bainae* in ticks collected from dogs across such a large geographic range, the authors suspect that the parasite may have been established decades ago but has gone undetected until recently.

We detected DNA of *C. bainae* in 5.7% of the brown dog ticks tested as part of the study. Based on molecular evidence alone, however, the authors cannot state whether brown dog ticks that tested PCR positive for *C. bainae* harbored developing stages of the parasite (L_1, L_2, L_3) or tested positive for DNA due to incidental ingestion of microfilariae with blood meal from an infected dog. Interestingly, there were no significant differences in proportions of PCR positive unengorged versus engorged stages, suggesting that more developed stages of *C. bainae* may have been present in the unengorged ticks, and that we were not merely detecting DNA of recently ingested microfilariae. The *C. bainae* sequences we obtained from ticks from other states were highly similar (95–100%) to those we amplified from ticks and dogs in a previous study in Oklahoma (Lineberry et al., 2020).

In Europe, larval brown dog ticks are not thought to play a role in the transmission of *C. bainae* to dogs. Nymphs are considered key in the life cycle; transstadial transmission of *C. bainae* from nymphs (fed on infected dogs) to adults has been demonstrated (Ramos et al., 2014b; Latrofa et al., 2017; Santos et al., 2017). We detected *C. bainae* DNA in all stages of ticks, including a few larvae, indicating that brown dog ticks may acquire *C. bainae* as larvae and support parasite development through molting to the nymphal and possibly adult stage. The finding of a significantly greater number of PCR positive female ticks than nymphs could be due to the fact the females had fed twice before (as larvae and nymphs), thus increasing the likelihood of acquiring the parasite (Aktas, 2014; Boulanger et al., 2019). Although certain infectious agents are more likely to be transmitted by brown dog ticks belonging to particular operational typing units, *C. bainae* DNA has been detected in both temperate and tropical lineages (Lineberry et al., 2020; Soares et al., 2020b). This is supported in the current study through the detection of *C. bainae* DNA in both temperate and

tropical lineages of brown dog ticks, further indicating that the parasite is well adapted to this tick group and is likely as geographically widespread (Dantas-Torres 2010; Jones et al., 2017; Saleh et al., 2021).

Overall, 17.6% of dogs from across the United States were infested with at least one *C. bainae* positive tick, possibly indicating that the filarioid is widespread but under-recognized in dogs in the region. In this study, the canine population does not represent all dogs in the geographic area surveyed, as we tested ticks collected from dogs, and thus these dogs had established tick infestations. However, dogs without observable ticks may still harbor *C. bainae* infections acquired previously (Lineberry et al., 2020). To the authors' knowledge, a significant difference in *C. bainae* infection status between dog age groups has not been reported, but younger dogs are more likely to be infested with brown dog ticks (Dantas-Torres, 2010). Here, we found dogs in the age range of 2–5 years of age had significantly more positive ticks than those <1 year old and >5 years old.

Travel and treatment history was not recorded for any animals with brown dogs tested for *C. bainae* DNA as part of this study. It is possible that all dogs with positive ticks were relocated from Florida and/or Oklahoma, where *C. bainae* has been previously documented (Boyd et al., 2018; Lineberry et al., 2020). Travel alone, however, seems unlikely to account for the total number of dogs from 9 additional states that were infested with at least one PCR positive brown dog tick. Brown dog ticks harboring *C. bainae* may also infest dogs in states not considered in this study, suggesting the canine filarioid is even more widespread than reported herein, but ticks from those areas were not available for testing.

5. CONCLUSION

This is the first national survey testing brown dog ticks for *C. bainae* DNA. This emerging parasite was recently recognized as enzootic in Florida and Oklahoma. Here, we demonstrate a greater geographic distribution of the parasite by documenting *C. bainae* DNA in ticks in nine additional states. As brown dog ticks thrive on dogs, and are therefore found anywhere there are dogs, *C. bainae* is possibly cycling between dogs and ticks across North America. Infections are often regarded as non-pathogenic, but clinical manifestations in dogs have been reported. Much remains to be learned about this emerging parasite. More survey work is needed to determine the potential geographic distribution and clinical impact *C. bainae* has on canine populations in the United States.

