HEPATOZOON SPECIES IN NORTH AMERICA:

PHYLOGENETIC DIVERSITY,

TRANSMISSION PATTERNS, AND OPPORTUNITES

FOR CONTROL

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CHAPTER I

INTRODUCTION

Members of the genus *Hepatozoon*, classified in phylum Apicomplexa, are unique hemogregarines in their oral routes of infection to vertebrate intermediate hosts and polysporocystic oocyst formation in invertebrate definitive hosts (Smith, 1996). Domestic dogs (*Canis familiaris*) have been recognized hosts of *Hepatozoon* spp. since 1905 (Baneth et al., 2000; Potter and Macintire, 2010). *Hepatozoon canis*, first described in India, is now documented in many areas of the world including Africa, Southeast Asia, the Middle East, southern Europe, and South America (Vincent-Johnson, 2003; Baneth et al., 2007; Little et al., 2009). *Rhipicephalus sanguineus* is recognized as the primary definitive host and tick vector of *H. canis,* and is found in most temperate and tropical regions of the world (Vincent-Johnson, 2003; Baneth et al., 2007; Little et al., 2009). Despite the presence of its definitive host throughout North America, *H. canis* was not definitively identified in canids in the United States until 2008 (Allen et al., 2008; Little et al., 2009).

The first natural *Hepatozoon* sp. infection in a canid in the United States was reported in a single coyote (*Canis latrans*) in Texas near the Gulf Coast in 1978 (Davis et al., 1978; Vincent-Johnson, 2003; Ewing and Panciera, 2003). Reports in domestic dogs soon followed, but the novel etiological agent, *Hepatozoon americanum*, was not recognized as distinct from *H. canis* until 1997 (Vincent-Johnson, 1997a; Vincent-

Johnson, 2003; Potter and Macintire, 2010). The accepted natural definitive host and primary tick vector of *H. americanum* is *Amblyomma maculatum*, or the Gulf Coast tick (Mathew et al., 1998; Ewing et al., 2002; Vincent-Johnson, 2003; Potter and Macintire, 2010). Originally, this tick was commonly found in states bordering the Gulf of Mexico and several states along the Atlantic coast including Georgia, Florida, and the southern portion of South Carolina. Current data document the Gulf Coast tick in states further inland including Arizona, Arkansas, Indiana, Kansas, Kentucky, Missouri, Oklahoma, and Tennessee, and additional states along the Atlantic coast including Maryland, Virginia, and West Virginia (Vincent-Johnson, 2003; Paddock et al., 2008; Kasari et al., 2010). Reports of canine hepatozoonosis in the United States have historically correlated with the traditional south-central and southeastern geographic distribution of *A. maculatum* (Vincent-Johnson, 2003; Ewing and Panciera, 2003; Potter and Macintire, 2010). Although canine hepatozoonosis in North America is considered an emerging disease (Ewing and Panciera, 2003; Vincent-Johnson, 2003; Potter and Macintire, 2010), survey studies evaluating prevalence and genetic diversity of *Hepatozoon* species infecting domestic dogs in enzootic areas are lacking.

Canine hepatozoonosis is an incurable disease (Macintire et al., 2001; Panciera and Ewing, 2003; Potter and Macintire, 2010; Sasanelli et al., 2010). In general, clinical disease associated with *H. canis* infection is inapparent to mild unless patients have concomitant infections or are immune-compromised (Baneth et al., 2003a; Vincent-Johnson, 2003; Sasanelli et al., 2010). Fever, lethargy, anorexia, depression, and anemia are most often observed in symptomatic patients (Vincent-Johnson et al., 1997a; Baneth et al., 2007; Little et al., 2009). Imidocarb dipropionate has been experimentally

evaluated for efficacy in *H. canis* infections, and although the 2-3 month therapy, administered subcutaneously twice monthly, generally provides favorable clinical results, tissue stages of the parasite are not cleared and animals are subject to clinical relapse (Vincent-Johnson et al., 1997b; Sasanelli et al., 2010).

Clinical disease associated with *H. americanum* infection, or American canine hepatozoonosis (ACH), is often severe to fatal, with salient clinical features including fever, muscle atrophy, muscle and bone pain, recumbency, lameness, mucopurulent ocular discharge, and neutrophilic leukocytosis (Vincent-Johnson, 2003; Ewing and Panciera, 2003; Little et al., 2009; Potter and Macintire, 2010). Current Companion Animal Parasite Council (CAPC) recommendations for treatment of ACH entail a 14-day oral administration of a triple combination of trimethoprim-sulfadiazine, pyrimethamine, and clindamycin (TCP) or ponazuril (toltrazuril sulfone) as an initial parasiticide followed by a two year administration of decoquinate to prevent or delay clinical relapse (www.capcvet.org). TCP and decoquinate treatments have been evaluated in naturally infected domestic dogs, and, although parasite tissue stages are not eliminated, the drug combination is shown to benefit patients in both longevity and quality of life (Macintire et al., 2001; Vincent-Johnson, 2003). Ponazuril is a recommended alternative to TCP as an initial modality of ACH treatment (www.capcvet.org) and has shown efficacy against other tissue cyst-forming apicomplexans (Gottstein et al., 2001; Mitchell et al., 2004; Mitchell et al., 2005; Charles et al., 2007), but has not been evaluated experimentally as a treatment of *H. americanum* infection.

The range of hosts parasitized by *Hepatozoon* species is broad. Over 300 species of *Hepatozoon* have been reported world-wide in poikilotherms, mammals, and some

birds (Smith, 1996; Baneth et al., 2007; Potter and Macintire, 2010). In North America, 24 species of *Hepatozoon* have been reported from snakes, and species documented in mammals include *H. americanum* in canids (*Canis familiaris* and *C. latrans*) (Vincent-Johnson et al., 1997a; Kocan et al., 2000), *H. muris* in rodents (*Rattus norvegicus*) (Eyles, 1952), *H. procyonis* in raccoons (*Procyon lotor*) (Richards, 1961; Clark et al., 1973), and *H. griseisciuri* in grey squirrels (*Sciuris carolinensis*) (Clark, 1958; Redington and Jachowski, 1971; Davidson and Calpin, 1976).

Only nine of the over 300 species of *Hepatozoon* reported have genetic sequence available in the National Center for Biotechnology Information database, GenBank, the majority of which are 18S rRNA gene data. The remaining *Hepatozoon* sequences listed in GenBank are published as *Hepatozoon* spp. with the hosts from which they were collected identified. *Hepatozoon* spp. sequence contributions from the United States are particularly limited. Prior to 2007, sequence data were available from domestic dogs only (Mathew et al., 2000; Paludo et al., 2005), despite the recognition of natural *Hepatozoon* spp. infections in other vertebrate hosts (Eyles, 1952; Clark, 1958; Richards, 1961; Redington and Jachowski, 1971; Clark et al., 1973; Davidson and Calpin, 1976; Davis et al., 1978). Focused wildlife survey studies in Oklahoma have since contributed two 18S rDNA sequences from rodents (*Sigmodon hispidus* and *Peromyscus leucopus*) and two 18S rDNA sequences from rabbits (*Sylvilagus floridanus* and *Sylvilagus aquaticus*) (Johnson et al., 2008a; Johnson et al., 2009). *Hepatozoon* sequence GU344682 from a turkey vulture (*Cathartes aura*) in Oklahoma was added in 2010. Additional *Hepatozoon* spp. sequences obtained from known and novel vertebrate hosts

would provide data for more comprehensive sequence comparisons in assessing phylogenetic relationships of *Hepatozoon* spp. cycling in nature in the United States.

The accepted primary route of natural *Hepatozoon* spp. transmission to intermediate hosts is by the ingestion of definitive hosts containing sporulated parasite oocysts (Smith, 1996; Baneth et al., 2007). However, paratenic routes have been reported and experimentally demonstrated in certain *Hepatozoon* species (Smith, 1996; Baneth et al., 2007; Johnson et al., 2008b; Johnson et al., 2009). A tertiary route of transmission, documented to occur in natural *H. griseisciuri* and *H. canis* infections, occurs congenitally, presumably across the placenta (Clark, 1958; Murata et al., 1993; Vincent-Johnson, 2003; Baneth et al., 2007; Little et al., 2009). Although speculated to occur in dogs infected with *H. americanum*, transplacental transmission of this parasite has not been confirmed (Vincent-Johnson, 2003; Little et al., 2009; Potter and Macintire, 2010). In current literature, the youngest ACH patient reported is 11 weeks of age (Ewing and Panciera, 2003), which, based on experimental data, is an age allowing adequate time from parasite exposure to observable clinical disease and parasite patency in canid intermediate hosts via established oral routes of infection (Mathew et al., 1998; Johnson et al., 2008b; Johnson et al., 2009). However, experimental evaluation of transplacental transmission of *H. americanum* has not been reported.

Specific objectives of this research include:

1. Estimate the prevalence of *Hepatozoon* spp. infections in naturally infected domestic dogs in the United States using a PCR method designed to amplify a hypervariable region of the 18S rRNA gene, and to compare amplicon sequences collected to assess phylogenetic diversity of organisms found.

2. Evaluate the efficacy of ponazuril (Marquis™) (Bayer HealthCare LLC, Shawnee Mission, USA), administered twice daily for four weeks at the onset of clinical disease, in a dog experimentally infected with *H. americanum* to assess parasite clearance and clinical improvement of the infected dog.

3. Compare 18S rRNA gene sequences of *Hepatozoon* spp. infecting known and novel vertebrate hosts in the southern United States to examine phylogenetic relationships and document diversity.

4. Assess transplacental transmission of *H. americanum* in an experimentally, chronically infected dam by evaluating whelped pups for clinical signs, parasitemia, and competence to infect *A. maculatum* ticks.

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CHAPTER II

REVIEW OF LITERATURE

THE GENUS *HEPATOZOON*

History

Hepatozoon species are obligate heteroxenous apicomplexan parasites that are documented in a wide variety of vertebrate and invertebrate hosts (Smith, 1996; Smith and Desser, 1997). The genus was first erected by Miller in 1908 based on his observations of *Hepatozoon muris* (syn. *Hepatozoon perniciosum*) in laboratory rats and mites (*Laelaps echidninus*) (Smith, 1996; Mathew et al., 2000). The genus was classified in family Haemogregarinidae by Le`ger in 1911, and reclassified in Hepatozoidae by Wenyon in 1926 (Mathew et al., 2000). The revision was based on two aspects of the *Hepatozoon* life cycle that distinguish the genus from other hemogregarines: the production of polysporocystic oocysts in hematophagous invertebrate definitive hosts and transmission to vertebrate intermediate hosts via the ingestion of definitive hosts carrying sporulated oocysts (Levine, 1982; Desser, 1990; Smith, 1996; Smith and Desser, 1998; Smith et al., 1999; Mathew et al., 2000).

Transmission

Hematophagous invertebrate definitive hosts of *Hepatozoon* spp. support parasite syngamy, sporogony, and oocyst formation, while vertebrate intermediate hosts support parasite merogony and gametogony (Smith, 1996; Smith and Desser, 1998). It is conventionally accepted that most *Hepatozoon* spp.infections are acquired by the consumption of invertebrate hosts carrying sporulated oocysts (Smith, 1996; Smith and Desser, 1998). This may occur when vertebrates ingest invertebrates as sustenance (Smith, 1996), while grooming self or companions (Ewing et al., 2003; Baneth et al., 2007), or accidentally during predation and/or scavenging (Ewing et al., 2003; Johnson et al., 2009a). After ingestion and excystation, sporozoites disseminate to extra-intestinal tissues and develop into meronts (Smith, 1996). Released merozoites may undergo further cycles of merogony or develop into gamonts within blood cells. Gamonts, present in circulating leukocytes or erythrocytes, are the infective stage to invertebrate definitive hosts (Smith, 1996; Smith and Desser, 1997).

Some species of *Hepatozoon* utilize obligate or facultative paratenic hosts (Smith, 1996). Paratenic hosts that consume sporulated oocysts or invertebrate definitive hosts containing them develop parasite monozoic or dizoic cyst stages, or cystozoites. Cystozoites, sequestered in the tissues of paratenic hosts, are infective to vertebrate intermediate hosts ingesting them (Desser, 1990; Smith, 1996; Johnson et al., 2009b). For example, *Hepatozoon sipedon* has seemingly lost its ability to establish infections in snake intermediate hosts without cystozoite development in obligate, paratenic frog hosts first, which are infected by consuming mosquito definitive hosts (Smith et al., 1994; Smith, 1996). *Hepatozoon americanum,* a parasite of canids, experimentally has been

shown to form cystozoites in alternate vertebrate host species which are infectious to dogs if ingested; however, these alternate hosts are not required for *H. americanum* transmission to canid intermediate hosts (Johnson et al., 2008a; Johnson et al., 2009b). It is thought that with *Hepatozoon* spp. infecting paratenic hosts, predatory habits of intermediate hosts are important for parasite transmission and life cycle completion (Desser, 1990; Smith, 1996; Johnson et al., 2008a; Johnson et al., 2009a; Johnson et al., 2009b).

HEPATOZOON SPECIES INFECTING CANIDS

Hepatozoon canis

Hepatozoon canis was discovered by S. P. James in 1905 in the blood of domestic dogs (*Canis familiaris*) in India (Mathew et al., 2000; Baneth et al., 2007). The novel organism was named *Leucocytozoon canis*, but was later reclassified in the genus *Hepatozoon* (Mathew et al., 2000; Baneth et al., 2007). Since its discovery, this parasite has been reported in dogs in many areas of the world, including Europe, Asia, Africa, South America, and more recently, North America (Baneth et al., 2007; Allen et al., 2008; Sasanelli et al., 2010, Little et al., 2009).

H. canis in its Definitive Host

The primary definitive host and tick vector of *H. canis* was identified by Christophers in 1907 as *Rhipicephalus sanguineus*, the brown dog tick (Mathew et al., 2000; Baneth et al., 2007). *Rhipicephalus sanguineus* nymphs have been experimentally demonstrated to support *H. canis* syngamy, sporogony, and oocyst formation after

repletion feeding on infected dogs or after percutaneous injection with buffy coat from infected dogs (Baneth et al., 2001; Baneth et al., 2007). Larvae are apparently refractory to infection (Ewing et al., 2002a; Ewing et al., 2002b). Mature oocysts are found approximately 53 days post-repletion in 66-85% of molted adult cohorts infected with *H. canis* as nymphs (Baneth et al., 2007). Although oocysts are readily observable in hemocoel preparations from dissected ticks, it is not entirely clear where zygote formation and sporogony occur within the tick, or whether these processes take place intracellularly or extracellularly (Baneth et al., 2007). Experiments assessing transovarial transmission of *H. canis* in *R. sanguineus* indicate this route does not occur (Baneth et al., 2001).

Geographic Distribution of *R. sanguineus*

Rhipicephalus sanguineus is most often an ectoparasite of dogs, as its common name, the brown dog tick, implies. Although *R. sanguineus* has been documented on other hosts, this three-host tick preferentially feeds on dogs during each instar (Dantas-Torres, 2008; Dantas-Torres, 2010). The brown dog tick is capable of establishing in a variety of climates with regards to temperature, relative humidity, and precipitation (Dantas-Torres, 2010). Also known as the kennel tick, *R. sanguineus* is infamous for its ability to infest indoor facilities (Dantas-Torres, 2008). As its preferred host is found world-wide and it is able to adapt to various environmental conditions, *R. sanguineus* is cosmopolitan in its geographic distribution (Dantas-Torres, 2010).

