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Diversity of *Hepatozoon* species in naturally infected dogs in the southern United States

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Abstract

Hepatozoon americanum is a protozoan that causes American canine hepatozoonosis (ACH) in the southern United States; *Hepatozoon canis*, the causative agent of canine hepatozoonosis in Africa, Asia, Europe, and South America, has not previously been definitively identified in dogs in the United States. To characterize the diversity of *Hepatozoon* spp. in domestic dogs from Oklahoma, blood samples collected from dogs residing in an endemic area of the state, clinical cases presented to veterinarians with symptoms of ACH, and dogs housed at a local shelter were evaluated by a nested PCR designed to amplify a variable region of the 18S rRNA gene of blood ampicomplexa, including *Hepatozoon* spp. *Hepatozoon* sequences recovered from a dog from an area where ACH is endemic, from clinically ill dogs, and from one shelter dog most closely resembled *H. americanum*. However, two other shelter dogs had evidence of infection with *H. canis* or a closely related organism. A subsequent review of real-time PCR results from the Molecular Diagnostics Laboratory at Auburn University revealed that the majority of samples submitted from dogs from across the United States which tested positive for *Hepatozoon* spp. had *H. americanum*. However, some submissions were also found which contained DNA sequence of *H. canis*. Mixed *H. americanum* and *H. canis*-like infections also were detected. Our data suggest that *H. americanum*, *H. canis*, as well as *H. canis*-like organisms are present and may cause disease in dogs in the southern U.S.

1. Introduction

Hepatozoon americanum was officially recognized as the causative agent of American canine hepatozoonosis (ACH) in 1997 (Vincent-Johnson et al., 1997). The organism was initially thought to be a more pathogenic strain of *Hepatozoon canis* but was later recognized as a distinct species based on 18S rRNA gene sequence, infection in the tick definitive host, *Amblyomma maculatum*, as well as the pathogenesis and severity of disease induced in infected dogs (Vincent-Johnson et al., 1997; Mathew et al., 2000; Panciera et al., 2001; Ewing et al., 2002). Hepatozoonosis in North America was first recognized in Texas, but the disease has since been

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reported in dogs from several other states, including Louisiana, Alabama, Georgia, Florida, Tennessee and Oklahoma (Cummings et al., 2005).

Dogs become infected with *H. americanum* when they ingest ticks harboring infective oocysts, and the infection is thought to occur during the act of grooming or ingestion of tick-infested prey (Ewing et al., 2003); infection may also follow ingestion of cystozoites in rodent paratenic hosts (Johnson et al., 2007a). The only known invertebrate host experimentally shown to harbor and transmit the agent of ACH is the Gulf Coast tick, *A. maculatum* (Mathew et al., 2000; Ewing et al., 2002), a species originally found in the United States primarily along the Gulf Coast but now established in several other areas including eastern and central Oklahoma and Kansas (Barker et al., 2004). Adults of this tick have been documented to feed on a variety of mammals and birds (Teel et al., 1998; Goddard and Paddock, 2005).

Dogs with ACH often present with extreme neutrophilia, reluctance to move and generalized pain, fever, lethargy, weight loss and ocular discharge (Ewing and Panciera, 2003). Parasitemia, recognized as gamonts in blood smears, is generally low in cases of ACH, but characteristic mucopolysaccharide "onion skin cysts" in striated muscle tissue, which contain merogonic stages of the parasite within a host leukocyte, are consistently apparent upon histological examination of patient muscle biopsies (Ewing and Panciera, 2003; Cummings et al., 2005). Also, by an unexplained mechanism, periosteal proliferation of long bones occurs in many infected dogs, giving bones a roughened appearance with abnormal thickenings evident on radiographs (Ewing and Panciera, 2003). Diseased dogs are thought to be in a great deal of discomfort from the muscular and osteal manifestations of ACH. Although chronic infections with *H. americanum* have been documented in dogs, severity of disease leading to death is not uncommon (Panciera et al., 1998; Ewing et al., 2003).

Old World hepatozoonosis caused by *H. canis* is described, in general, as a milder disease than ACH (Paludo et al., 2003); this disease was first described in India in 1905 and has since been reported in South America, Europe, Africa, and the Far and Middle East. The brown dog tick, *Rhipicephalus sanguineus* is the accepted primary definitive host of *H. canis*, although other ticks have also been implicated as potential vectors (Murata et al., 1995; O'Dwyer et al., 2001; Forlano et al., 2005). With a strong preference for canine hosts and a tolerance for low-humidity, indoor climate-controlled environments, this tick is typically found in kennels and homes throughout the United States and in tropical, sub-tropical and temperate regions all over the world (Ewing et al., 2000, Ewing et al., 2002). As in the case of *H. americanum*, *H. canis* transmission to dogs occurs by the ingestion of the arthropod vector harboring the parasite. All stages of *R. sanguineus* preferentially feed on dogs; perhaps as a consequence, no other vertebrates have been implicated or documented to serve as potential intermediate hosts of *H. canis* (Baneth et al., 2007).

