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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.

Keywords: Apicomplexa; *Hepatazoon americanum*; *Hepatozoon canis*; Hepatozoonosis

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