Other Potential Invertebrate Hosts of *H. canis*

Although *R. sanguineus* is the accepted definitive host of *H. canis*, other tick species have been reported as potential hosts of this parasite. Oocysts of *Hepatozoon*

spp. have been identified in *Haemaphysalis longicornis* and *H. flava* collected from naturally infected dogs with *H. canis* in Japan (Murata et al., 1995; Baneth et al., 2001). Also, a molecular survey of organisms in wild-caught *Ixodes ricinus* in Luxumbourg revealed *H. canis* DNA in an unfed adult female (Reye et al., 2010). In Brazil, an adult *Amblyomma ovale* collected from a naturally infected dog was reported to contain *Hepatozoon* sp. oocysts. Sporozoites liberated from these oocysts were injected into an uninfected dog intra-peritoneally and circulating gamonts were observed in the animal 84 days after inoculation (Forlano et al., 2005). Another study demonstrated transstadial transmission of *H. canis* by *A. ovale* to susceptible dogs (Rubini et al., 2009). These findings implicate *A. ovale* as a definitive host and vector of *H. canis* in parts of South America.

H. canis in its Intermediate Host

The preponderance of knowledge regarding *H. canis* infections in canids has been gleaned from observations in naturally infected domestic dogs (Baneth et al., 2007). Canid intermediate hosts are thought to primarily become infected by ingesting *R. sanguineus* ticks that contain *H. canis* oocysts (Baneth et al., 2007). Oocysts are reportedly fragile, and likely rupture during canid mastication or when introduced into the stomach (Baneth et al., 2001). It remains unclear whether sporozoites released from sporocysts in the alimentary tract penetrate the gut lining and migrate to target organs or if they are engulfed by phagocytic cells and carried hematogenously to tissues (Baneth et al., 2007).

Typical sites of merogony in *H. canis* infections include bone marrow, lymph nodes, and spleen (Baneth and Weigler, 1997; Baneth et al., 2000; Baneth et al., 2007).

In a study conducted by Baneth et al. (2007), two morphologically distinct populations of meronts were observed in the bone marrow of experimentally infected dogs after 26 days. One form contained only two to four large zoites, termed macromerozoites, randomly arranged within the meront. The role of macromerozoites in *H. canis* infections remains to be elucidated, but they are documented to give rise to micromerozoites and perpetuate merogony in other species of *Hepatozoon* (Baneth et al., 2007). The second type of meront contained 20-30 smaller zoites arranged in a "wheel-spoked" configuration similar to that documented in other species of *Hepatozoon* (Baneth et al., 2007; Vincent-Johnson, 2003). These zoites, termed micromerozoites, were thought to be the progenitors of gamonts (Baneth et al., 2007).

Mature gamonts of *H. canis* in experimentally infected dogs may be observed in peripheral neutrophils four weeks after infection (Baneth et al, 2001; Baneth et al., 2007). *H. canis* infections are often associated with high levels of parasitemia, with gamonts sometimes reported in as many as 100% of neutrophils on blood films (Baneth and Vincent Johnson, 2005).

<u>Clinical Signs and Diagnosis of *H. canis* Infections in Domestic Dogs</u>

Disease associated with *H. canis* infection may range from sub-clinical and chronic, especially in the absence of concurrent infections, to severe and life-threatening (Baneth et al., 2001; Baneth et al., 2007). Severity of disease tends to correlate with patient immune status, which may be impacted by age, genetic disorder, immune therapy, or coinfection with another etiological agent such as *Ehrlichia canis*, *Leishmania canis*, *Babesia canis*, and *Toxoplasma gondii* (Harmelin et al., 1992; Baneth et al., 1997; Vincent Johnson et al., 1997; Baneth et al., 2001; Mylonakis et al., 2005; Baneth et al.,

2007). In patients with overt disease, symptoms including fever, anemia, lethargy, anorexia, and depression may be observed (Baneth et al., 2001; Baneth et al., 2007; Vincent-Johnson et al., 1997). *Hepatozoon canis* infections are classically diagnosed by microscopic observation of gamonts in blood films, which sometimes are incidental findings (Sasanelli et al., 2010; Baneth et al., 2007; Vincent-Johnson, 2003). Polymerase chain reaction (PCR) methods have recently been developed to detect parasite DNA in peripheral blood (Baneth et al., 2000; Oyamada et al., 2005; Criado-Fornelio et al., 2007a; Li et al., 2008).

Other Potential Vertebrate Hosts of *H. canis*

As disease associated with *H. canis* infection is sub-clinical to mild in most patients, domestic dogs are thought to be well-adapted hosts of this parasite (Vincent-Johnson, 2003). Canine tolerance to infection and the partiality of *R. sanguineus* for feeding on dogs during each instar suggest *H. canis* is chiefly cycled between dogs and ticks. Monozoic cysts of *H. canis* have been reported in the spleens of naturally and experimentally infected dogs that are morphologically similar to cystozoites observed in alternate hosts of other *Hepatozoon* species (Smith, 1996; Baneth and Shkap, 2003; Baneth et al., 2007). However, in dogs infected with *H. canis*, these cysts are present in addition to meronts, which may indicate that dogs serve as both intermediate and paratenic hosts (Baneth et al., 2003b; Baneth et al., 2007).

Although *H. canis* is mainly identified in domestic dogs, this parasite has been reported in jackals, hyenas, and palm civets; however, the species of *Hepatozoon* infecting these wild carnivores have not been confirmed (Levine, 1985). Recently, genetic sequences most identical to that documented as *H. canis* were obtained from red

foxes (*Vulpes vulpes*) in Italy (Gabrielli et al., 2010) and Croatia (Dezdek et al., 2010)

and domestic cats (*Felis catus*) in France (Criado-Fornelio et al., 2009), Thailand

(Jittapalapong et al., 2006), and Brazil (Rubini et al., 2006).

Hepatozoon americanum

Hepatozoon americanum was first reported in a coyote (*Canis latrans*) in Texas in

1978 (Davis et al., 1978). Over the next two decades, *H. americanum* was reported in

domestic dogs in several states including Alabama, Georgia, Louisiana, Mississippi,

Oklahoma, and Texas (Craig et al., 1978; Vincent-Johnson et al., 1997; Panciera et al.,

1997). At that time, these infections were attributed to a particularly virulent strain of *H.

canis*. Further research examining the novel North American parasite's morphology,

tissue tropism, and clinical disease caused indicated that it was an organism distinct from

H. canis. In 1997, *H. americanum* was recognized as the etiological agent of canine

hepatozoonosis in the United States, and the syndrome associated with infection was

dubbed American canine hepatozoonosis (ACH) (Vincent-Johnson et al., 1997; Mathew

et al., 2000; Panciera et al., 2001; Ewing et al., 2002a). Subsequent experiments

elucidated additional differences between *H. canis* and *H. americanum* including primary

definitive host required for parasite life cycle completion and disparities in regions of 18S

rRNA gene sequence (Baneth et al., 2000; Ewing et al., 2002a: Ewing et al., 2002b).

H. americanum in its Definitive Host

Although *Hepatozoon* spp. oocysts have been recognized in feeding *A. maculatum*

removed from canids in enzootic areas of ACH, such reports are scarce, and the species

of parasites were not determined (Vincent-Johnson et al., 1997; Ewing et al., 2002b).

However, *A. maculatum*, commonly known as the Gulf Coast tick, has experimentally been demonstrated to be an excellent definitive host of *H. americanum*, while other common tick species in ACH enzootic areas, including *R. sanguineus*, *A. americanum*, and *Dermacentor variabili*s, have empirically been refractory to infection (Mathew et al., 1999; Ewing et al., 2002b). Experiments characterizing the development of *H. americanum* in *A. maculatum* infected via blood meal acquisition have revealed that parasite is transstadially maintained in the tick from larvae to nymph, nymph to adult, and larva to adult (Ewing et al., 2002b). Molted cohorts are demonstrated to harbor sporulated oocysts infective to canine hosts after approximately 33 to 42 days post-repletion in the majority (96%-99%), if not all, of those dissected (Mathew et al., 1999; Ewing et al., 2002b). Intermittent microscopic examination of experimentally infected ticks has shown evidence of parasite syngamy, sporogony, and oocyst formation occurring within gut cells of tick hosts (Mathew et al., 1999). Upon tick dissection, oocysts are readily apparent free in the hemocoel, but some are often closely associated with midgut tissues (Mathew et al., 1999; Ewing et al., 2002b). Experiments assessing transovarial transmission of *H. americanum* in *A. maculatum* have not been reported; this route is not suspected, as it has not been documented in other known definitive hosts of *Hepatozoon* spp. (Levine, 1985; Baneth et al., 2001).

<p align="center">Geographic Distribution of A. maculatum</p>

In the United States, *A. maculatum* was traditionally endemic in states bordering the Gulf Coast and several states bordering the Atlantic coast including Georgia, Florida, and the southern portion of South Carolina (Kasari et al., 2010). However, current data report the Gulf Coast tick in states further inland including Arizona, Arkansas, Indiana,

Kansas, Kentucky, Missouri, Oklahoma, and Tennessee and additional states along the Atlantic coast including Maryland, Virginia, and West Virginia (Vincent-Johnson, 2003; Paddock et al., 2008; Kasari et al., 2010). *Amblyomma maculatum* is also documented in Central and South American regions that border the Gulf of Mexico and Caribbean Sea including Mexico, Guatemala, Belize, Nicaragua, Honduras, Costa Rica, Colombia, Venezuela, and parts of Ecuador and Peru (Sumner et al., 2007), although recent evaluations of historical records in these regions from the past 50 years indicate that Gulf Coast ticks had sometimes been confused with *Amblyomma triste* (Mertins et al., 2010).

<u>Other Potential Invertebrate Hosts of *H. americanum*</u>

Unlike with *H. canis*, other invertebrate definitive hosts of *H. americanum* have not been implicated. The exceptional ability of *A. maculatum* to acquire and support parasite development in experimental settings argues its role as the most important definitive host in nature (Mathew et al., 1999; Ewing et al., 2002b). Furthermore, prior to 2008, reports of ACH generally correlated with the geographic distribution of *A. maculatum* in the United States (Allen et al., 2008; Li et al., 2008; Potter and Macintire, 2010). Newly reported cases of ACH in areas where *A. maculatum* is not established are thought to be instances of patient relocations from confirmed *H. americanum* enzootic areas (Allen et al., 2008; Little et al., 2009).

<u>*H. americanum* in its Intermediate Host</u>

The primary documented route of *H. americanum* transmission to canid intermediate hosts is by the ingestion of infected *A. maculatum* (Vincent-Johnson, 2003; Ewing et al., 2003; Johnson et al., 2009a). Once introduced into the canine host's alimentary tract, sporozoites are liberated from oocysts and sporocysts in response to

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gastric juices, a phenomenon demonstrated *in vitro* using canine bile (Ewing et al., 2000).

Freed sporozoites are thought to penetrate the gut lining and disseminate to other tissues,

but as with *H. canis*, the route of dispersion remains unclear (Cummings et al., 2005). As

soon as 3½ weeks after exposure, parasite meronts are found within host cells, likely

monocytes, that are principally located between individual fibers of skeletal and cardiac

muscle tissues (Ewing et al., 2000; Cummings et al., 2005). Maturing meronts of *H.*

americanum do not have a characteristic "wheel-spoked" arrangement of zoites, but

rather, exhibit blastophore formation (Vincent-Johnson, 2003), and appear to transform

host cells (Panciera and Ewing, 2003; Ewing and Panciera, 2003; Cummings et al.,

2005). In histological preparations of muscle tissue, parasitized cells are surrounded by

concentric strata of a mucopolysaccharide-rich material reminiscent of onion skin layers

(Panciera and Ewing, 2003; Ewing and Panciera, 2003). The lesions are aptly termed

"onion skin" cysts (Panciera et al., 1999; Ewing and Panciera, 2003; Cummings et al.,

2005). Over time, meronts overtake and rupture host cells, thereby liberating merozoites

which breach degenerating cyst walls. Merozoites incite local influxes of inflammatory

cells that often progress to granulomata (Panciera et al., 1998; Ewing and Panciera, 2003;

Cummings et al., 2005). Distinct populations of macromerozoites and micromerozoites

as are seen in *H. canis* infections have not been observed (Panciera et al., 1999; Panciera

et al., 2001; Cummings et al., 2005). It is hypothesized that some merozoites develop

into gamonts after invading new leukocytes while others distribute hematogenously to

new sites and continue to reproduce asexually (Panciera et al., 1998; Ewing et al., 2000;

Ewing and Panciera, 2003; Cummings et al., 2005). Gamonts, usually present in less

than 0.1% of circulating white blood cells, are observable on blood smears as soon as

four to five weeks after infection, primarily during the acute stage of disease (Ewing and Panciera, 2003; Johnson et al., 2009b).

Clinical Signs and Diagnosis of *H. americanum* Infections in Domestic Dogs

In experimental infections, dogs often present with symptoms of ACH four to five weeks after ingesting *H. americanum* oocysts (Panciera and Ewing, 2003). Salient clinical features of ACH include fever, lethargy, mucopurulent ocular discharge, pain and reluctance to move, altered gait, and muscle atrophy (Vincent-Johnson et al., 1997; Ewing et al., 2000; Macintire et al., 2001; Panciera and Ewing, 2003; Vincent-Johnson, 2003). Laboratory findings may reveal neutrophilic leukocytosis, which is sometimes profound, and anemia (Vincent-Johnson et al., 1997; Panciera and Ewing, 2003; Vincent-Johnson, 2003). In severe cases, symmetric periosteal bone proliferation, particularly of the long bones, is evident on radiographs (Vincent-Johnson et al., 1997; Panciera et al., 2000; Panciera and Ewing, 2003; Vincent-Johnson, 2003). Dogs infected with *H. americanum* may exhibit waxing and waning courses of clinical disease over time, with clinical relapses attributed to the periodic release of merozoites from tissue meronts and associated inflammation (Panciera et al., 1998; Macintire et al., 2001; Vincent-Johnson, 2003). Although chronically infected animals have been reported, ACH patients often die within 12 to 24 months without supportive therapies (Macintire et al., 2001; Ewing et al., 2003; Vincent-Johnson, 2003).

Clinical signs, blood count abnormalities (particularly neutrophilia), observation of rare gamonts in blood smears, and characteristic osteal lesions on radiographs are findings that often lead to a diagnosis of ACH (Vincent-Johnson et al., 1997; Ewing and Panciera, 2003; Holman and Snowden, 2009; Potter and Macintire, 2010). Muscle

biopsy, although invasive, is considered the gold-standard method for achieving a definitive diagnosis, as parasite or parasite-induced lesions can readily be observed in histopathologic stained sections of the biopsied sample (Ewing and Panciera, 2003; Holman and Snowden, 2009; Bowman, 2009; Potter and Macintire, 2010). PCR methods have been developed for detecting circulating *Hepatozoon* spp., but may lack sensitivity in *H. americanum* infections due to low levels of parasitemia (Allen et al., 2008; Li et al., 2008; Potter and Macintire, 2010). Xenodiagnosis is a dependable, sensitive method used experimentally to detect *H. americanum* infections (Ewing and Panciera, 2003).

<div align="center">Other Vertebrate Hosts of H. americanum</div>

Experiments conducted by Johnson et al. (2008a, 2009b) to establish the susceptibility of several preferred hosts of immature instars of *A. maculatum* demonstrated development of cystozoites in the tissues of mice (*Mus musculus*) and New Zealand white rabbits (*Oryctolagus cuniculus*) four and 24 weeks, respectively, after ingestion of *H. americanum* oocysts. The cyst-laden tissues were, in turn, infective to dogs ingesting them. Parasite development and clinical disease in dogs occurred as described in infections resulting from sporozoite ingestion (Johnson et al., 2008a; Johnson et al., 2009a; Johnson et al., 2009b). The susceptibility of other hosts to *H. americanum* infection, and parasite development of cystozoites within these hosts, although experimental, suggests that paratenic hosts for *H. americanum* could be a source of infection for dogs and confirms that predation, either of infected prey or prey infested with infected ticks, is a possible significant epidemiologic factor in natural transmission cycles (Johnson et al., 2009a; Johnson et al., 200b). Focused wildlife survey studies conducted in ACH enzootic areas in Oklahoma have documented *Hepatozoon* spp.

infections in trapped rodents and hunted rabbits (Johnson et al, 2007; Johnson et al., 2009a), but thus far, confirmed natural *H. americanum* infections as evidenced by microscopic and molecular data have only been reported in domestic dogs and coyotes (Macintire et al., 1997; Panciera et al., 1997; Kocan et al., 2000; Allen et al., 2008; Li et al., 2008).