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Table 1. States and dogs with *Cercopithifilaria bainae* PCR positive brown dog ticks.

States with PCR positive ticks[A]	PCR positive ticks (%; 95% CI) (stages)	Dogs with PCR positive ticks (%; 95% CI)
Arkansas	3/26 (11.5%; 3.2–29.8) (2 F, 1 M)	3/7 (42.9%; 15.8–75)
Arizona	16/116 (13.8%; 8.6–21.3) (13 F, 3 M)	8/48 (16.7%; 8.4–29.8)
California	2/166 (1.2%; 0.1–4.6) (1 L, 1 N)	1/29 (3.4%; <0.01–18.6)
Colorado	6/38 (15.8%; 7.1–30.8) (1 F, 5 M)	2/7 (28.6%; 7.6–64.8)
Florida	4/63 (6.3%; 2.1–15.7) (1 F, 3 M)	2/19 (10.5%; 1.7–32.6)
Kentucky	1/3 (33.3%; 5.6–79.8) (1 F)	1/3 (33.3%; 5.6–79.8)
New Mexico	7/70 (10%; 4.7–19.5) (1 L, 1 N, 2 F, 3 M)	6/14 (42.9%; 21.3–67.5)
Oklahoma	2/175 (1.1%; 0.1–4.3) (1 N, 1 F)	2/36 (5.6%; 0.6–19.1)
Texas	35/659 (5.3%; 3.8–7.3) (1 L, 4 N, 20 F, 10 M)	27/120 (22.5%; 15.9–30.8)
Utah	3/6 (50%; 18.8–81.2) (2 F, 1 M)	2/4 (50%; 15–85)
Wisconsin	1/7 (14.3%; 0.5–53.4) (1 M)	1/5 (20%; 2–64)
Total	80/1329 (6%) (3 L, 7 N, 43 F, 27 M)	55/292 (18.8%)

[A] The 71 ticks submitted from Hawaii, Idaho, Indiana, Michigan, Minnesota, North Carolina, Nevada, New York, Ohio, Oregon, South Carolina, and Tennessee were PCR negative.

CHAPTER V

DIVERSITY AND GEOGRAPHIC DISTRIBUTION OF RICKETTSIAL AGENTS IDENTIFIED IN BROWN DOG TICKS FROM ACROSS THE UNITED STATES

Lineberry, M.W., Grant, A.N., Sundstrom, K.D., Little, S.E., Allen, K.E. To be submitted to Ticks and Tick-borne Diseases

ABSTRACT

Rhipicephalus sanguineus sensu lato, or brown dog ticks, transmit a variety of pathogens of veterinary and public health importance globally. Pathogens vectored by brown dog ticks and identified in the United States include *Babesia canis vogeli*, *Ehrlichia canis*, and several spotted fever group *Rickettsia* spp. (SFGR). Due to the challenge of collecting canine blood samples nationwide to screen for exposure to these pathogens, we took an indirect approach and tested brown dog ticks for molecular evidence of infection. Brown dog ticks (n=681) collected from dogs and cats across the nation were tested by separate PCR assays detecting *Babesia* spp., *E. canis*, and SFGR. While no *Babesia* sp. was found, we identified rickettsial agents in 3.5% (24/681; 95% CI 2.4–5.2) of the ticks. Pathogens detected in ticks include 1 *E. canis*, 3 *Rickettsia amblyommatis*, 11 *R. massiliae*, 3 *R. monacensis*, 5 *R. montanensis*, and 1 undefined *Rickettsia* species. These data demonstrate a wider geographic distribution of *R. massiliae* than previously known, and to the authors' knowledge, reports *R. monacensis* in brown dog ticks for the first time. Due to the close association that brown dog ticks have with domestic dogs and humans, more research is needed to understand the full array of organisms, some of which are zoonotic, potentially transmitted by this widespread tick complex.

Keywords: Brown dog tick, Canine, *Ehrlichia canis*, *Rickettsia* spp.