Unlike in ACH, dogs infected with *H. canis* often, although not always, develop detectable parasitemia with gamonts readily seen in routine blood smear preparations (O'Dwyer et al., 2001; Eiras et al., 2007; Baneth and Vincent-Johnson, 2005). Merogonic stages of the parasite are found in neutrophils, and dogs infected with *H. canis* do not develop cysts in muscle tissue. *H. canis* undergoes merogony in a variety of sites other than muscle tissue, i.e. hemolymphatic tissues and visceral organs, giving rise to characteristic "wheel spoke" merozoite arrangements, with parasite invasion often leading to anemia (Baneth et al., 2001; Baneth et al., 2007). Similar to infections with *H. americanum*, dogs suffering from *H. canis* infections may present with elevated white blood cell counts, stiffness, pain, weight loss, lethargy, and fever; however, clinical signs are generally less pronounced in Old World hepatozoonosis. Although not typical of the disease, infection by *H. canis* can be life-threatening (Baneth et al., 2000). It has been observed that *H. canis* is often found in dogs with concurrent *Babesia* spp. or *Ehrlichia* spp. infections, which may incite immunosuppression leading to exacerbation of disease (Panciera et al., 1997; Ewing et al., 2000; Ewing et al., 2003).

The purpose of the present study was to gain a better understanding of the frequency of infection with *Hepatozoon* spp. and determine the phylogenetic diversity of *Hepatozoon* strains occurring in domestic dogs in the southern United States. To achieve this goal, blood samples for standard and real-time PCR were collected from dogs in Oklahoma residing in ACH-endemic areas, dogs presented to veterinarians with clinical signs of ACH, and dogs awaiting adoption in local shelters. In addition, real-time PCR results for the detection of *Hepatozoon* spp. were reviewed from dogs throughout the United States.

2. Material and methods

2.1. Sample collection

Blood was collected in 3 ml EDTA tubes from dogs residing in an ACH-endemic area of Oklahoma

(Muskogee County; $n = 16$), those presenting with consistent clinical signs of ACH ($n = 2$), and random-source dogs housed at an animal shelter in Payne County, OK ($n = 200$). Anticoagulated whole blood, mostly EDTA-whole blood, from dogs presented to veterinarians throughout the U.S. was also submitted to the Molecular Diagnostics Laboratory at Auburn University (Auburn University College of Veterinary Medicine, Auburn, Alabama).

2.2. DNA extraction

For standard PCR, DNA was obtained from blood samples using the GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Approximately 100 μl of whole blood was extracted following the protocol provided by the manufacturer. Nucleic acid was eluted with 100 μl nuclease-free water. For real-time PCR, nucleic acid was extracted from fresh or refrigerated blood samples by the Molecular Diagnostics Laboratory at Auburn University (Auburn University College of Veterinary Medicine, Auburn, AL).

2.3. PCR

Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination. DNA extraction, primary amplification, secondary amplification, and product analyses were performed in separate dedicated laboratory areas. A negative water control was included in each set of DNA extractions and one water control was included in each set of primary and secondary PCR reactions. Nested PCR was performed as previously described (Gubbels et al., 1999; Yabsley et al., 2005) using primers 5.1, 3.1, RLBH-F, and RLBH-R, which amplify regions of the 18S rRNA gene of Hepatozoon species and other apicomplexans. For each primary reaction, 5 μl of extracted DNA was used as template in a 25 μl reaction containing 1X Thermophilic DNA Polymerase Magnesium Free Buffer, 2.5 mM MgCl$_2$, 0.125u Taq DNA Polymerase in Storage Buffer A (the last three reagents from Promega, Madison, WI), 0.2 mM dNTP, and 0.8 μM each of primers 3.1 and 5.1. Primary PCR was carried out in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) according to the following parameters: 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min ending with an extension step at 72 °C for 5 min.