Feeding behaviors of *A. maculatum* instars also argue predator-prey relationships are significant in the transmission of *H. americanum* in nature. Although *A. maculatum* larvae and nymphs will feed on canids in a laboratory setting (Ewing et al., 2003), in nature, immature instars of *A. maculatum* tend to be found predominantly on small mammals and ground-dwelling birds, especially cotton rats (Barker et al., 2004) and meadow larks (Teel et al., 1998; Ketchum et al., 2006). Adults are more commonly ectoparasites of larger mammals including cattle, deer, and pigs, but are occasionally found on dogs and coyotes (Kocan et al., 1999; Barker et al., 2004; Ketchum et al., 2006). As development of *H. americanum* within its definitive host requires up to 42 days and occurs through tick ecdysis, and evidence of transovarial transmission in the tick is lacking (Mathew et al., 1999; Ewing et al., 2002b), adult *A. maculatum* must acquire the parasite as larvae or nymphs, most likely by feeding on smaller, not yet identified, vertebrate carriers in nature (Johnson et al., 2009b). Additionally, small non-reservoir hosts may be infested with *H. americanum*-infected *A. maculatum*, and canids preying or scavenging on these animals may inadvertently ingest infective ticks while consuming prey species (Johnson et al., 2009b). Interestingly, although *A. maculatum* is found in areas of Central and South America (Sumner et al., 2007), confirmed reports of

ACH have not been documented in these areas (Little et al., 2009), which suggests the absence of a key natural reservoir host of *H. americanum* in these regions.

In ACH enzootic areas of Oklahoma, approximately half of surveyed coyotes have had muscle stages of a parasite that resemble those seen in domestic dogs infected with *H. americanum* (Kocan et al., 1999; Kocan et al., 2000; Ewing et al., 2000; Ewing et al., 2003). Based on the prevalence of *H. americanum* in coyotes in enzootic areas, some researchers suspect coyotes are an important reservoir host of the parasite (Kocan et al., 1999; Garrett et al., 2005). However, others argue that domestic dogs and coyotes are most likely accidental hosts of *H. americanum* and the parasite is probably cycled between *A. maculatum* and an unidentified vertebrate reservoir host (Ewing et al., 2002b; Panciera and Ewing, 2003; Vincent-Johnson, 2003).

Severity of disease observed in domestic dogs suggests that they are not well-adapted hosts to this parasite (Vincent-Johnson, 2003). Although coyotes are reported to tolerate infection with *H. americanum* somewhat better than their domesticated counterparts, naturally infected coyotes develop pathognomonic muscle and bone lesions, and experimentally infected animals display clinical disease consistent with ACH (Kocan et al., 1999; Ewing et al., 2000; Kocan et al., 2000; Ewing et al., 2003; Panciera and Ewing, 2003; Garrett et al., 2005). Severity of disease may be greater in younger animals or those ingesting high numbers of *H. americanum* oocysts (≥ 100) (Kocan et al., 2000; Garrett et al., 2005). It is also possible that coyotes with mild or inapparent symptoms of *H. americanum* infection are acutely ill at one time, but clinically improve, as is the case with *H. americanum* infections in chronically infected domestic dogs (Ewing et al., 2003).

TREATMENT OF CANINE HEPATOZOONOSIS

Hepatozoon canis infections are most commonly treated with imidocarb dipropionate twice monthly, administered subcutaneously at 5-6 mg/kg, until gamonts are no longer evident in patient blood smears for 2-3 consecutive months (Macintire et al., 2001; Sasanelli et al., 2010). The mechanism of action of this compound is not well understood (Sasanelli et al., 2010). Although clinical improvement of patients may occur, this drug does not clear *H. canis* at its currently recommended dose (Sasanelli et al., 2010). *Hepatozoon canis* DNA is detectable in peripheral blood by PCR for weeks following treatment end, even though gamonts have not been microscopically observable on blood or buffy coat smears for several months (Sasanelli et al., 2010). Still, clinical signs due to *H. canis* infection can be well-controlled in many patients with this drug compound, although relapses may occur (Macintire et al., 2001; Sasanelli et al., 2010).

Currently, the Companion Animal Parasite Council (CAPC) (www.capcvet.org) recommends presenting ACH patients be treated with either a triple combination of trimethoprim-sulfadiazine (15 mg/kg, twice daily), clindamycin (10 mg/kg, thrice daily), and pyrimethamine (0.25 mg/kg, once daily) or ponazuril (10 mg/kg, twice daily) for 14 days to kill merozoites in tissues. It is advised that initial parasiticide treatment be followed by two years of twice daily decoquinate administration (10 to 20 mg/kg), which serves to prevent or delay clinical relapse by continually arresting zoites as they are released from meronts (Macintire et al., 2001; Vincent-Johnson, 2003). Supplemental non-steroidal anti-inflammatory drugs may be given for fever and pain control (Ewing et al., 2000; Macintire et al., 2001; Vincent-Johnson, 2003).

Triple therapy with Trimethoprim-sulfadiazine, clindamycin, and pyrimethamine, or TCP, is aimed at inhibiting parasite folic-folinic acid metabolism, and is used to treat toxoplasmosis in dogs and cats. Decoquinate is classified as a coccidiostat, but in higher concentrations it is cocciocidal, and targets parasite mitochondria. It is commonly used as a preventative against coccidiosis in chickens, sheep, goats, and rabbits (Macintire et al., 2001). Combined TCP and decoquinate treatment was evaluated in naturally infected dogs in a study conducted by Macintire et al. (2001). Although this treatment regimen is not curative, it does extend life expectancy and increase quality of life for many ACH patients (Macintire et al., 2001). Should clinical relapse occur, it is recommended that TCP or ponazuril treatments be repeated and again followed by long-term decoquinate administration (Macintire et al., 2001; Vincent-Johnson, 2003).

Ponazuril (toltrazuril sulfone) is the active ingredient in Marquis™ (Bayer HealthCare LLC, Shawnee Mission, USA), which is approved by the Food and Drug Administration only for the treatment of equine protozoal myeloencephalitis (EPM) (Charles et al., 2007; Mackay et al., 2008). In Europe, toltrazuril (Baycox® 5%, Bayer HealthCare, Animal Health, Monheim, Germany) is labeled for the treatment of coccidiosis in piglets and poultry, and in Switzerland and Denmark, the drug is labeled for piglets and cattle (Mundt et al., 2007). Also, a toltrazuril derivative (Vecoxan®, Janssen Animal Health, Beerse, Belgium) is registered for the treatment of coccidiosis in sheep, cattle, and poultry in Europe (Mundt et al., 2007).

In the United States, although ponazuril (toltrazuril sulfone) is labeled solely for EPM treatment, this drug is widely used as an effective treatment of *Cystoisospora* spp. infections in young dogs and cats (www.capcvet.org; Charles et al., 2007) as well as a

preventative against coccidiosis in chickens (Mitchell et al., 2005; Charles et al., 2007). Ponazuril has been shown to inhibit development of other tissue-cyst forming protozoans including *Toxoplasma gondii* and *Neospora caninum* in mice and *in vitro* systems (Mitchell et al., 2004; Mitchell et al., 2005). Although a currently accepted alternative to TCP for treatment of ACH patients, ponazuril had not been evaluated experimentally in *H. americanum* infections for clinical sign alleviation or clearance of parasite prior to the research reported in this dissertation.

OTHER SPECIES OF *HEPATOZOON*

Collectively, more than 300 species of *Hepatozoon* have been documented world-wide, but the naming of new species has predominately been based on discovery and characterization in novel vertebrate hosts (Smith, 1996). Complete life cycle descriptions are available for very few of the *Hepatozoon* species documented (Smith, 1996). Experimental evidence has demonstrated the plasticity of some *Hepatozoon* spp. in their abilities to infect alternate hosts (Smith, 1996; Smith and Desser, 1997; Moço et al., 2002; Johnson et al., 2008a; Johnson et al., 2008b; Johnson et al., 2009b). Thus, the estimated number of distinct *Hepatozoon* species is thought by some to be exaggerated (Smith, 1996).

In his review of the genus, Smith (1996) lists documented *Hepatozoon* spp. according to vertebrate host group. The different species numbered 1 in amphibians and salamanders, 6 in crocodilians, 19 in birds, 42 in anurans, 74 in lizards, 46 in mammals,

and 121 in snakes. Invertebrate hosts of *Hepatozoon* spp. include ticks, mites, lice, fleas, reduviid bugs, sand flies, tse tse flies, mosquitoes, and leeches.

GENETIC CHARACTERIZATION OF *HEPATOZOON* SPECIES

Due to the abilities of certain *Hepatozoon* species to infect multiple hosts, genetic characters are now considered important by some in descriptions of new species, in addition to classical criteria (Baneth et al., 2000; Mathew et al., 2000; Forlano et al., 2007). Currently, there are approximately 200 genetic sequences listed in the National Center for Biotechnology Information database (NCBI), GenBank, the majority of which are reported from areas other than North America. The predominance of data are18S rRNA gene sequences, although a few internal transcribed spacer (ITS) (Boulianne et al., 2007; Smith et al., 1999), 5.8S rRNA gene (Smith et al., 1999), and plastid RNA gene sequences are available (Lang-Unnasch et al., 1998). Only nine of the over 300 different *Hepatozoon* species documented have genetic sequence reported in GenBank, which are listed in Appendix A (pp. 147-152). Many sequences of unidentified *Hepatozoon* spp. are listed in GenBank with the hosts in which they were collected identified. These include sequences gathered from invertebrates, reptiles, marsupials, rodents, felids, and canids, and are also listed in Appendix A.

HEPATOZOON SPECIES INFECTING VERTEBRATES

IN NORTH AMERICA

To date, there are 29 species of *Hepatozoon* reported from North America. Species documented in snakes include *H. bradfordi, H. brumpti, H. crotali, H. digueti, H. eurytopis, H. fasciatae, H. fusifex*, *H. guttata, H. horridus, H. karyolysi, H. lahillei, H. mansoni, H. mocassini, H. pictiventris, H. pituophis, H. polytopis, H. rarefaciens*, *H. rexi, H. sauritus, H. seminatrici, H. sipedon, H sistruri, H. thamnophis,* and *H. wardi* (Smith, 1996; Telford et al., 2001; Telford et al., 2002; Telford et al., 2005a; Telford et al., 2005b; Telford et al., 2008; Telford, 2010). *Hepatozoon* species reported in mammals include *H. muris* in rats (*Rattus norvegicus*) (Eyles, 1952), *H. procyonis* in raccoons (*Procyon lotor*) (Clarke et al., 1973; Schaffer et al., 1978; Pietrzak et al., 1998), *H. griseisciuri* in grey squirrels (*Sciurus carolinensis*) (Davidson and Calpin, 1976), *H. americanum* in domestic dogs and coyotes (Vincent-Johnson et al., 1997; Kocan et al., 2000), and more recently, *H. canis* in domestic dogs (Allen et al., 2008; Little et al., 2009). Undetermined species have been reported in a domestic cat (*Felis catus*) (Lane and Kocan, 1983), bobcats (*Lynx rufus*), and ocelots (*Leopardus pardalis*) (Lane and Kocan, 1983; Mercer et al., 1988).

Genetic Sequences of Vertebrate *Hepatozoon* Species

in the United States

Hepatozoon spp. sequence contributions from the United States are particularly limited; less than 20 of the approximately 200 *Hepatozoon* spp. sequences currently listed in GenBank are reported from this country. All data reported from the United States are 18S rRNA gene sequences. Prior to 2007, only *Hepatozoon* sequences obtained from

domestic dogs were available in the NCBI database (Mathew et al., 2000; Baneth et al., 2000; Paludo et al., 2005). Johnson et al. (2007) contributed one sequence collected from cotton rats (*Sigmodon hispidus*) and one sequence collected from white-footed mice (*Peromyscus leucopus*) to the database in 2007. Allen et al. (2008) added six more sequences obtained from domestic dogs in 2008, and in 2009, Johnson et al. (2009a) added a sequence from a cottontail rabbit (*Sylvilagus floridanus*) and a swamp rabbit (*Sylvilagus aquaticu*s). Sequence GU344682 gathered from a turkey vulture (*Cathartes aura*) was contributed to the database in 2010. To date, all *Hepatozoon* spp. sequences added to GenBank from the United States after 2007 have been collected from animals in Oklahoma.

TRANSPLACENTAL TRANSMISSION OF *HEPATOZOON* SPECIES

Although accepted routes of *Hepatozoon* spp. transmission to intermediate hosts are by the ingestion of sporozoites or cystozoites, transmission has been reported to occur across the placenta in certain species (Clark, 1958; Murata et al., 1993). In 1958, Clark documented the presence of circulating *H. griseisciuri* gamonts in 19 of 21 suckling grey squirrels born of infected mothers. A pup euthanitized at 36 hours of age was found to have shizonts in visceral organs in addition to peripheral gamonts, suggesting the animal had been infected prior to birth. Previous work on *H. muris* documented peripheral gamonts in rats 12 to 14 days after infection by the oral route (Clark, 1958). Clark suspected that had the neonatal squirrel been infected by the conventional route, the interim between infection and parasite patency would likely require longer than three

days. Thus, he deduced that the parasite was congenitally acquired, likely via the placenta.

Murata et al. (1993) monitored *H. canis* infections in six litters of beagle pups born of three naturally infected dams. Animals were maintained in acaracide treated cages during monitoring periods. Fourteen pups comprising five litters from two infected dams were positive for circulating parasite after 21 to 31 days. Initially, gamonts were present in 0.02%-0.04% of leukocytes observed, but after several months were present in as many as 3.3%. Although it is possible that gamonts were transplacentally transferred from dams to pups and transiently circulated, the increase in gamont numbers in pups in the months after birth suggests active parasite merogony and gamont production. In the same study study, meronts were observed in the main visceral organs of one of two pups that died. Also, four pups whelped from a third infected dam were positive for peripheral parasite four weeks after birth, indicating *H. canis* establishment and development in these animals

TRANSPLACENTAL TRANSMISSION OF *H. AMERICANUM*

Although speculated to occur, transplacental transmission of *H. americanum* in naturally infected dogs has not been reported. Most ACH cases are of singly presenting patients residing in rural areas (Ewing et al., 2003; Johnson et al., 2009a). Patient histories often include behaviors of roaming and predatory tendencies (Johnson et al., 2009a). The youngest reported age of *H. americanum* infection is 11 weeks, which is an age allowing enough time from exposure to clinical presentation by either of the two

established routes of infection (Ewing and Panciera, 2003; Johnson et al., 2009a; Johnson et al., 2009b). Documented outbreaks of ACH have also occurred in rural settings, in animals old enough to roam at will or to be used in recreational hunting pursuits (Johnson et al., 2009a), not in young litter mates still relying heavily on their mothers for survival. Experiments assessing the occurrence of transplacental transmission in *H. americanum* infections have not been reported.

SUMMARY OF RESEARCH

Canine hepatozoonosis caused by *H. canis* was first recognized more than a century ago in India, and has since been recognized in many areas of the world (Mathew et al., 2000; Baneth et al., 2007). However, interestingly, canine hepatozoonosis in North America was not documented until 1976, but the etiological agent was misidentified as *H. canis* until 1997 (Davis et al., 1978; Craig et al., 1978; Vincent-Johnson et al., 2003). After the recognition of *H. americanum* as the causative agent of American canine hepatozoonosis, *H. canis* was generally not thought to be in the United States (Baneth et al., 2007; Allen et al., 2008; Little et al., 2009). Survey studies evaluating genetic sequences of *Hepatozoon* species infecting domestic dogs in North America have traditionally been lacking, which was the impetus behind the research presented in the third chapter of this dissertation.