1. INTRODUCTION

Rhipicephalus sanguineus sensu lato, commonly called brown dog ticks, are found in most parts of the world. Unlike other tick species, *R. sanguineus* thrive indoors, commonly infesting homes and kennels. Although all motile stages of this tick complex preferentially feed on dogs, they also feed on other species including humans (Dantas-Torres, 2010; Saleh et al., 2021). Brown dog ticks are important vectors of canine disease agents across the globe, some of which are zoonotic and are of public health concern (Dantas-Torres and Otranto, 2015). In the United States, brown dog ticks transmit *Babesia canis vogeli, Ehrlichia canis*, *Rickettsia rickettsii*, considered possible vectors of *B. conradae, B. gibsoni*, *Cercopithifilaria bainae*, *Hepatozoon canis,* and other spotted fever group *Rickettsia* spp. (SFGR), and are implicated as secondary vectors of *E. chaffeensis* and *E. ewingii* (Yamane et al., 1993; Ndip et al., 2007; Wikswo et al., 2007; Little, 2010; Ndip et al., 2010; Baneth, 2018; Lineberry et al., 2021; Saleh et al., 2021).

Babesia canis vogeli is a protozoan that infects erythrocytes of dogs. Infections with this parasite may cause mild to moderate disease including anemia, fever, hemoglobinuria, lethargy, and vomiting (Lira–Amaya et al., 2017; Baneth et al., 2018). *Ehrlichia canis* is an obligate intracellular bacterium that infects monocytes of canine hosts and rarely humans (Little, 2010; Almazán et al., 2016). The disease caused in dogs, canine monotropic ehrlichiosis (CME), can be mild to fatal. Clinical signs observed in dogs with acute or chronic infections include anorexia, bleeding diatheses, lethargy, lymphadenopathy, myalgia, and splenomegaly (Little, 2010). *Rickettsia* spp. are intracellular bacteria that can be highly pathogenic. In North America, *Dermacentor* spp. are thought to be the primary vector for *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF). However, brown

dog ticks have been recognized as a vector in Arizona and California (Wikswo et al., 2007; Yaglom et al., 2018), and this relationship is well known throughout Latin America (Labruna et al., 2011). Additionally, *R. massiliae* has been reported in brown dog ticks from Arizona, California, and Virginia. Both of these *Rickettsia* spp. can cause disease in dogs and humans (Fornadel et al., 2013; Yaglom et al., 2018). Due to the challenges associated with obtaining canine blood samples nationwide to test for these various pathogens, we screened brown dog ticks collected across the United States as an indirect way of determining which canine and zoonotic infectious agents these ticks may carry in different geographic regions.

2. MATERIALS AND METHODS

2.1. Ticks

Ticks were collected from dogs and cats by veterinarians and staff and sent to researchers at Oklahoma State University's College of Veterinary Medicine (OSU-CVM) through a national tick survey (showusyourticks.org) as previously described (Saleh et al., 2019). Ticks in the current study were morphologically identified as *R. sanguineus* s.l. using standard entomological keys (Clifford et al., 1961; Strickland et al., 1976). Ticks and DNA extractions of ticks were stored at -20°C until dissection and PCR, respectively.

Due to the high submission numbers of ticks from some individual animals or from certain geographic locations (within Arizona, California, Oklahoma, and Texas), up to 5 ticks were tested per animal and up to 50 ticks were tested per city. Ticks were selected for testing at random using a random number generator (www.calculator.net). If present, multiple stages of ticks were tested from a single animal.

2.2. Molecular assays

Ticks were dissected and all internal contents were removed as previously described (Jones et al., 2017), with some modifications, including an incubation of 10 minutes per extraction kit instructions and immature ticks were dissected instead of pulverized; extractions were carried out using either the Illustra™ genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA) or QIAamp DNA Blood Kit (Qiagen, Germantown, MD, USA) protocol. Nested PCR assays were performed to detect an *18S rRNA* region of *Babesia* spp., a *16S rRNA* region of *E. canis*, and *17-kDa* gene targets of *Rickettsia* spp. as previously described (Chen et al., 1994; Dawson et al., 1996; Heise et al., 2010; Kim et al., 2013; Qurollo et al., 2017). Ticks testing positive for zoonotic and/or emerging *Rickettsia* spp. were re-tested by separate assays amplifying two additional *Rickettsia* spp. gene targets (*gltA* and *ompA*) to confirm molecular identity as previously described (Eremeeva et al., 2006; Summer et al., 2007). A negative control was included at each step (extraction, primary PCR, and secondary PCR) to confirm samples were not contaminated. To ensure PCR methods were carried out properly, a known positive control was used for each PCR, including *B. conradae*, *E. canis*, and *Rickettsia amblyommatis*.