For each nested reaction, 1 μl of the primary reaction was used as template in a second, 25 μl reaction containing 1X Thermophilic DNA Polymerase Magnesium Free Buffer, 2.5 mM MgCl$_2$, 0.125u Taq DNA Polymerase in Storage Buffer A, 0.2 mM dNTP, and 0.8 μM each of primers RLBH-F and RLBH-R. Secondary PCR was performed for 1 min at 94 °C followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1.5 min and a final extension step of 72 °C for 10 min. The resulting PCR products (560–570 bp) were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Real-time PCR for detection of Hepatozoon spp. was performed in the Molecular Diagnostics Laboratory at Auburn University (Auburn University College of Veterinary Medicine, Auburn, AL). Copy numbers of the Hepatozoon spp. 18S rRNA gene (18S rDNA) in positive samples were quantified by use of H. americanum and H. canis standards, respectively, and the two species were differentiated by melting curve analysis.

2.4. Sequence analyses

Amplicons from positive PCR samples were prepared for sequencing by purification and concentration using Amicon® Microcon®-PCR Centrifugal Filter Devices (Millipore Corporation, Bedford, MA) per the manufacturer's protocol. Amplicons were sequenced in both directions with internal primers RLBH-F and RLBH-R using an ABI3730 capillary sequencer. Sequences obtained from this study (EU146062–EU146067) were compared to those available in the National Center for Biotechnology Information database, including 15 Hepatozoon spp. sequences previously reported in GenBank (AY864676, AY461378, DQ519357, DQ439544, DQ439542, DQ439540, AY150067, AY471615, DQ111754, DQ439543, AY731062, DQ519358 and AY461375). MacVector 8.1 software was used for multiple sequence alignments. Percent similarity matrices were constructed to evaluate sequence diversity of H. americanum and other Hepatozoon spp. present in the domestic dogs sampled.

3. Results

One of 16 dogs (6.25%) residing in an area of Oklahoma where ACH is endemic was found to harbor evidence of circulating H. americanum; the 18S rDNA fragment amplified from this organism (EU146062) was 99.6% identical to previously published H. americanum sequence (AY864676; Fig. 1). Two dogs that presented with clinical signs of ACH to the Oklahoma State University Veterinary Teaching Hos-

	H. americanum (AY864676)	A.C. 3-23	Muskogee	Clinic Dog 1	Clinic Dog 2	H. canis (AY461378)	A.C. 1-119	A.C. 939
H. americanum (AY867646)	-	95.5	99.6	95.7	97.2	92.3	92.3	92.3
A.C. 3-23		-	95.7	92.7	93.7	88.6	87.4	87.4
Muskogee			-	95.7	96.8	92.7	92.5	92.5
Clinic Dog 1				-	94.5	88.5	88.3	88.3
Clinic Dog 2					-	90.2	89.3	89.3
H. canis (AY461378)						-	98.8	98.8
A.C. 1-119							-	100.0
A.C. 939								-

Fig. 1. Percent similarities of 18S rDNA sequences from *Hepatozoon* spp. of dogs.

pital were also infected with *H. americanum*, based on 18S rDNA sequence (EU146066 and EU146067). Respectively, these sequences were 95.7% and 97.2% similar to previously published *H. americanum* sequence (AY864676; Fig. 1).

Evidence of circulating *Hepatozoon* spp. was detected in three of 200 shelter dogs surveyed, or 1.5%. In one of these dogs, sequence (EU146065) analysis revealed the 18S rDNA fragment most closely resembled that previously reported as *H. americanum*. Sequence recovered from the other two shelter dogs (EU146063 and EU146064) shared 98.8% identity with sequence reported from *H. canis* (AY461378) and 100% identity with each other (Fig. 1). Further evaluation of this region of the 18S rDNA of *H. canis* from sequences available in the National Center for Biotechnology Information database ($n = 13$) from dogs in Africa, Asia, Europe, and South America showed previously reported *H. canis* sequences are 98.6% to 99.8% identical to one another.

Real-time PCR assays performed at Auburn University through the Molecular Diagnostics Laboratory (Auburn University, Auburn, Alabama) confirmed the Oklahoma State University results, identifying sequence of either *H. americanum* or an organism closely resembling *H. canis* in the samples from Oklahoma. In addition, a review of real-time PCR results on blood samples submitted from a total of 274 dogs from throughout the U.S. revealed *H. americanum*, *H. canis*, and a mixture of *H. americanum* and *H. canis* in 68 (24.8%), 2 (0.7%), and 7 (2.6%), respectively, of 77 canine blood samples found positive for *Hepatozoon* spp.