In domestic dogs, disease associated with *H. americanum* infection is often clinically severe and ultimately fatal (Macintire et al., 2001; Ewing et al., 2003; Vincent-Johnson, 2003). Studies examining the efficacies of drug compounds against this parasite are relatively limited. The currently recommended 14-day triple therapy with TCP as an initial parasiticide followed by two-year decoquinate administration has been evaluated experimentally in naturally infected dogs, and is documented to rescue moribund patients and control clinical relapse (Macintire et al., 2001; Vincent-Johnson, 2003). However, ponazuril (toltrazuril sulfone), the alternative recommended initial parasiticide for the treatment of ACH, had not been experimentally evaluated for efficacy in clearance of parasite and resolution of clinical disease in dogs infected with *H. americanum* until the pilot treatment trial presented in Chapter 4.

Over 300 species of *Hepatozoon* have been reported world-wide in poikilotherms, mammals, and some birds (Smith, 1996). Genetic characterization has become a contemporary criterion in the complete description of established *Hepatozoon* species and the proposition of novel species (Baneth et al., 2000; Mathew et al., 2000; Forlano et al., 2007; Johnson et al., 2007). Reported genetic data of *Hepatozoon* species cycling in the United States are few. The research detailed in the fifth chapter of this dissertation was conducted to genetically characterize *Hepatozoon* spp. infecting previously recognized and novel vertebrate hosts in the United States, as well as to assess the phylogenetic diversity of the species collected and interpret possible transmission patterns in nature from the genetic relationships observed.

Although ingestion of infective zoite stages is conventionally considered the requisite mode of *Hepatozoon* spp. transmission to intermediate hosts, transplacental transmission is a documented alternate route in several species of *Hepatozoon*, including *H. canis* (Clark, 1958; Murata et al., 1993). This particular mode of transmission is also suspected in *H. americanum* infections, but experiments assessing its occurrence are lacking (Ewing and Panciera, 2003; Potter and Macintire, 2010). To better understand the ecology of this parasite and epidemiology of ACH, an experiment designed to assess transplacental transmission in a chronically infected dam is outlined in Chapter 6.

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CHAPTER III

DIVERSITY OF *HEPATOZOON* SPECIES IN NATURALLY INFECTED DOGS

IN THE SOUTHERN UNITED STATES[1]

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. **Key Words**: Apicomplexa; *Hepatozoon americanum*; *Hepatozoon canis*; Hepatozoonosis, Polymerase Chain Reaction (PCR).

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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 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., 2007). 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.

MATERIALS AND METHODS

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).

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).

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.

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.

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; Figure. 1, p. 81). Two dogs that presented with clinical signs of ACH to the Oklahoma State University Veterinary Teaching Hospital were also infected with *H. americanum*, based on 18S rDNA sequence (EU146066 and

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EU146067). Respectively, these sequences were 95.7% and 97.2% similar to previously published *H. americanum* sequence (AY864676; Figure 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 (Figure. 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.

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., 2008). 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 shelter 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., 2003a; Ewing and Panciera, 2003). However, our data suggest that these earlier infections could also represent either *H. canis* infection or coinfections 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

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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 nine 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 coinfections, 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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	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								-

Figure 1. **Percent Similarities of 18S rDNA Sequences from *Hepatozoon* Spp. of Dogs.**

*A.C. = Random-source dog residing in an animal control facility (Payne County)
*Clinic Dog = Dog presented to OSU Veterinary Teaching Hospital with clinical ACH
*Muskogee = Dog residing in ACH-endemic area (Muskogee County)

CHAPTER IV

TREATMENT OF *HEPATOZOON AMERICANUM* INFECTION:

REVIEW OF THE LITERATURE AND EXPERIMENTAL EVALUATION OF EFFICACY[1]

ABSTRACT

There is no labeled treatment for dogs with American canine hepatozoonosis (ACH), but the drug therapies discussed in this article, although not rapidly curative, may be successful in alleviating acute clinical signs, prolonging life, reducing the number of clinical relapses, and enhancing quality of life. This article also describes a pilot trial conducted to assess the efficacy of a novel treatment approach with ponazuril as a stand-alone parasiticide administered for four weeks without follow-up decoquinate treatment. Although extended ponazuril treatment in combination with NSAID administration did ameliorate acute clinical signs associated with ACH, the parasite was not completely cleared with this treatment protocol alone. Long-term decoquinate therapy remains a critical component of successful treatment of ACH. **Keywords:** Apicomplexa; American canine hepatozoonosis (ACH); Polymerase chain reaction (PCR).

INTRODUCTION

Hepatozoon americanum is an apicomplexan that causes American canine hepatozoonosis (ACH), a painful disease in dogs in North America (Panciera et al., 1998; Ewing et al., 2000). Dogs become infected with *H. americanum* by ingesting ticks infected with sporulated oocysts or prey infected with quiescent cystozoite stages of the protozoan parasite (Johnson et al., 2008; Johnson et al., 2009; Little et al., 2009). The natural reservoir host(s) has not been clearly identified, but coyotes, rabbits, rodents, and other vertebrate prey species may play a role in maintaining a cycle of infection in nature (Kocan et al., 2000; Johnson et al., 2008; Little et al., 2009). Clinical signs of ACH include fever, mucopurulent ocular discharge, reluctance to move, wasting, gait

disturbance, and abnormal blood profiles revealing extreme neutrophilia (Vincent-Johnson et al., 1997; Ewing et al., 2000; Macintire et al., 2001; Vincent-Johnson, 2003 Little et al., 2009). Without supportive drug therapy, dogs often worsen in clinical condition and die within one to two years (Macintire et al., 2001).

The only known tick vector of *H. americanum*, *Amblyomma maculatum*, was historically restricted to US states bordering the Gulf Coast, but recent data indicate its establishment in central states as far inland as Iowa and Illinois and in states bordering the Atlantic Coast as far north as Maryland and Delaware (Ewing et al., 2002; Paddock et al., 2008). At the same time, the distribution of *H. americanum* infections in domestic dogs, previously considered enzootic in the southeastern United States, has expanded to include several south-central, southeastern, midwestern, and mid-Atlantic states. A recent nationwide survey of real-time polymerase chain reaction (PCR) assay results revealed *H. americanum* infections in areas of the country where cases of ACH have not previously been documented (Li et al., 2008). Domestic travel of pet owners and relocation of rescue dogs have likely contributed to the introduction of *H. americanum* into previously unreported areas. Thus, ACH is becoming an increasingly important disease to the health of domestic dogs throughout the United States. However, despite increasing national recognition of ACH as an emerging disease in the field of veterinary medicine, a rapidly curative treatment has not yet been identified (Macintire et al., 2001; Vincent-Johnson et al., 2003).

No drug therapies are labeled for the treatment of *H. americanum* infection. Patients with ACH are prescribed palliative drug therapies, such as carprofen, to aid in pain relief and parasiticides to control the infection (Macintire et al., 2001; Vincent-

Johnson et al., 2003). A study conducted by Macintire et al. (2001) assessed the efficacies of toltrazuril and a combination of trimethoprim-sulfadiazine, clindamycin, and pyrimethamine (TCP) in naturally infected, client-owned dogs with histologically confirmed merozoite stages of *H. americanum* in muscle tissue. The dogs were divided into groups that received toltrazuril for 5 days or 10 days, TCP for 14 days without follow-up decoquinate treatment, or TCP for 14 days with follow-up decoquinate treatment. Dogs were followed for up to two years. Results of the study indicated that the 14-day TCP treatment period followed by long-term daily decoquinate administration, although not curative, was the drug regimen that often gave the most favorable prognosis.

The Companion Animal Parasite Council (CAPC) (www.capcvet.org) currently recommends 14-day initial parasiticide treatments with either a triple combination regimen of TCP or ponazuril (toltrazuril sulfone) alone, followed by a minimum of two years of twice-daily decoquinate to prevent relapse. Clinical signs often recur if treatment is not consistently administered as directed (Macintire et al., 2001). Data supporting current recommendations for ACH drug therapy protocols are gathered from naturally occurring cases. Although such cases are of clinical value, patient histories almost invariably lack detailed information regarding time, route, and dose of parasite inoculation. Also, clinical follow-up data often depend on pet owner compliance. Convalescing dogs may not be monitored in a controlled setting with consistent clinical evaluation and testing. Thus, it is difficult to broadly assess parasite response to different treatments in naturally infected dogs over time.

In this paper, we review evidence supporting currently recommended treatments

for ACH and describe a pilot trial that evaluated a four-week regimen of oral ponazuril for its ability to limit infection in an experimentally infected dog. For this purpose, one experimentally infected dog was treated with ponazuril and carprofen (for pain), one experimentally infected dog was treated with carprofen only, and one uninfected dog was administered ponazuril and carprofen and served as a negative control. The dogs were evaluated for parasite presence by observation of clinical signs, muscle biopsy histology, and PCR assay of whole blood and muscle tissue.

MATERIALS AND METHODS

Dogs

All animals in this study were cared for according to the principles outlined by the Institutional Animal Care and Use Committee at Oklahoma State University, College of Veterinary Health Sciences, Laboratory Animal Resources, Stillwater, Oklahoma. Three dogs obtained from a commercial vendor were housed in the animal resources facility at Oklahoma State University for several months before the start of this study. Before experimental infection, whole blood from all three dogs was collected in EDTA and evaluated by complete blood count (CBC) to verify normal blood profiles. PCR assay of whole blood, performed as described elsewhere (Allen et al., 2008), was carried out to confirm negative PCR status for *H. americanum* and *H. canis* of dogs. Muscle biopsy samples, collected under anesthesia, were examined histologically and tested by PCR to ensure that none of the dogs harbored muscle stages of *H. americanum*.

Experimental Infection of Dogs

Two of the three dogs used in this study were infected by the administration of approximately 40 mature oocysts harvested from molted adult *A. maculatum* ticks that had fed as nymphs on an experimentally and chronically infected carrier dog. The oocysts were enumerated, suspended in physiologic saline, and mixed with wet, canned dog food. The two dogs were observed to eat the entire serving of dog food containing the oocyst suspensions. The third dog served as a negative control to evaluate drug safety only and was not exposed to oocysts.

Sample Collection and Clinical Monitoring

After inoculation of the two dogs, whole blood from all three dogs was collected in EDTA for PCR assay and CBC every 2 weeks for 6 weeks, then weekly for 4 weeks until the infected dogs were observed to both display clinical signs of acute disease and test positive by PCR for DNA of parasites circulating in whole blood. Muscle biopsy samples were collected from all three dogs at 10 weeks post exposure to confirm the presence of established parasite stages in muscle tissue of experimentally infected dogs by histopathology and PCR before beginning treatment. All three dogs were evaluated weekly by PCR, CBC, and observation of clinical signs during the treatment period, and at 2, 3, and 8 weeks after treatment end. The uninfected negative-control dog was then released from the study. The infected dogs were evaluated by PCR assay of whole blood on seven more occasions and a final CBC analysis conducted 15 weeks after ponazuril treatment end. The dogs were euthanitized and necropsied 43 weeks after experimental infection, and blood and muscle tissue samples were harvested for testing by PCR.

Treatments

One of the two experimentally infected dogs was treated with 10 mg/kg ponazuril (Marquis™, Bayer HealthCare LLC), donated by Bayer HealthCare LLC, administered orally every 12 hours for 4 weeks. Ponazuril treatment was withheld from the second experimentally infected dog, which served as a control to monitor disease progression without parasiticide intervention. Both infected dogs were given 2.2 mg/kg carprofen (Rimadyl®, Pfizer Animal Health) to control pain during the ponazuril treatment period and until the study ended. The third, uninfected dog served as a drug safety control and was administered identical treatments to the infected, ponazuril-treated dog.

Complications

One of the dogs had several epileptic seizures at the beginning of the study after initial sedation for radiography. The condition was noted before experimental infection by oocyst administration and before administration of any treatment and thus was deemed incidental. The dog was managed daily with anticonvulsive medications. No more seizures were observed, and the dog later served as the infected, untreated (no ponazuril) control in the study.

RESULTS

Clinical Signs Displayed by the Experimentally Infected Dogs

The infected dog designated to receive ponazuril treatment began to display early signs of ACH seven weeks after inoculation with *H. americanum* oocysts. During the time between first clinical signs displayed and ponazuril treatment, this dog's clinical signs consistently included malaise, inappetence, and mucopurulent ocular discharge.

Elevated body temperatures were noted for this dog in weeks 7 through 9 post exposure (Table 1, p. 98). At 10 weeks post inoculation, the dog was observed to have a stiff and painful gait characteristic of dogs infected with *H. americanum*.

After one week of treatment with ponazuril and carprofen, this dog's fever subsided and other signs of disease (ocular discharge, inappetence, stiffness) were observably less pronounced. Signs associated with acute ACH did not return during the 29-week monitoring period after ponazuril treatment ended.

The infected dog that did not receive ponazuril treatment began to display early clinical signs of ACH 10 weeks post inoculation. Signs included mucopurulent ocular discharge and neutrophilia, but not malaise, inappetence, or stiffness in gait. This dog's body temperature was only marginally elevated in the seventh week post inoculation (Table 1). The dog was administered carprofen only and never developed more severe signs of ACH.

The unexposed dog designated to serve as the drug safety control showed no clinical signs of disease during the interim between the inoculation of infected dogs and start of treatments. The ponazuril and carprofen administered had no apparent ill effect on the health of this dog during the treatment period. The dog remained healthy after treatment was ceased through its remaining time in the study.

Neutrophil Counts

The infected dog treated with ponazuril showed slight neutrophilia seven weeks after exposure to oocysts (Table 1). This dog's neutrophil count continued to rise for the next three weeks, reaching 27,378 cells/µl at 10 weeks post inoculation, the week ponazuril and carprofen treatment commenced. After one week of treatment and for the

next seven sampling times over a period of 14 weeks, this dog's neutrophil count was within the normal reference range (2060 to 10,600 cells/μl). However, 15 weeks after ponazuril treatment cessation (29 weeks after experimental infection), marked neutrophilia (18,056 cells/μl) was again observed.

The infected dog treated only with carprofen had a neutrophil count of 13,970 cells/μl nine weeks after exposure. At the time of ponazuril treatment of the other infected dog (week 10), this dog's neutrophil count had increased to 21,725 cells/μl and remained elevated for the next four weeks. Neutrophil counts decreased to within normal range after four weeks of carprofen treatment but were found to be high for the next three sampling intervals over a seven-week period. After 19 weeks of carprofen treatment (29 weeks after experimental infection), this dog's neutrophil count was 23,408 cells/μl.

The unexposed control dog maintained normal neutrophil counts throughout the study.

Biopsies

Muscle biopsy samples taken from all three dogs one week before inoculation were negative for *H. americanum* by PCR and negative for parasite stages and characteristic inflammatory lesions by histology. Samples were taken again at 9 weeks post inoculation (one week before treatment commencement) and confirmed parasite presence in the experimentally exposed dogs and parasite absence in the uninfected control dog. Muscle tissue samples taken at necropsy (43 weeks post inoculation) from the two experimentally infected dogs were tested by PCR and examined histologically. Parasite was detected by PCR and was observed microscopically in samples collected from both dogs.