All PCR products were electrophoresed through a 2% agarose gel using standard methods. Positive amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and bi-directionally sequenced using the Sanger method at the Oklahoma State University Molecular Core Facility (Stillwater, OK). Chromatograms were visually inspected and sequences annotated. Sequences were then compared to those available in the National Center for Biotechnology Information Database (GenBank™).

2.3. Statistical Analyses

Statistical analyses were performed using QuickCalcs-GraphPad (www.graphpad.com). For all analyses, the p-value was set at 0.05. Fisher's Exact Test was performed to compare the significance of positive ticks and submissions by state, geographic region previously described by Little et al., 2009 (Midwest, Northeast, South, and West), and tick stage (female, male, and nymph). Confidence intervals (CI) were calculated for all proportions.

3. RESULTS

3.1. Ticks

A total of 681 brown dog ticks (335 females, 281 males, 65 nymphs) were tested from 306 animals, including 300 dogs and 6 cats. Ticks were collected March 2018–September 2021, with submissions occurring in all months of the year. Ticks were submitted from all 4 geographic regions and 25 of 50 states.

No *Babesia* spp. DNA was amplified in these ticks. Rickettsial agents were amplified from 3.5% (24/681; 95% CI 2.4–5.2) of the ticks. *Ehrlichia canis* was identified in 0.2% (1/681; 95% CI 0–0.01) of ticks; sequence was 99.7% identical to the Oklahoma strain of *E. canis* (NR_118741) reported in GenBank. Spotted fever group *Rickettsia* spp. were detected by *17-kDa* sequence in 3.4% (23/681; 95% CI 2.2–5) of ticks including 3 *R. amblyommatis*, 10 *R. massiliae*, 1 *R. monacensis*, 5 *R. montanensis*, and one undefined *Rickettsia* and were 99.5–100% identical to accessions MN885532, KT032120, LC379454, DQ402377, and

HF935072, respectively. Sequences of rickettsial *glta* and *ompA* from ticks with *R. massiliae* by *17-kDa* were 100% identical to those reported from *R. massiliae* (MH064440 and KU498298, respectively). Sequences of rickettsial *glta* from ticks with *R. monacensis* by *17-kDa* were 99.7% identical to that reported from *R. monacensis* (MH589997), and sequences of rickettsial *ompA* from ticks with *R. monacensis* by *17-kDa* were 95.7% identical to that reported from *R. monacensis* (LC388792). Additionally, a single tick tested positive for *R. massiliae* and 2 ticks tested positive for *R. monacensis* by the *17-kDa* gene target, but neither of the other gene targets were amplified in these organisms. No co-infections were identified in individual ticks. Rickettsial agents were amplified from 4.2% (14/335; 95% CI 2.5–7) of female ticks and 3.6% (10/281; 95% CI 1.9–6.5) of male ticks. The nymphs tested were not positive for any pathogens. *Rickettsia massiliae* and *R. monacensis* sequences obtained from this study were deposited in GenBank as ON843654– ON843660.

3.2. Submissions

Pathogens were only detected in brown dog ticks removed from dogs. Of the dogs, 6.7% (20/300; 95% CI 4.3–10.1) had at least one PCR positive tick. Two dogs had three positive ticks removed from each of them; all three ticks from one dog were positive for *R. massiliae* and two ticks and one tick from the other dog were positive for *R. massiliae* and *R. montanensis,* respectively. When comparing states, Idaho and Colorado had significantly more positive submissions than Arizona, California, and Florida ($p \leq 0.05$).

3.3. Geographic distribution

Positive ticks were collected in two of four regions: 3.5% (13/375; 95% CI 2–5.9) of ticks from the South and 3.9% (11/279; 95% CI 2.1–7) of ticks from the West (Table 1).