4. Discussion

Although *H. americanum* and ACH were recognized fairly recently, this disease has become increasingly important in veterinary medicine in the southern United States. Heightened awareness of the disease has coincided with an increase in reported cases in endemic areas (Ewing et al., 2003). However, an estimation of the prevalence of *H. americanum* in naturally infected dogs has not been previously reported. This study suggests that *H. americanum* and *H. canis*, or a closely related organism, are both present in domestic dog populations in Oklahoma; sequence-confirmed PCR evidence of infection with *Hepatozoon* spp. was detected in 1.5% of our shelter dogs. This value is likely lower than the actual infection prevalence because muscle biopsy, rather than whole blood, is considered ideal for detecting infection (Ewing et al., 2003), although even when using muscle biopsy samples, parasitemia levels in experimentally infected dogs fluctuate over time and sometimes wane to undetectable levels (Ewing et al., 2003).

H. americanum was identified in free ranging dogs in Oklahoma as well as in dogs that presented with suspicion of ACH. Prior to the present work, only two sequences of the 18S rRNA gene of *H. americanum* had been described (Mathew et al., 2000; Paludo et al., 2005). The additional sequences we report here are 92.7–96.8% identical to one another, and 95.5–99.6% similar to those previously published, suggesting that there may be multiple strains of *H. americanum* infecting dogs in the southern U.S. Novel *Hepatozoon* spp. continue to be described from wildlife in North America (Johnson et al., 2007b). Indeed, our comparison of reported 18S rDNA sequences from different strains of *H. canis* revealed they are 98.6–99.8% identical to one another.

In the present study, we also found two dogs that were infected with a *Hepatozoon* spp., the 18S rDNA sequence fragment of which was 98.8% identical to a previously reported *H. canis* sequence; this degree of similarity is within the range of the innate variation between strains of *H. canis* and suggests that the organism in these two dogs was most likely *H. canis* or a closely related species. This finding is important

because *H. canis* was long thought not to be present in North America although infections are commonly reported in dogs in Europe, Asia, and throughout Central and South America (Forlano et al., 2007). Previous reports of *H. canis* in the southern U.S. are widely thought to be *H. americanum* infections which were misidentified prior to recognition of the latter organism as an etiologic agent of hepatozoonosis in North America (Nordgren and Craig, 1984; Macintire et al., 1997; Baneth et al., 2003; Ewing and Panciera, 2003). However, our data suggest that these earlier infections could also represent either *H. canis* infection or co-infections with both *H. canis* and *H. americanum*. Review of results from the Molecular Diagnostics Laboratory at Auburn University supports our assertion that *H. canis* is present in dogs in the U.S.; *H. canis* sequence was identified in nine of 77 (11.7%) dogs with PCR evidence of *Hepatozoon* spp. infection.

Finding dogs with evidence of *H. canis* infection in North America is not entirely surprising; both the dog reservoir host and the tick vector, *R. sanguineus*, are common, and travel of dogs could certainly result in introduction of this pathogen from areas where *H. canis* is endemic. Unfortunately, patient histories of the two shelter dogs from Oklahoma infected with *H. canis* or a similar organism were not available; clinical information was not gathered at the time of blood collection for the survey, and the origin of neither dog prior to their stay at the animal shelter is clear. These dogs may have been previously infected and brought into the United States from other regions of the world where *H. canis* is endemic. However, despite the lack of previous documentation of *H. canis* in naturally infected dogs in North America, it is also possible that the two dogs from Oklahoma and the 9 identified at Auburn University, became infected with *H. canis* or an *H. canis*-like organism in the United States. Further study is required to determine whether travel to and from the United States resulted in the introduction of this agent and subsequent establishment of endemic cycles of infection in domestic dogs in the U.S. In the absence of co-infections, disease due to *H. canis* is relatively mild (Ewing et al., 2000).

H. canis infection differs from that of *H. americanum* in both disease presentation and treatment approach (Baneth et al., 1995; Shaw and Day, 2005); veterinarians practicing in North America should be aware that both organisms may be present and causing disease in domestic dogs in this region. Our identification of *H. canis* or an *H. canis*-like organism in dogs in North America, together with the significant variation of 18S rRNA gene sequences seen among strains of *H.*

americanum evaluated in the present study, suggest that the *Hepatozoon* spp. infecting dogs in the United States are quite diverse. In addition, although complete patient histories were not available for the random source dogs evaluated in this study, it is possible that some of these infections were subclinical. Continued analysis of the diversity of *Hepatozoon* spp. infecting dogs in North America is needed to fully characterize the strains and species present.

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