Polymerase Chain Reaction (PCR) Assay of Whole Blood

The first positive PCR assay result in the infected dog designated for ponazuril treatment was obtained two weeks after inoculation (Table 1), but parasitemia subsequently decreased to undetectable levels for four weeks. A positive PCR result was again observed for this dog at 7 weeks post exposure. For the next seven consecutive weeks, including the four weeks of ponazuril treatment, this dog tested positive for circulating parasite. Positive PCR results were observed for this dog twice more between treatment cessation and study end and on the final day of the study, 43 weeks after experimental exposure to *H. americanum* oocysts.

The infected dog treated with carprofen only also had a positive PCR assay result two weeks after inoculation. Subsequent PCR results were negative until 7 weeks post infection; they then remained (intermittently) positive for 15 weeks of the study. However, whole blood from this dog tested negative for circulating parasite on the final day of the study.

The unexposed control dog had negative PCR assay results for *H. americanum* infection throughout the entire time used in the study.

DISCUSSION

Current therapies for ACH often result in prolonged survival and relief of clinical signs in carefully managed patients (Macintire et al., 2001; Vincent-Johnson, 2003). However, recommended parasiticides administered for a period of 14 days (as indicated) do not clear muscle cyst stages of *H. americanum* (Macintire et al., 2001). Ponazuril is a major metabolite of toltrazuril, which is a coccidiosis preventive widely used in poultry

(Mitchell et al., 2005; Charles et al., 2007). Ponazuril (toltrazuril sulfone) is approved by the US Food and Drug Administration for the treatment of horses with equine protozoal myeloencephalitis (EPM) caused by *Sarcocystis neurona* (Mackay et al., 2008). *Sarcocystis neurona*, another oocyst-forming protozoan, disseminates systemically after ingestion and sequesters in neural tissue similar to the manner in which ingested *H. americanum* disseminates in its intermediate host and localizes in muscle tissue (Dubey et al., 2001; Cummings et al., 2005). In horses with EPM, the recommended ponazuril dose of 5 mg/kg administered orally for 28 days results in neurologic improvement in a large number of patients (Mackay et al, 2008). Although *S. neurona* may not be cleared from equine neurologic tissue, infections can often be controlled by ponazuril administration after clinical signs are evident or if ponazuril is administered prophylactically (Dubey et al., 2001; Furr et al., 2006).

Ponazuril is labeled only for use in horses with EPM, but it is highly effective against apicomplexans other than *S. neurona*. *In vitro* and *in vivo* studies have demonstrated its inhibitory effect on *Toxoplasma gondii* development (Mitchell et al., 2004). Studies in cell culture systems also indicate that *Neospora caninum* is vulnerable to ponazuril treatment (Mitchell et al., 2005). Ponazuril is often used as an off-label treatment for coccidiosis in dogs and cats. It may also be used as an alternative to TCP in initial treatment of ACH (www.capcvet.org). Although no clear advantage to the patient is reported for ponazuril compared to TCP treatment, the treatment regimen is considerably easier for pet owners. Rather than the recommended one, two, or three times daily administration of the three compounds in the TCP regimen, ponazuril is

administered only twice a day and, as a paste, can be diluted to the correct concentration and mixed with food.

Ponazuril has proved safe in juvenile dogs treated for coccidiosis at doses as high as 250 mg/kg (Charles et al., 2007). Also, to our knowledge, no reported studies have demonstrated that ponazuril is unsafe if administered for prolonged periods. Many researchers believe this compound shows promise as an effective preventive against EPM, toxoplasmosis, and coccidiosis when administered prophylactically (Mitchell et al., 2004; Furr et al., 2006). Although we did not evaluate it, the prophylactic activity of ponazuril (if any) could be of value for dogs in areas where they are likely to be exposed to *H. americanum*. However, we speculate that the use of ponazuril after ACH diagnosis is probably not rapidly curative because parasite meront stages are already encysted in muscle tissue (Macintire et al., 2001). *H. americanum* induces a waxing and waning course of disease, possibly due to repeat parasite merogonic cycles, merozoite release, and merozoite invasion of new muscle and cardiac tissue (Panciera et al., 1998; Macintire et al., 2001; Vincent-Johnson, 2003).

Currently recommended parasiticide compounds may initially control *H. americanum* infections by destroying circulating merozoites, but they are ineffective against parasite stages protected in muscle tissue cysts. It is possible that continued ponazuril treatment, thus far shown to have no deleterious effects on animal health, would continue to control disease in patients with ACH, but prolonged decoquinate administration is already recommended by CAPC for this purpose (Macintire et al., 2001).

In this study, we demonstrate that *H. americanum* is not cleared in an experimentally infected dog treated with ponazuril for a four-week period based on PCR assay of whole blood and PCR assay and histologic examination of biopsied muscle tissue. Although clinical signs of ACH resolved in the experimentally infected dog treated with ponazuril and carprofen, carprofen alone may have resulted in clinical improvement in the second experimentally infected dog. Neutrophil counts of both experimentally infected dogs were within the normal reference range after beginning treatment with ponazuril and carprofen or carprofen alone; however, values became elevated in the weeks after ponazuril treatment ended. Given that carprofen was administered from ten weeks post exposure until the end of study, neutrophil levels of infected dogs were likely not affected by this drug's administration, although it appears to have alleviated pain associated with inflammatory lesions in muscle tissue. Although clinical *H. americanum* infection did temporarily resolve in the ponazuril-treated dog, the dog's blood tested positive consistently by PCR for presence of parasite during and after the treatment periods. Blood and muscle tissue from this dog were PCR positive at necropsy, 31 weeks after treatment end.

CONCLUSIONS

The results from this pilot study indicate that ponazuril administration, together with NSAID administration, alleviates clinical signs associated with acute ACH. They also confirmed the necessity of long-term follow-up decoquinate administration as currently recommended. Without continued decoquinate management, ACH patients

with chronic disease may relapse; relapse probably results from recurrent parasite merogonic cycles.

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Table 1. **Temperature, Neutrophil Count, and PCR Assay Results of Dogs throughout Study.**

| | Experimental Infection Status | | | | | | | | |
| | Infected | | | Infected | | | Uninfected | | |
WPE	Temperature (°F)	Neutrophils/µl	PCR result	Temperature (°F)	Neutrophils/µl	PCR result	Temperature (°F)	Neutrophils/µl	PCR result
0	102.1	8384	–	101.8	7936	–	101.3	7412	–
1	101.2			101.6			102.1		
2	102.5	5452	+	102.3	7749	+	102.3	7705	–
4	102.3	7448	–	102.3	4932	–	102.9	7384	–
5	101.7			102.0			102.8		
6	102.5	6431	–	102.8	4185	–	102.5	6489	–
7	103.3	11,256	+	103.2	6345	+	102.8	6090	–
8	104.3	14,342	+	102.8	6885	+	102.4	6077	–
9	103.3	27,639	+	100.3	13,970	+	100.9	7458	–
10	103.0	27,378	+	101.1	21,725	+	101.4	10,295	–
Treatment	Ponazuril and carprofen			Carprofen			Ponazuril and carprofen		
11	102.5	9577	+	101.8	14,495	–	101.5	5394	–
12	100.8	9720	+	101.6	15,696	–	101.3	6630	–
13	102.3	9856	+	102.4	15,744	+	101.5	6392	–
14	100.6	6844	+	101.1	11,830	+	100.5	5820	–

Table 1 Continued. **Temperature, Neutrophil Count, and PCR Assay Results of Dogs throughout Study.**

	Carprofen			Carprofen			No treatment		
16	100.3	6954	+	102.4	5600	−	100.7	4312	−
17	101.1	7888	+	102.4	7198	+	100.7	5452	−
22	101.2	5472	−	102.6	17,384	−	102.3	6222	
25		8384	−		25,360	−			
28			−			−			
29		18,056	−		23,408	+			
30			−			−			
31			−			−			
32			−			−			
43			+			−			

*WPE = week post exposure, PCR = polymerase chain reaction.

CHAPTER V

NOVEL *HEPATOZOON* SPECIES IN VERTEBRATES

FROM THE SOUTHERN UNITED STATES[1]

ABSTRACT

Novel *Hepatozoon* spp. sequences collected from previously unrecognized vertebrate hosts in North America were compared with documented *Hepatozoon* 18S rRNA sequences in an effort to examine phylogenetic relationships between the different *Hepatozoon* organisms found cycling in nature. An approximately 500 base pair fragment of 18S rDNA common to *Hepatozoon* spp. and some other apicomplexans was amplified and sequenced from the tissues or blood of 16 vertebrate host species from the southern United States, including one opossum (*Didelphis virginiana*), 2 bobcats (*Lynx rufus*), a domestic cat (*Felis catus*), 3 coyotes (*Canis latrans*), a gray fox (*Urocyon cinereoargenteus*), 4 raccoons (*Procyon lotor*), a pet boa constrictor (*Boa constrictor imperator*), a swamp rabbit (*Sylvilagus aquaticus*), a cottontail rabbit (*Sylvilagus floridanus*), 4 woodrats (*Neotoma fuscipes* and *Neotoma micropus*), 3 white-footed mice (*Peromyscus leucopus*), 8 cotton rats (*Sigmodon hispidus*), a cotton mouse (*Peromyscus gossypinus*), an eastern grey squirrel (*Sciurus carolinensis*), and a woodchuck (*Marmota momax*). Phylogenetic analyses and comparison with sequences in the existing database revealed distinct taxonomic groups of *Hepatozoon* spp., with clusters formed by sequences obtained from scavengers and carnivores (opossum, raccoons, canids, and felids) and those obtained from rodents. Surprisingly, *Hepatozoon* spp. sequences from wild rabbits were most closely related to sequences obtained from carnivores (97.2% identical), and sequence from the boa constrictor was most closely related to the rodent cluster (97.4% identical). These data are consistent with recent work identifying prey/predator transmission cycles in *Hepatozoon* spp., and suggest this pattern may be more common than previously recognized. **Key Words:** Apicomplexa; DNA

sequencing*; Hepatozoon* spp.; 18S rRNA; Phylogenetics; Polymerase chain reaction (PCR).

INTRODUCTION

Members of the apicomplexan genus *Hepatozoon* are obligate heteroxenous parasites of a variety of vertebrate intermediate and invertebrate definitive hosts (Smith, 1996; Smith and Desser, 1997; Vincent-Johnson et al., 1997). Characteristics distinguishing *Hepatozoon* from other hemogregarines include the development of large, polysporocystic oocysts within invertebrate definitive hosts (Desser, 1990; Smith, 1996; Smith et al., 1999) with transmission to vertebrate intermediate hosts usually occurring by ingestion of infected invertebrate hosts (Smith, 1996; Baneth et al., 2007). After ingestion, zoite stages disseminate within vertebrate hosts and localize within host tissues, forming either cyst stages or meronts (Smith, 1996; Johnson et al., 2008). In intermediate vertebrate hosts, after one or a number of merogonic cycles, merozoites invade blood cells and develop into gamonts, which are found circulating in host erythrocytes or leukocytes (Smith, 1996; Baneth et al., 2007). Gamonts are the infective stage to blood-sucking invertebrates, which include various lice, fleas, ticks, mites, triatomids, and mosquitoes (Smith, 1996, Smith and Desser, 1997).

More than 300 species of *Hepatozoon* have been individually named, but less than fifty of these are from mammals (Smith, 1996). Complete life cycle descriptions are available for very few of the *Hepatozoon* species documented (Smith, 1996). Confounding taxonomic classifications, certain well-studied *Hepatozoon* species have been observed to establish infections in multiple vertebrate species in experimental

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studies (Smith, 1996; Smith and Desser, 1997; Moço et al., 2002; Johnson et al., 2009a). Experiments demonstrating transmission of *Hepatozoon* spp. to documented intermediate hosts via the ingestion of parasite cyst stages within other vertebrate tissues suggest predation as an alternate transmission route in nature (Desser, 1990; Smith, 1996; Johnson et al., 2009b).

There is a dearth of information regarding *Hepatozoon* species cycling in North American wildlife, exotic pets, and domestic animals. At the time of this study's undertaking, only 14 of the over 160 sequences listed in GenBank originated from animals in North America, all of which were obtained from domestic dogs with the exception of two sequences from rodents contributed in 2007 (Johnson et al., 2007). Sequence data from known and novel vertebrate hosts of *Hepatozoon* in the southern United States were analyzed to document the diversity of *Hepatozoon* spp. in North America and examine phylogenetic relationships among the known species and the species detected in North American vertebrates of the present study.

MATERIALS AND METHODS

Samples, DNA Extraction, and PCR

The host, geographic location, and tissue type for the samples included in this study are described in Table 2 (pp. 119-120). For standard PCR, DNA was extracted from blood or tissues using the GFX™ Genomic Blood DNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK) or the Illustra™ blood genomic Prep Mini Spin Kit (GE Healthcare UK Limited Little Chalfront, Buckinghamshire, UK), respectively. DNA was extracted from approximately 100-200 µl of whole blood or 15-

20 mg of tissue following the protocols provided by the manufacturers. Briefly, for

tissues, sterile scalpels were used to shave frozen sections which were placed in lysis

buffer with Proteinase K for one hour before following the extraction protocol.

For the domestic cat, only a blood smear was available. The smear was placed in

xylene overnight to remove the sealed cover slip and a sterile scalpel was used to scrape

the stained cell layer off of the slide into a small amount of sterile Tris-EDTA (TE) buffer

(Promega Corporation, Madison, WI). The scraped material was placed in a

microcentrifuge tube, brought to a total volume of 200 ul in sterile TE, and was extracted

for DNA from blood with the Illustra™ kit. Nested PCR was performed on DNA

extractions using primers 5.1 and 3.1 followed by RLBH-F and RLBH-R as previously

described (Allen et al., 2008).

Sequencing

Amplicons from positive PCR samples were prepared for direct sequencing and

sequenced as previously described (Allen et al., 2008). Briefly, amplicons obtained from

nested PCR reactions were purified and concentrated using Amicon® Microcon® -PCR

Centrifugal Filter Devices (Millipore Corporation, Bedford, MA), and were sequenced in

both directions with primers RLBH- F and RLBH-R using an ABI3730 capillary

sequencer.

Due to difficulties encountered with direct sequence attempts, cloning was

performed on amplicons obtained from coyotes, the domestic cat, and the blood of a

naturally infected dog maintained by Oklahoma State University as an *H. americanum*

reservoir (Panciera et al., 1999) using the TOPO® TA Cloning Kit For Sequencing

(Invitrogen, Carlsbad, CA) and plating on OptiGrow™ Luria broth agar (Fisher

BioReagents, Fair Lawn, NJ) supplemented with 50 ug/ml ampicillin (EMD Chemicals

Inc., Gibbstown, NJ) 1 mMol IPTG (Invitrogen, Carlsbad, CA), and 0.8 mg B-galactose

(Invitrogen, Carlsbad, CA). Colonies harboring plasmids with an insert were inoculated

into 3 ml Luria broth (Fisher BioReagents, Fair Lawn, NJ) with ampicillin (50 ug/ml) and

incubated for 12-18 hours at 37ºC while shaking at 150 rpm. Turbid cultures were

centrifuged at 1500 rpm for ten minutes to pellet cells, and the Promega PureYield™

Plasmid Miniprep System (Promega Corporation, Madison, WI) was used according to

the manufacturer's protocol to harvest plasmids from transformed cells. Plasmids with

inserts were sequenced using M13 forward and reverse primers.

Sequence Analyses

Sequences obtained from this study were compared to all available sequences in

the National Center for Biotechnology Information (NCBI) database. Five *Hepatozoon*

spp. sequences previously reported in GenBank (A864676, AY461378, AY620232,

AEF157822, and AF494058) were selected for use in the phylogenetic analyses based on

identity with the novel sequences reported here and diversity within the genus. Also, two

sequences obtained from the Oklahoma State University (OSU)-maintained reservoir

dog, designated *H. americanum* OSU-1 and OSU-2, were used in our comparisons.