When examining data according to state, 36% (9/25; 95% CI 20.2–55.6) of states had at least one positive brown dog tick submitted. The pathogens and geographic distribution are detailed in Figure 1. Idaho had significantly more positive ticks than Arkansas, Arizona, California, Florida, New Mexico, New York, Oklahoma, South Carolina, Texas, and Wisconsin ($p \leq 0.0455$); Colorado had significantly more positive ticks than Arizona, California, Florida, New Mexico, and Texas ($p \leq 0.0361$); California had significantly fewer positive ticks than Kentucky and North Carolina ($p = 0.0435$).

4. DISCUSSION

Determining the geographic distribution of known and emerging pathogens is important to canine and public health in the United States. While canine serologic testing has been performed at a national level for many brown dog tick pathogens (Birkenheuer et al., 2005; Beall et al., 2012), it can be challenging to obtain species-level identification for some organisms based on serology alone; *Ehrlichia* spp. and *Babesia* spp. can cross-react or have lower sensitivity depending on the stage of infection (acute or chronic) when performing antibody tests such as immunofluorescent assays (IFA) and enzyme linked immunosorbent assays (ELISA) (Beall et al., 2012; García-Quesada et al., 2021). Cross-reactivity commonly occurs when testing for SFGR, so there is a lack of certainty about which species are responsible for the antibodies detected (Moncayo et al., 2010; Fordanel et al., 2013; Drexler et al., 2016). Testing ticks is an alternative way to obtain species-level surveillance data on tick-borne pathogens (Dantas-Torres, 2010). Here we tested brown dog ticks for select agents, including *Babesia* spp., *E. canis*, and SFGR, as an indirect approach to assessing

where these organisms may be circulating in the United States and to identify emerging pathogens.

Babesia gibsoni and *B. c. vogeli* are present in dogs in some regions of the United States (Birkenheuer et al., 2005; Cannon et al., 2016), but this study did not identify any *Babesia* spp. in the brown dog ticks tested. Prevalence of *Babesia* spp. in brown dog ticks in other areas of the world tends to be low (<2%) (Chao et al., 2017; Prakash et al., 2018; Onyiche et al., 2021). It is possible that certain strains of ticks have greater vectorial capacity and, similar to what is known about transmission of *B. gibsoni* in the United States, direct transmission may play a role in the dispersal of other *Babesia* spp. (Baneth et al., 2018; Prakash et al., 2018). In the present study, *E. canis* was identified in a single male tick from Galveston, Texas. While *E. canis* has been identified from pools of ticks removed from a dog in Oklahoma known to be infected with *E. canis* (Murphy et al., 1998), and serologic testing shows antibodies to *E. canis* are commonly detected in dogs from some focused regions of the southcentral and southwestern United States (Beall et al., 2012), this is, to our knowledge, the first report of a randomly sampled, individual tick testing positive by PCR for *E. canis* in the United States. Of the *Ehrlichia* spp. infecting dogs in the United States, *E. canis* appears the least common (Beall et al., 2012). Serologic or PCR testing of the dog for *Ehrlichia* spp. was not performed at the time of visit, so infection status of the dog was not known, but only one tick (out of 5) tested positive for *E. canis.* The prevalence of *E. canis* in this study is lower than reports from brown dog ticks in other areas of the world, which range from 2.3–6% (Aguiar et al., 2007; Ndip et al., 2010), but have been reported as high as 51.5% when ticks are removed from dogs harboring active infections (Koh et al., 2016).

Testing the tick vector can be especially informative for understanding distribution of SFGR; serologic assays for SFGR cross-react among *Rickettsia* spp., making it difficult to determine the agent responsible from antibody tests alone, and PCR assays for SFGR on peripheral blood of hosts have poor sensitivity (Kidd et al., 2008; Moncayo et al., 2010). Molecular testing of vectors allows identification of specific *Rickettsia* species that may be circulating in a given area (Saito et al., 2019; Duncan et al., 2021). While there have been case reports and localized studies identifying *Rickettsia* spp. in brown dog ticks in the United States, data on a national level are lacking (Fornadel et al., 2013; Yaglom et al., 2018).