MacVector 8.1 software was used for sequence analyses. A phylogenetic tree (Neighbor

Joining; Best Tree, Jukes-Cantor calculated evolutionary distance with *Adelina* outgroup

rooting) and a percent similarity matrix were constructed to determine genetic

relationships and identities of sequences gathered in this study based on the ~ 500 base

pair 18S rDNA hypervariable region amplified.

RESULTS

Hepatozoon spp. sequences collected in this study are listed in Table 2 (p. 119-120). When sequences were compared, distinct taxonomic groupings were evident in the phylogenetic tree (Figure 2, p. 121). In general, *Hepatozoon* sequences from carnivores and the opossum grouped together, while those from rodents formed a separate clade. Exceptions included *Hepatozoon* sequences from the cottontail and swamp rabbits, which did not group with sequences found in other herbivores but instead were included within a separate clade that was most closely related to sequences obtained from canids in North America (Figure 2). Also, *Hepatozoon* sequence from the pet boa constrictor in Oklahoma and a previously documented *H. ayorgbor* sequence from a snake from Ghana (EF157822) grouped with sequences obtained from rodents trapped in Texas, Oklahoma, Georgia, and California. A sequence from a gray fox grouped with a sequence documented as *H. canis* from a domestic dog in Spain (AY461378) and the sequence obtained from the blood smear of the domestic cat grouped with sequences from coyotes and domestic dogs from the United States (Figure 2) rather than those previously reported from felids (AY620232, DQ315566, EU028344, EU267606, EU622910, FJ213775, and GQ377216).

When *Hepatozoon* spp. sequences from each cluster represented in the phylogenetic tree were compared to determine percent identities, they were found to be 90.3-97.2% identical (Appendix B, pp. 153-154). The sequences obtained from the cottontail and swamp rabbit were 99.2% identical to each other and up to 97.2% identical to sequences from a coyote, the domestic dog *H. americanum* sequences OSU-1 and OSU-2, and published *H. americanum* sequence AY86467.

Among the rodent *Hepatozoon* spp. sequences, percent identities ranged from 97.2-99.8%. Sequences from the boa constrictor and the previously documented *H. ayorgbor* sequence (EF157822) grouped within the rodent cluster; the sequences from the snakes were 95.9-97.4% and 97.8-99.6% similar to collected rodent sequences, respectively.

The sequences from coyotes were 96.2-99.8% identical to each other and were most similar to *Hepatozoon* sequences documented in domestic dogs in North America (96.0-99.8%). Two distinct *Hepatozoon* spp. sequences were obtained from each of two coyotes from Oklahoma. Coinfected coyotes harbored *Hepatozoon* organisms that were 97.0% or 99.8% identical.

The sequence collected from the gray fox was 90.9-94.6% identical to *Hepatozoon* sequences obtained from coyotes and domestic dogs in North America. As indicated by the phylogenetic tree, the parasite found in the gray fox was most closely related (98.0%) to *H. canis* found in a domestic dog in Spain (AY461378).

The sequences from the four raccoons trapped in Oklahoma were 100% identical, and the sequence was 90.9-95.2% identical to other sequences obtained in the present study.

The bobcat *Hepatozoon* spp. sequences shared an identity of 94.9% and were 91.7-94.6% and 92.4-96.7% similar to *Hepatozoon* sequences collected from other animals. The sequence from the domestic cat was 91.7% and 92.4% identical to sequences collected from the two bobcats, but was 99.4% identical to *H. americanum* sequence AY864676 previously documented in Alabama in a domestic dog and 99.8% identical to a coyote sequence reported in the current study.

DISCUSSION

Although *Hepatozoon* spp. are found in a wide range of hosts, and have been reported in several vertebrate species in North America, genetic data from *Hepatozoon* spp. infecting animals in North America are extremely limited. Until two sequences collected from rodents were added to the database in 2007 (Johnson et al., 2007), *Hepatozoon* spp. data available in GenBank from the United States had been exclusively from domestic dogs. The present study is the first to document *Hepatozoon* spp. 18S rDNA sequence data from a variety of vertebrate hosts in North America.

At a broad level, relationships of the *Hepatozoon* sequences collected in this study appeared to be associated with intermediate vertebrate host taxonomy. Phylogenetic analyses revealed two distinct groupings; *Hepatozoon* spp. sequences collected from carnivores predominantly fell into one assemblage while the second cluster was comprised chiefly of sequences obtained from rodents. However, there were some surprising exceptions to this generalization. A rather interesting and unexpected finding in this study was the phylogenetic relatedness of *Hepatozoon* sequences from rabbits to sequences collected from carnivores. Previous work has documented the transmission of various *Hepatozoon* species to vertebrate hosts by the ingestion of monozoic or dizoic cyst stages of parasite in tissues from other vertebrates (Desser, 1990; Smith, 1996; Baneth and Shkap, 2003; Johnson et al., 2009a). Some species of *Hepatozoon* have been shown capable of utilizing novel paratenic hosts, including rabbits, in experimental trials (Smith, 1996; Johnson et al., 2008; Johnson et al., 2009a). The two infected rabbits found in this study may have been incidentally infected with a genetically uncharacterized species of *Hepatozoon* by ingestion of infected arthropod hosts or

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ingestion of contaminating oocysts from mechanically disrupted arthropod hosts. Conversely, undescribed species of *Hepatozoon* may be cycling in rabbits and unidentified arthropod and carnivorous hosts. To the authors' knowledge, the rabbit sequences are unique and are the first *Hepatozoon* sequences reported from lagomorphs.

Data of *Hepatozoon* spp. published from other areas of the world from pine martens (*Martes martes*; EU686690, EF222257), bank voles (*Clethrionomys glareolus*; AY600625, AY600626), a wild rat (*Bandicota indica*; AB181504), and a red squirrel (*Sciurus vulgaris*; EF222259) were available in GenBank at the time of this study. Data contributed from North America were deposited in GenBank in 2007 from cotton rats and white-footed mice trapped in Oklahoma (EF620026 and EF620027, respectively) (Johnson et al., 2007). Rather than sharing highest similarities with previously published sequences from rodents from other parts of the world, interestingly, rodent *Hepatozoon* sequences collected in North America were most similar to those documented in a grass mouse (*Abrothrix olivaceus*) from Chile (FJ719818) (Merino et al., 2009) and a python from Ghana (EF157822) (Sloboda et al., 2007). Additionally, sequence obtained from the boa constrictor in the current study which had observable parasites in a blood film was most similar to rodent sequences collected from North America, and was 97.4% identical to that from a woodrat (*Neotoma micropus*) trapped in California.

Predator-prey cycles have been documented in *Hepatozoon* spp. infecting snakes, but these cycles generally involve frog or lizard intermediate or paratenic hosts (Smith, 1996; Smith et al., 1999), although some researchers have suspected rodents' involvement in the natural history of certain snake *Hepatozoon* species (Sloboda et al., 2008). The data presented in the present study implicate rodents as possible mammalian

hosts in the transmission cycles of *Hepatozoon* spp. infecting snakes in North America. Unfortunately, only a single *Hepatozoon* 18S rDNA sequence from a snake (EF157822) was available in GenBank for comparison with our sequences; the remaining sequence data from snakes were ITS-1 (internal transcribed spacer 1) and 5.8S or 18S sequences that did not overlap with our snake amplicon sequence (Smith et al., 1999; Ujvari et al., 2004).

Sequences from coyotes in the present study were closest in identity to those previously documented in domestic dogs in North America. Although sequence data of *H. americanum* are available from domestic dogs only, coyotes have long been recognized as infected with this parasite in enzootic areas (Kocan et al., 1999; Garrett et al., 2005). In one study, approximately half of surveyed coyotes in American canine hepatozoonosis enzootic areas in Oklahoma harbored characteristic skeletal lesions indicative of *H. americanum* infection, and coyotes have been experimentally confirmed to be capable of infecting ticks (Kocan et al., 1999; Kocan et al., 2000).

Interestingly, two coyotes surveyed in the present study were found to each harbor two distinct sequences of *H. americanum*, which suggests coinfections with separate, but very similar organisms. Coinfections of *Hepatozoon* species in domestic dogs have been documented in North America based on genetic data (Allen et al, 2008; Li et al., 2008). Thus, it is not surprising that coyotes would be susceptible to infection with more than one strain of *H. americanum* if multiple strains were cycling in nature in enzootic areas.

Hepatozoon spp. sequence from the gray fox was most closely related to that documented as *H. canis*. *H. canis* was not thought to be present in North America until

2008, after two separate PCR survey studies identified infections in domestic dogs (Allen et al., 2008; Li et al., 2008). Sequence-confirmed infections with *H. canis* have been reported in foxes in other areas of the world (Criado-Fornelio et al., 2006; Criado-Fornelio et al., 2007a; Gimenez et al., 2009), but this organism has not been previously documented in wild canids in the United States. Comparisons of data available in GenBank reveal that 18S rDNA sequences among strains of *H. canis* may differ by 0.2% to 1.4% (Allen et al., 2008). Sequence from the gray fox collected in this study was 98.0% identical to *H. canis* documented in a domestic dog (AY461378), suggesting that a *Hepatozoon* species in addition to *H. americanum* infects wild canids in North America.

Although raccoons are recognized hosts of *H. procyonis* in North America (Clark et al., 1973; Schaffer et al., 1978), the present study is the first report of sequence data for *Hepatozoon* species in raccoons. The sequences from the four raccoons collected from Payne County, Oklahoma were identical, suggesting they are the same species. Invertebrate species transmitting *Hepatozoon* to raccoons have not yet been identified, although raccoons have been shown to host *Amblyomma americanum* (Clark et al., 1973; Pung et al., 1994; Kollars et al., 2000). Interestingly, a *Hepatozoon* sequence from a field-collected, unfed *A. americanum* adult was 100% identical to the sequence collected from the four raccoons (M. Reichard, unpublished data).

Hepatozoon spp. have been documented in several wild felids including lions (*Panthera leo*), leopards (*Panthera pardus*), cheetahs (*Acinonyx jubatus*), genet cats (*Genetta trigrina*), ocelots (*Felis pardalis*), palm civets (*Paradoxurus hermaphrodites*), and Pallas cats (*Felis manul*) (Lane and Kocan, 1983; Baneth et al., 1998; Metzger et al., 2008). In North America, circulating gametocytes of undetermined *Hepatozoon* species

have been reported in bobcats and ocelots (Lane and Kocan, 1983; Mercer et al., 1988).

However, until the present study, no *Hepatozoon* sequence data from North American

wild felids have been deposited in GenBank. Infection with *Hepatozoon* spp. was first

described in domestic cats nearly a century ago, but, as in wild felids, the organism(s)

associated with infection is poorly understood (Rubini et al., 2006; Baneth et al., 1998;

Metzger et al., 2008). Aside from an isolated report in 1977 in a cat relocated from

Hawaii to California, *Hepatozoon* spp. have not been reported in domestic cats in the

United States until the present study (Lane and Kocan, 1983; Baneth et al., 1998).

Sequence data from domestic cats from other areas of the world are available in

GenBank and are 99% or 96% identical to sequences documented as *H. canis* (Criado-

Fornelio et al., 2006; Criado-Fornelio et al., 2007b; Majláthová et al., 2007; Criado-

Fornelio et al., 2009). The sequence obtained from the domestic cat in this study was

nearly identical (99.4%) to *H. americanum* AY86467. Some researchers speculate

Hepatozoon species infecting cats are opportunists, and take advantage of

immunocompromised feline hosts that are otherwise resistant to infection (Baneth et al.,

2007). The domestic cat included in this study, a patient at the OSU Veterinary Teaching

Hospital, had been diagnosed with lymphoma and was euthanitized prior to identification

of a *Hepatozoon* spp. gamont in a blood smear.

The paucity of information regarding *Hepatozoon* species cycling in vertebrate

hosts in North America was the inspiration for the present collaborative study. Here,

Hepatozoon sequences were gathered from a variety of vertebrate hosts in the southern

United States, novel vertebrate hosts of *Hepatozoon* spp. were identified, and sequences

evaluated appear to support the existence of predator/prey cycles as previously suggested

(Desser, 1990; Smith, 1996; Johnson et al., 2008; Johnson et al., 2009a: Johnson et al., 2009b). Genetic data collected in this survey contribute to the base of knowledge supporting research aimed at characterizing *Hepatozoon* species present in North America, although further work is needed to better describe those organisms found

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Table 2. Host, Accession Number, Tissue Type, and State of Host Origin for *Hepatozoon* spp. included in Comparison.

Host	Accession number	Tissue type	US state of host origin
Virginia opossum, *Didelphis virginiana*	Pending	whole blood	Georgia
Bobcat, *Lynx rufus**	Pending	spleen	Georgia
	Pending		
Domestic cat, *Felis catus*	Pending	blood smear	Oklahoma
Coyote, *Canis latrans*	Pending	whole blood	Texas
Coyote, *Canis latrans**	Pending	whole blood	Oklahoma
	Pending		
Coyote, *Canis latrans**	Pending	whole blood	Oklahoma
	Pending		
Swamp rabbit, *Sylvilagus aquaticus*	Pending	heart	Oklahoma
Eastern cottontail rabbit, *Sylvilagus floridanus*	Pending	heart	Oklahoma
Raccoon, *Procyon lotor* (n = 4)[†]	Pending	heart	Oklahoma

*A single sample contained multiple sequences of *Hepatozoon* sp., [†]Amplicons from all animals tested had identical sequence.

Table 2 Continued. **Host, Accession Number, Tissue Type, and Sate of Host Origin for** *Hepatozoon* **spp. included in Comparison.**

Gray fox, *Urocyon cinereoargenteus*	Pending	whole blood	Georgia
White-footed mouse, *Peromyscus leucopus*	Pending	liver	Oklahoma
Cotton mouse, *Peromyscus gossypinus*	Pending	whole blood	Georgia
Dusky-footed woodrat, *Neotoma fuscipes**	Pending	whole blood	California
	Pending		
Southern plains woodrat, *Neotoma micropus**	Pending	whole blood	Texas
	Pending		
Hispid cotton rat, *Sigmodon hispidus*	Pending	liver	Oklahoma
Eastern gray squirrel, *Sciurus carolinensis*	Pending	spleen	Georgia
Woodchuck, *Marmota monax*	Pending	whole blood	Missouri
Boa constrictor, *Boa constrictor imperator*	Pending	whole blood	Oklahoma

*A single sample contained multiple sequences of *Hepatozoon* **sp., [†]Amplicons from all animals tested had identical sequence.**

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Figure 2. Phylogenetic Tree (Neighbor Joining; Best Tree, Jukes-Cantor Calculated Evolutionary Distance with *Adelina* Outgroup Rooting) Constructed to Evaluate Genetic Relationships among 18S rDNA Hypervariable Region Sequences of *Heptozoon* spp. from Various Vertebrate Hosts.
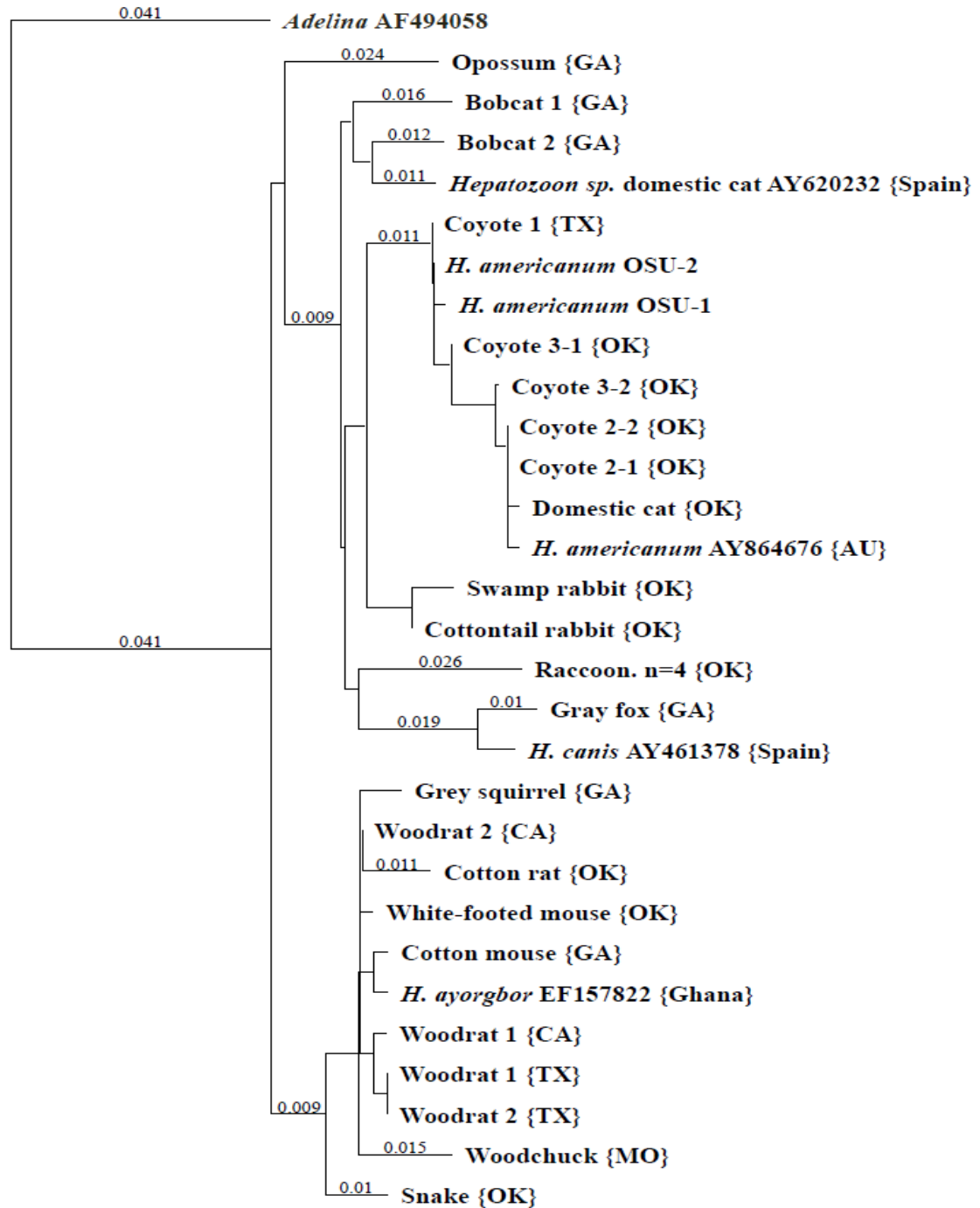
CHAPTER VI

LACK OF EVIDENCE FOR TRANSPLACENTAL TRANSMISSION

OF *HEPATOZOON AMERICANUM* IN AN EXPERIMENTALLY INFECTED DOG[1]

ABSTRACT

Hepatozoon americanum is the causative agent of American canine

hepatozoonosis, a disease of domestic dogs in North America. Established routes of

transmission to dogs include ingestion of *H. americanum* oocysts within *Amblyomma*

maculatum and ingestion of paratenic hosts harboring cystozoites. Although

transplacental transmission is speculated to occur, and has been documented in other

species of *Hepatozoon*, this alternate mode of transmission has not been previously

investigated in *H. americanum* infections. Our objective was to determine if a dam

experimentally infected with *H. americanum* transmitted the parasite transplacentally to

her pups. To this end, an adult female research hound experimentally infected with *H.*

americanum two years prior to this study was mated with an uninfected adult male

research hound. Eight pups born to the infected dam were housed in an acaricide-treated

room and monitored for *H. americanum* infection over a 12-week period by clinical

signs, complete blood count analysis, blood smear examination, and PCR of whole blood.

Amblyomma maculatum nymphs were fed to engorgement on pups at 12 weeks of age,

held through the molting period, and dissected after 70 days to examine for *H.*

americanum oocysts. In this study, transplacental transmission of *H. americanum* was

not demonstrated. Pups remained clinically normal and PCR and blood smear negative

over the 12-week period, and parasite oocysts were not observed in dissected adult *A.*

maculatum. **Key words:** Apicomplexan; American canine hepatozoonosis (ACH);

Hepatozoon americanum; *Hepatozoon canis*; Polymerase chain reaction (PCR);

Transplacental transmission; Vertical transmission.

INTRODUCTION

American canine hepatozoonosis (ACH) is an emerging, potentially devastating disease affecting domestic dogs in North America that is caused by *Hepatozoon americanum* (Ewing and Panciera, 2003; Allen et al., 2008; Potter and Macintire, 2010). This apicomplexan is transmitted to canids either by ingestion of *Amblyomma maculatum* (Gulf Coast tick) harboring sporulated oocysts of parasite (Ewing and Panciera, 2003; Allen et al., 2008; Johnson et al., 2009; Little et al., 2009; Potter and Macintire, 2010) or ingestion of infected paratenic hosts carrying cystozoites (Johnson et al., 2009). Wild and domestic canids serve as the intermediate host, with asexual stages of *H. americanum* found predominantly in skeletal and cardiac muscle tissues (Ewing and Panciera, 2003; Allen et al., 2008; Potter and Macintire, 2010). Gamonts, present in circulating white blood cells several weeks into infection, are infective to larval and nymphal feeding ticks and genetically recombine within the acarine host to form zygotes which give rise to polysporocystic oocysts that are transstadially maintained (Ewing and Panciera, 2003; Little et al., 2009; Potter and Macintire, 2010).

Experiments demonstrating cystozoite development in mice (*Mus musculus*), cotton rats (*Sigmodon hispidus*), and New Zealand white rabbits (*Oryctolagus cuniculus*) suggest rodents and lagomorphs may serve as paratenic hosts *of H. americanum* in ACH-enzootic areas, although natural infections in these animals have not yet been identified (Johnson et al., 2009). Acute ACH is observed as early as 4-6 weeks after ingestion of *H. americanum* zoites (Johnson et al., 2009), and is typically characterized by fever, lethargy, mucopurulent ocular discharge, neutrophilia, myalgia, wasting, abnormal gait, and reluctance to move (Ewing and Panciera, 2003; Allen et al., 2008; Little et al., 2009; Potter and Macintire, 2010). Acute ACH patients may clinically improve with non-

steroidal anti-inflammatory drugs and parasiticide treatments, but often episodically relapse without long-term decoquinate therapy (Ewing and Panciera, 2003; Potter and Macintire, 2010). Death may result within one year without treatment, although chronically infected survivors have been documented (Ewing and Panciera, 2003; Allen et al., 2008; Potter and Macintire, 2010).

Diagnosis of ACH is sometimes difficult, and relies on assessment of clinical signs, observation of rare parasite gamonts on blood films, and finding pathognomonic lesions in biopsied muscle tissues (Ewing and Panciera, 2003; Allen et al., 2008; Potter and Macintire, 2010). Polymerase chain reaction (PCR) methods may be used to detect circulating parasites (Allen et al., 2008; Li et al., 2008; Potter and Macintire, 2010) but may lack sensitivity depending on parasitemia levels in presenting patients (Li et al., 2008). In our research, we have found xenodiagnosis to be a very reliable, sensitive experimental method of identifying *H. americanum* infections (Ewing and Panciera, 2003).

Vertical transmission has been documented in other species of *Hepatozoon*, including *H. canis*, and although speculated to occur in natural *H. americanum* infections, experiments examining this particular mode of transmission for *H. americanum* have not been reported (Ewing and Panciera, 2003; Little et al, 2009; Potter and Macintire, 2010). Here, a dog experimentally infected with *H. americanum* two years prior to this study was bred to assess transplacental transmission of parasite.

MATERIALS AND METHODS

Dogs

Two years prior to this study, an intact female research hound was experimentally infected with *H. americanum* by ingestion of sporulated oocysts collected from *A. maculatum* acquisition fed on a naturally, chronically infected dog. The infected female was administered carprofen for pain at the onset of clinical signs, which she has been maintained on daily ever since. At the time of breeding, she was approximately 5 years of age, harbored parasite stages in biopsied muscle tissue, and had consistently transmitted parasite to feeding *A. maculatum* over the previous 2 years. The sire was a research hound approximately one year of age, was a principal in an unrelated study, and had not been experimentally infected with any disease agent. The dam delivered 8 pups, which were monitored for clinical signs daily over the course of the study. All animals in this study were cared for according to the principles outlined by the Institutional Animal Care and Use Committee at Oklahoma State University, College of Veterinary Health Sciences, Laboratory Animal Resources, Stillwater, Oklahoma.

Sample Collection, Processing, and Testing

Whole blood in EDTA was collected from the dam and sire the day before the first mating and again 6 weeks later, and from each pup at 2, 4, 6, 8, 10, and 12 weeks of age and used for blood smear preparation (Romanowski staining), complete blood count analysis to assess neutrophil levels, and total DNA extraction. Placental and umbilical tissues were taken at the time of whelping and were frozen at -20˚C.

For standard PCR, total DNA was extracted from blood or tissues using the Illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare UK Limited Little Chalfront, Buckinghamshire, UK). Whole blood (200 ul) was extracted following the

protocol provided by the manufacturer. Frozen tissue shavings (15-20 mg) were extracted by placing them in lysis buffer with Proteinase K for one hour, then following the Illustra™ kit's protocol for DNA extraction from blood. PCR was performed as previously described using primers 5.1 and 3.1 followed by nested primers RLBH-F and RLBH-R (Allen et al., 2008).

Xenodiagnosis Experiment

At 12 weeks of age, pups were assessed for infection by xenodiagnosis. Briefly, 50 *A. maculatum* nymphs were placed beneath stockinette wraps fitted to each animal and secured with tape. Pups were housed in separate metabolism cages over water moats until tick repletion. Ticks were collected from water pans or from removed stockinettes, and were placed in sealed paper cups in a humidity chamber for a period of at least 70 days after engorgement. Molted adults were dissected in 100 ul PBS under 30X magnification to examine for *H. americanum* oocysts.

RESULTS

At the time of mating and six weeks into the study, both sire and dam were clinically sound and free of circulating parasite as determined by PCR and blood film examination. No parasite was detected by PCR in placental or umbilical tissues. One pup died four days after birth and was necropsied. Routine tissue samples (lung, liver, spleen, kidney, heart and skeletal muscle) that were collected for histopathologic examination and PCR analysis revealed neither parasite or associated lesions nor parasite DNA, respectively. The pup was determined to have died from aspiration pneumonia.

Surviving pups remained free of clinical signs over the 12-week monitoring period, and collected blood samples were consistently PCR and blood smear negative. One pup developed a head tilt at approximately three weeks of age that resolved spontaneously without complications. Rectal temperatures for all pups remained within normal range. Neutrophil counts remained below the normal reference range maximum (10,600/ul) for all pups, although several pups had sporadically higher levels (10, 920/ul - 13,910/ul) on some sampling dates consistent with a stress leukogram due to over-excitement. Neutrophil levels were within normal range for these animals on subsequent bleed dates. Pups continued to remain healthy and free of clinical signs indicative of *H. americanum* infection well after the study end, and were adopted out at approximately 16 weeks of age.

Replete nymphs recovered from each of the seven pups in the xenodiagnosis experiment were held through ecdysis. A total of thirty molted adult ticks were dissected and examined microscopically. *Hepatozoon americanum* oocysts were not observed in any of the ticks.

DISCUSSION

Transplacental transmission has been speculated to occur in natural *H. americanum* infections (Ewing and Panciera, 2003; Potter and Macintire, 2010), but to the authors' knowledge, no experiments assessing this transmission pattern have been reported. Here, a dam in clinical remission that was experimentally infected with *H. americanum* two years prior to this study was bred to an uninfected male. Although the dam was asymptomatic prior to breeding, during pregnancy, and after whelping, ticks

consistently acquired *H. americanum* infection from repletion feeding on the dam both prior to breeding and after completion of this study. Successful experimental infection of laboratory-raised *A. maculatum* suggests continuous merogonous development of the parasite.

During this study, neither clinical disease nor parasitemia was detected in any of the dam's seven surviving pups over the course of the 12-week monitoring period. Xenodiagnoses further confirmed the animals were free of circulating parasite. PCR and histopathologic examination of tissues from a pup that died soon after birth were negative for parasite DNA and parasite-associated lesions, respectively. Placenta and umbilical cord collected after birthing were also negative for parasite DNA.

Experiments demonstrating vertical transmission of *Hepatozoon canis* reported gamonts in blood smears or meronts in tissues from pups whelped from infected dams (Murata et al., 1993). As *Hepatozoon* spp. gamonts are thought to be infective only to invertebrate hosts (Smith et al., 1996), these congenitally acquired *H. canis* infections occurred presumably as a result of sporozoites or merogonic stages crossing the placenta.

In contrast to infections with *H. canis*, dogs with ACH exhibit extremely low levels of parasitemia (Ewing and Panciera, 2003; Allen et al., 2008; Little et al., 2009). Gamonts of *H. americanum* are rarely observed in blood smears from dogs with histologically and xenodiagnostically confirmed infections (Ewing and Panciera, 2003; Allen et al., 2008), and in our experience, parasitemia levels even in known infected dogs at times can be below the threshold for PCR detection. Dogs with ACH often exhibit waxing and waning clinical courses of disease, and clinical relapses are thought to result

from inflammatory processes associated with the periodic release of encysted zoites (Ewing and Panciera, 2003, Potter and Macintire, 2010).

The clinical phase of *H. americanum* infection may be an important factor in determining if transplacental transmission will occur in an infected dam. In the chronically infected, clinically stable dam used in this study, circulating levels of parasite may have been so low that very few, if any, organisms crossed the placenta, or those transmitted were the incorrect stage to establish infection. Alternatively, if a recently infected dam were to become pregnant while in an acute state of clinical disease, zoites actively disseminating throughout the dam's tissues might migrate across the placenta to infect fetal pups. In animals with persistent infections with other apicomplexans, endogenous transplacental transmission due to reactivation of parasite during pregnancy has been reported (Trees and Williams, 2005). Here, we did not observe a decline in clinical condition of the dam during pregnancy to indicate recrudescence of disease, nor did we detect clinical signs or parasite in her pups. Nonetheless, *de novo* or exogenous infection of a pregnant dam could lead to successful transplacental transmission of infective zoites, as is the case with congenitally acquired *Toxoplasma gondii* infections in humans (Trees and Williams, 2005).

In addition to clinical disease state and timing of infection with respect to pregnancy, the parasite stage by which a dam is infected may also determine if transplacental transmission of *H. americanum* will occur. *Hepatozoon americanum* infection in domestic dogs has been experimentally demonstrated by both the oral administration of parasite sporozoite and cystozoite stages (Allen et al., 2008; Johnson et al., 2009; Little et al., 2009). These stages may be distinct in dissemination and cellular

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invasion behaviors and thus not equal in their abilities to cross the placenta to establish infections in fetal pups. However, ingestion of either stage leads to indistinguishable lesions and clinical manifestations in domestic dogs, and both result in the circulation of gamonts that are infective to feeding ticks (Johnson et al., 2008), suggesting the ultimate nature of the infections are similar.

No reports of *H. americanum* infections in pups less than 11 weeks of age are found in current literature (Ewing and Panciera, 2003). Based on experimental evidence, 11 weeks of age is adequate time for these animals to have acquired *H. americanum* infections by sporozoite or cystozoite ingestion after weaning (Johnson et al., 2009). Outbreaks of ACH have only been documented in dogs allowed to roam in tick-infested areas or engage in predatory behaviors (Johnson et al., 2009), not in recently whelped pups from the same litter. In support of our experimental finding, six clinically normal pups approximately 6-8 weeks of age that were born to a recently infected dam involved in a natural, multiple dog ACH outbreak documented by Johnson et al. (2009) also tested negative for circulating parasite by PCR; xenodiagnoses or muscle biopsies to further rule out infection, however, were not performed in these pups.