In other areas of the world, *R. massiliae* appears to be the predominant SFGR detected in brown dog ticks, with a prevalence of 4.7% and 18% in ticks in Morocco and Spain, respectively (Márquez et al., 2008; Sarih et al., 2008). In the present study, *R. massiliae* was the most commonly identified SFGR with 11 ticks testing positive, demonstrating greater geographic distribution in the United States than previously known. This agent has been reported previously in dogs and/or brown dog ticks in Arizona, California, and Virginia (Eremeeva et al., 2006; Beeler et al., 2011; Fornadel et al., 2013); the present study identified *R. massiliae* in ticks submitted from 6 additional states, including Colorado, Idaho, New Mexico, North Carolina, Oklahoma, and Texas. To the authors' knowledge, *R. monacensis* has only been reported in the United States once from an *Ixodes* sp. larva collected from a migratory bird in Galveston, Texas. Typically, this pathogen is associated with *I. ricinus* and distributed in Africa, Asia, and Europe (Cohen et al., 2015). Here, we found sequences of *R. monacensis* in 3 brown dog ticks submitted from Kentucky, Oklahoma, and Texas.

While *Amblyomma americanum* are the primary host for *R. amblyommatis* in the United States, this pathogen has also been identified in *Dermacentor* spp. and *Rhipicephalus* spp., and thus it is not surprising that we identified this pathogen in 3 *R. sanguineus* (Snellgrove et al., 2021). *Dermacentor* spp. are thought to be the primary vector for *R. montanensis*, but this SFGR has also been reported in *A. maculatum* and *R. sanguineus* collected from companion animals in Georgia (Duncan et al., 2021; Snellgrove et al., 2021; Stanley and Rhodes, 2021). Here, we also identified *R. montanensis* in 5 brown dog ticks; this finding could be due to the ticks feeding on infected dogs and more research is needed to understand the competence of brown dog ticks as vectors. We did not detect *R. rickettsii* in any ticks in the present study. This absence was surprising given this pathogen has been reported as documented in 1.6–3% of brown dog ticks tested in Arizona and California (Demma et al., 2005; Wikswo et al., 2007). Reported cases of RMSF have increased in the United States in the past two decades (CDC, 2021). However, this change has been attributed to cross-reactivity in serologic assays used for RMSF diagnosis, and other *Rickettsia* spp. are considered responsible for increased reports of disease (Moncayo et al., 2010; Drexler et al., 2016).

All ticks with detectable pathogens were adults, a finding that may be due to adults having an extra life stage to take a blood meal compared to immature ticks, increasing the possibility of acquiring pathogens (Dantas-Torres, 2010; Saleh et al., 2021). We cannot prove that brown dog ticks act as a vector for pathogens identified in this study, but the results demonstrate the variety of disease agents that may be circulating in certain geographic regions. Therefore, our findings are of importance to veterinary and public health (Dantas-Torres and Otranto, 2015).

5. CONCLUSION

Brown dog ticks are the most geographically widespread tick species complex, and although primarily associated with dogs, occasionally parasitize humans. As domestic dogs are typically closely associated with humans, brown dog tick populations supported by dogs pose a direct risk to human health. Results from this study indicate that brown dog ticks harbor a variety of pathogens in the United States, some of which are zoonotic. More research is needed to understand the role brown dog ticks play in pathogen transmission, and how known and novel infectious agents affect canine and human health.

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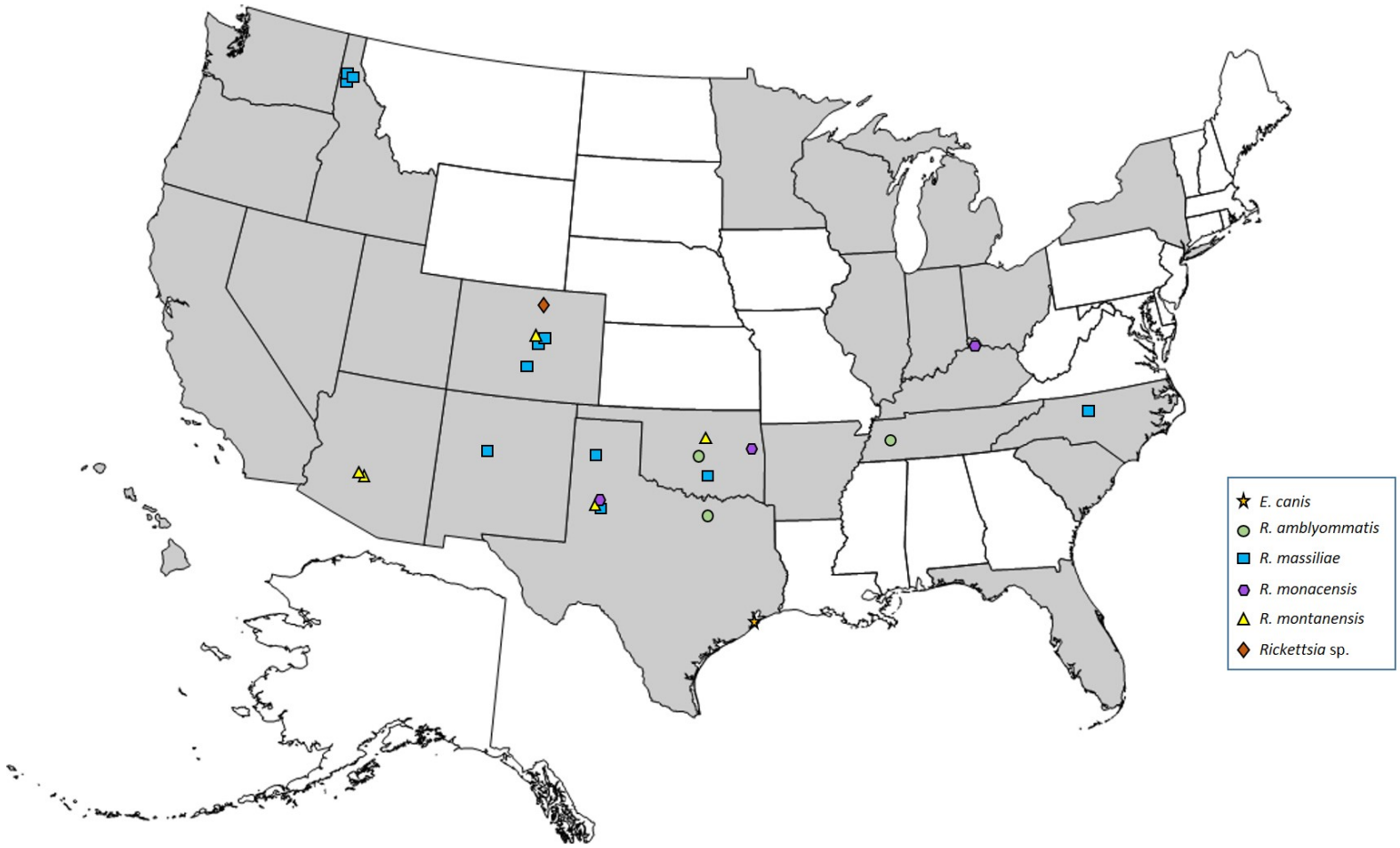
Table 1. Pathogens detected by at least one gene target in brown dog ticks in the United States by geographic region.

	E. canis **(%)**	***R. ambloymmatis*** **(%)**	***R. massiliae*** **(%)**	***R. monacensis*** **(%)**	***R. montanensis*** **(%)**	***Rickettsia* sp.** **(%)**	**Total (%)**
Midwest (n=20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Northeast (n=7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
South (n=375)	1 (0.3)	3 (0.8)	4 (1.1)	3 (0.8)	2 (0.5)	0 (0)	13 (3.5)
West (n=279)	0 (0)	0 (0)	7 (2.5)	0 (0)	3 (1.1)	1 (0.4)	11 (3.9)
Total (n=681)	1 (0.2)	3 (0.4)	11 (1.6)	3 (0.4)	5 (0.7)	1 (0.2)	24 (3.5)

Figure 1. Map of states from which brown dog ticks were submitted (grey shading) and specific geographic locations of ticks with pathogens detected by at least one gene target.



CHAPTER VI

CONCLUSIONS

Brown dog ticks transmit a variety of organisms globally, including bacteria, nematodes, and protozoa (Yamane et al., 1993; Wikswo et al., 2007; Little, 2010; Dantas-Torres and Otranto, 2015; Baneth et al., 2018; Saleh et al., 2021). While there was a case report of *Cercopithifilaria bainae* in a Florida dog, data are lacking from dogs and ticks across the United States that inform our understanding of the prevalence and geographic distribution of *Cercopithifilaria* spp. in the region (Boyd et al., 2019). Additionally, our understanding of the brown dog tick's role in transmission of other tick-borne pathogens, especially zoonotic rickettsial species including *Rickettsia massiliae* and *R. rickettsii*, remains deficient (Wikswo et al., 2007; Yabsley et al., 2008; Fornadel et al., 2013). Testing brown dog ticks can provide data on where different infectious agents may be circulating in a given region without the invasiveness or challenges of collecting samples from canines across a large geographic range. The overarching goals of the research presented within this dissertation aim to help address the above knowledge gaps by testing dogs and brown dog ticks in the United States for known and emerging infectious agents.

STUDY 1 (CHAPTER III)

The goal of the first study was to determine if *Cercopithifilaria* spp. were present in canine and tick populations in Oklahoma. Current survey data are available for canine and tick populations in other areas of the world, but there is only one case report of *C. bainae* in the United States that occurred in Florida in a young dog with no travel history (Gabrielle et al., 2014; Ionică et al., 2014; Latrofa et al., 2014; Otranto, 2015; Ramos et al., 2016; Boyd et al., 2019; Bezerra-Santos et al., 2022; Sazmand et al., 2022). Our study outlined in Chapter 3 was the first to document *C. bainae* in dogs from Oklahoma and from brown dog ticks within the United States. Of the 230 shelter dogs tested, 2.6% were positive for *C. bainae* microfilariae by saline sedimentation and/or amplification of the *12S rRNA* mitochondrial gene. While multiple tick species were collected from these dogs, only *R. sanguineus* s.l. tested positive for *C. bainae* DNA. The results of this research demonstrate that *C. bainae* may be more widespread within the United States than previously known and should be considered in diagnostic work-ups of dogs with dermatitis and polyarthritis.

STUDY 2 (CHAPTER IV)

In the second study, our aim was to determine if *C. bainae* was circulating in other areas of the United States by testing brown dog ticks for molecular evidence of infection. Brown dog ticks are the only tick species known to transmit canine *Cercopithifilaria* spp., and therefore testing them can be a non-invasive way to gain insight into the geographic distribution of *Cercopithifilaria* within the United States (Dantas-Torres et al., 2012; Ramos et al., 2014; Santos et al., 2017; Bezerra-Santos et al., 2022). Our study outlined in Chapter 4

was the first survey in the United States testing brown dog ticks for *Cercopithifilaria* spp. DNA at a national level. Of the 23 states with brown dog tick submissions, we identified *C. bainae* positive ticks collected from animals in 11 (47.8%) states. Of the brown dog ticks tested, 5.7% (80/1400) were PCR positive for *C. bainae* and 17.6% (55/312) of the dogs with brown dog ticks removed had at least one *C. bainae* positive tick. This research indicates that *C. bainae* may be more widespread in the United States than previously thought. More research is needed to understand the prevalence of *C. bainae* in dogs and the impact of these infections on dogs.

STUDY 3 (CHAPTER V)

The aim of the final study was to identify other infectious agents present in brown dog ticks sampled from across the United States. While *Babesia canis vogeli* and *Ehrlichia canis* are reported in dogs throughout the United States, and brown dog ticks are the known vector, prevalence data in ticks for these two organisms are lacking (Beall et al., 2012; Qurollo et al., 2014; Baneth, 2018; Birkenheuer et al., 2020). Additionally, various spotted fever group *Rickettsia* spp. (SFGR) have been identified in brown dog ticks, some of which are emerging and of zoonotic concern (Wikswo et al., 2007; Yabsley et al., 2008; Fornadel et al., 2013). To the authors knowledge, our study described in Chapter V is the first in the United States to document *E. canis* in randomly sampled individual brown dog ticks (i.e. not collected from a dog known to be infected with *E. canis*), to detect *R. monacensis* in brown dog ticks, and to document a broader geographic distribution of *R. massiliae* than previously known. Rickettsial agents were identified in 3.5% (24/681) of the ticks tested and distributed

across 9 states. More research is needed in the United States to understand the impacts these emerging and zoonotic rickettsial organisms have on canine and human populations.

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