Our failure to demonstrate vertical transfer of *H. americanum*, although a valuable finding, is obviously not conclusive. Further experiments are needed to better assess this possible transmission route in nature.

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CHAPTER VII

CONCLUSIONS

Twenty nine of the over 300 *Hepatozoon* species documented world-wide are reported from North America, with 24 of these in snakes (Smith, 1996; Telford et al., 2001; Telford et al., 2002; Telford et al., 2005a; Telford et al., 2005b; Telford et al., 2008; Telford, 2010). Five *Hepatozoon* species have been reported from non-poikilothermic vertebrates in North America including *H. muris* in rats (Eyles, 1952), *H. griseisciuri* in squirrels (Clarke et al., 1973), *H. procyonis* in raccoons (Davidson and Calpin, 1976), and *H. americanum* and *H. canis* in domestic dogs (Vincent-Johnson et al., 1997; Allen et al., 2008; Li et al., 2008). Other North American vertebrate species are documented hosts of unidentified *Hepatozoon* species (Lane and Kocan, 1983; Mercer et al., 1988). The overarching objectives of the research presented in this dissertation have been to gain a better understanding of the species of *Hepatozoon* present in vertebrates in the United States.

Study 1 (Chapter 3)

The goals of this study were to better understand the frequency of *Hepatozoon* spp. infections in domestic dogs in Oklahoma, and to assess the phylogenetic diversity of

the *Hepatozoon* spp. organisms found using PCR and 18S rDNA sequence analyses. Although the frequencies of *Hepatozoon* spp. infections were fairly low in the dog populations sampled, the sequences obtained, deposited in GenBank, were rather diverse. Four of these sequences were most identical to that of *H. americanum* collected from a dog in Alabama (Paludo et al., 2005), with identities to the previously documented sequence ranging from 99.6% to 95.5%. These sequence variations suggest several strains of *H. americanum* or other species of *Hepatozoon* not yet classified are infecting dogs in the United States. *Hepatozoon canis* has generally not been a suspected parasite of North American domestic dogs since the recognition of *H. americanum* in1997 (Vincent-Johnson et al., 1997), but interestingly, sequences obtained from two dogs in this study were 98.8% identical to a sequence of *H. canis* reported from Spain (Criado-Fornelio et al., 2006). A real-time PCR survey study conducted by Auburn University corroborated our results as well as provided data from dogs distributed throughout the United States that had presented to veterinarians with clinical signs of hepatozoonosis; melt-curve analyses revealed predominately *H. americanum* infections, but *H. canis* and *H. canis* and *H. americanum* coinfections were detected in several samples (Li et al., 2008). It remains unclear whether *H. canis* infections in North America are autochthonous or are the result of introduction through increased international travel practices. Regardless, the often mild clinical syndrome associated with *H. canis* infection (Baneth et al., 2001) has likely been prohibitive of its discovery. This study documented molecular evidence of *H. canis* infections in domestic dogs in the United States for the first time.

Study 2 (Chapter 4)

Hepatozoon americanum is the parasite causing the devastating, often fatal clinical syndrome American canine hepatozoonosis (ACH) in domestic dogs in North America (Macintire et al., 2001). As an alternative to TCP in the treatment of ACH, the Companion Animal Parasite Council (CAPC) recommends ponazuril (toltrazuril sulfone), although experiments evaluating this drug for efficacy have not previously been reported. In the pilot study presented in this chapter, ponazuril (Marquis™) (Bayer HealthCare LLC, Shawnee Mission, USA) was administered twice daily for four weeks to a dog experimentally infected with *H. americanum* at the onset of clinical signs. Ponazuril treatment did not clear parasite as evidenced by PCR detection of parasite DNA and microscopic confirmation of parasite in muscle tissue samples taken at necropsy. Although clinical disease appeared to resolve after one week of treatment, carprofen, administered for pain control, may have also resulted in the abatement of clinical signs. Still, as ponazuril has shown efficacy in the treatment and prevention of other protozoal diseases (Gottstein et al., 2001; Mitchell et al., 2005; Charles et al., 2007), further experimental evaluation of ponazuril as a possible initial parasiticide or prophylactic drug in ACH patients or at-risk dogs, respectively, is warranted.

Study 3 (Chapter 5)

The major objectives of the research presented in Chapter 5 were to genetically characterize *Hepatozoon* spp. from recognized and novel vertebrate hosts in the United States, and to compare *Hepatozoon* spp. sequence data obtained to assess phylogenetic relationships of the *Hepatozoon* spp. detected. To this end, PCR was performed on DNA extracts from whole blood or tissue samples collected from a variety of vertebrates in

Oklahoma, Texas, Missouri, Georgia, or southern California. 18S rDNA sequence

comparisons revealed that most *Hepatozoon* spp. clustered according to vertebrate host

taxonomy, but several interesting relationships were apparent in the constructed

phylogenetic tree. *Hepatozoon* spp. sequences amplified from two hunted rabbits in

Oklahoma were most related to those obtained from coyotes and domestic dogs. Also,

sequence collected from a boa constrictor in Oklahoma, along with a previously reported

Hepatozoon spp. sequence from a snake in Ghana, grouped within the rodent clade.

These two findings suggest predator/prey relationships of carnivorous intermediate and

prey paratenic hosts are more important in *Hepatozoon* spp. transmission patterns than

previously realized. *Hepatozoon* spp. sequences obtained from coyotes were most

identical to those collected from domestic dogs, and more than one distinct sequence was

amplified from two of the three coyotes, indicating coinfections in these animals. In

support of our assertion that *H. canis* or a highly similar species is present in the United

States (Allen et al., 2008; Li et al., 2008), a sequence most identical to *H. canis* (98.0%)

was collected from a gray fox from Georgia. Finally, a sequence from an immune-

compromised domestic cat was most identical to that previously reported in a dog from

Alabama (Paludo et al., 2005). This study not only provided valuable information

regarding the phylogenetic relationships of the *Hepatozoon* spp. present in the southern

United States, but also added to the genetic data available from North America to aid in

further sequence comparisons of *Hepatozoon* spp. collected from known and novel hosts.

Study 4 (Chapter 6)

Previous studies have demonstrated that in pregnant animals, *H. griseisciuri* and

H. canis are capable of transmitting across the placenta to gestating pups (Clark et al.,

1958; Murata et al., 1993). This transmission pattern is suspected in natural *H.*
americanum infections, yet no experiments assessing this particular route have been
reported until now (Vincent-Johnson, 2003; Little et al., 2009; Potter and Macintire,
2010). In this study, no evidence of transplacental transmission occurred in an
experimentally, chronically infected dam as determined by clinical signs, PCR of whole
blood, and xenodiagnosis of seven whelped pups. Although the experiment involved
only one infected dam, and is therefore not conclusive, reports of ACH have only
occurred in dogs old enough to have acquired infection via established routes of
sporozoite or cystozoite ingestion (Mathew et al., 1998; Ewing and Panciera, 2003;
Johnson et al., 2008; Johnson et al., 2009). The timing of infection, whether before or
during pregnancy, the stage of parasite by which infected, whether sporozoites or
cystozoites, and the clinical phase of disease, whether acute or chronic, may be factors
influencing the occurrence of transplacental transmission of *H. americanum* in naturally
infected dogs. More research is needed to assess this possible route of transmission in
nature to better understand the epidemiology of ACH.

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APPPENDIX A

HEPATOZOON SPP. SEQUENCES LISTED IN GENBANK ACCORDING TO

HOST(S), ACCESSION NUMBER(S), AND LITERATURE CITATION(S).

Documented Species of *Hepatozoon*			
Species	**Host(s)**	**GenBank Acc No(s).**	**Reference(s)**
H. americanum	Domestic dog (*Canis familiaris*)	AF206668	Baneth et al., 2000
		AY864676	Paludo et al., 2005
		EU146062	Allen et al., 2008
	Genomic DNA from sporozoites	AF176836	Mathew et al., 2000
H. ayorgbor	Snake (*Python regius*)	EF157822	Sloboda et al., 2007
H. canis	Genomic DNA from sporozoites	AF176835	Mathew et al., 2000
		AF206669	Baneth et al., 2000
	Domestic dog (*Canis familiaris*)	AF418558 and DQ111751 - 59	Oyamada et al., 2005
		DQ198378- 79	Rubini et al., 2005
		DQ060324 - 29	Karagenc et al., 2006
		AY461378	Criado-Fornelio et al., 2006
		DQ071888	Forlano et al., 2007
		EF650846	Criado-Fornelio et al., 2007a
		DQ519357 - 58	Criado-Fornelio et al., 2007b
		DQ439540 and DQ439543 - 44	Criado-Fornelio et al., 2007b
		AB365071	Sasaki et al., 2008
		FJ497009 - 22	Votja et al., 2009
		FJ743476	Rubini et al., 2009
		GQ395386	Götsch et al., 2009
		EU289222	Not published
		EU571737	Not published
		FJ608736	Not published
		GQ176285	Not Published

	Crab-eating fox (*Dusicyon thous)*	AY461375	Criado-Fornelio et al., 2006
	Pampas Fox (*Pseudalopex gymnocercus*)	AY461376 and AY471615	Criado-Fornelio et al., 2006
	Red Fox (*Vulpes vulpes*)	AY150067	Criado-Fornelio et al., 2003
		DQ439541 - 42	Criado-Fornelio et al., 2007b
		DQ869309	Majláthová et al., 2007
		GU371446 - 58	Gabrielli et al., 2010
		HM212625 - 26	Dezdek et al., 2010
	Domestic cat (*Felis catus*)	AY731062	Not published
		DQ315565 - 66	Rubini et al., 2006
		FJ213775 and EU622909 - 10	Criado-Fornelio et al., 2009
	Jaguar (*Panthera onca*)	EF222256	Not published
	Castor bean tick (*Ixodes ricinus*)	GU827130	Reye et al., 2010
	Genomic DNA	GU386283 - 84	Not Published
H. catesbianae	Bull frog (*Rana catesbeiana*)	AF041438 and AF040972	Lang-Unnash et al., 1998
	Green frog (*Rana clamitans*)	AF110241 - 42	Smith et al., 1999
	Genomic DNA	AF130361	Carreno et al., 1999
	Genomic DNA from sporozoites	AF176837	Mathew et al., 2000
	Genomic DNA	AF206670	Baneth et al., 2000
	Green frog (*Rana clamitans*)	DQ856589 - 90	Boulianne et al., 2007
H. clamatae	Green frog (*Rana clamitans*)	DQ856584 - 88	Boulianne et al., 2007
H. felis	Domestic cat (*Felis catus*)	AY620232 and AY628681	Criado-Fornelio et al., 2006
	Ocelot	EU028344 and	Metzger et al., 2008

	(*Leopardus pardalis*) and Little Spotted Cat (*Leopardus tigrinus*)	EU267606	
	Korean leopard cat (*Prionailurus bengalensis*)	GQ377216 - 17	Kubo et al., 2010
H. sipedon	Northern Ribbon Snake (*Thamnophis saruitus septentrionalis*)	AF112049 - 48	Smith et al., 1999
	Eastern Garter Snake (*Thamnophis sirtalis sirtalis*)	AF110243 and AF110245 - 47	Smith et al., 1999
	Northern Water Snake (*Nerodia sipedon sipedon*)	AF110244	Smith et al., 1999
	Snake	AF206671	Baneth et al., 2000
H. tuatarae	Tuatara (*Sphenodon Punctatus*)	GU385470 - 71	Herbert et al., 2010
H. ursi	Japanese black bear (*Ursus thibetanus japonicus*)	AB586028	Not published
		EU041717 -18	Kubo et al., 2010

Species of *Hepatozoon* not Identified			
	Host(s)	GenBank Acc. No(s).	Reference(s)
Hepatozoon spp. in reptiles	Slatey-grey snake (*Stegonotus cucullatus*)	AY252110 - 11	Ujvari et al., 2004
	Spotted tree monitor (*Varanus scalaris*)	AY252106 - 09	Ujvari et al., 2004
	Water python (*Liasis fuscus*)	AY252104 - 05	Ujvari et al., 2004
	Brown tree snake (*Boiga irregularis*)	AY252103	Ujvari et al., 2004
	Brown tree snake (*Boiga irregularis*)	AF29708	Not published
	Sand lizard (*Lacerta agilis*)	EU908289	Not published
Hepatozoon spp. in marsupials	Monito del monte (*Dromiciops gliroides*)	FJ719813 - 14	Merino et al., 2009

	Brown bandicoot (*Isoodon obesulus*)	EF152218 - 30	Wicks et al., 2006
***Hepatozoon* spp. in felids**	Flat-headed cat (*Prionailurus planiceps*)	GQ926901	Salakij et al., 2008
	Leopard cat (*Prionailurus bengalensis*)	GQ926902	Salakij et al., 2010
***Hepatozoon* spp. in canids**	Domestic dog (*Canis familiaris*)	EU146063 - 67	Allen et al., 2008
		FJ497023 - 24	Votja et al., 2009
	Crab-eating fox (*Dusicyon thous*)	AY461377 and AY471614	Criado-Fornelio et al., 2006
	Red Fox (*Vulpes vulpes*)	AY471616	Criado-Fornelio et al., 2006
		HM212627	Dezdek et al., 2010
	Spotted hyena (*Crocuta crocuta*)	EF188809	East et al., 2008
	Bush dog (*Speothos venaticus*)	FJ876444 - 48	Andre et al., 2010
***Hepatozoon* spp. in rodents**	European pine marten (*Martes martes*)	EU686690	Simpson et al., 2005
		EF222257	Criado-Fornelio et al., 2009
	Japanese Marten (*Martes melampus melampus*)	FJ595127 - 34	Kubo et al., 2009
	Wild rat (*Bandicota indica*)	AB181504	Not published
	Bank vole (*Clethrionomys glareolus*)	AY600625 - 26	Criado-Fornelio et al., 2006
	Grass mouse (*Abrothrix sanborni*)	FJ719816 and FJ719819	Merino et al., 2009
	Grass mouse (*Abrothrix olivaceus*)	FJ719815 and FJ719817 - 18	Merino et al., 2009
	Cotton rat (*Sigmodon hispidus*)	EF620026	Johnson et al., 2007
	White-footed mouse (*Peromyscus leucopus*)	EF620027	Johnson et al., 2007
	Red squirrel (*Sciurus vulgaris*)	EF222259	Not published

Hepatozoon **spp. in lagomorphs**	Cotton tail rabbit (*Sylvilagus floridanus*)	FJ895406	Johnson et al., 2009
	Swamp rabbit (*Sylvilagus aquaticus*)	FJ895407	Johnson et al., 2009
Hepatozoon **sp. in aves**	Turkey Vulture (*Cathartes aura*)	GU344682	Not published
Hepatozoon **spp. in invertebrates**	Snake tick (*Amblyomma moreliae*)	EU430233	Vilcins et al., 2009
	Amblyomma fimbriata	EU430231 - 32 and EU430234 - 35	Vilcins et al., 2009
	Opossum tick (*Ixodes tasmani*)	EU430237 - 40	Vilcins et al., 2009

APPENDIX B

PERCENT SIMILARITY MATRIX OF 18S rDNA *HEPATOZOON* SPP.SEQUENCES

COLLECTED FROM VERTEBERATE SPECIES IN THE SOUTHERN

UNITED STATES.

	Opossum	Bobcat 1 (GA)	Bobcat 2 (GA)	Domestic cat (OK)	Coyote 1 (TX)	Coyote 2-1 (OK)	Coyote 2-2 (OK)	Coyote 3-1 (OK)	Coyote 3-2 (OK)	Swamp rabbit (OK)	Cottontail rabbit (OK)	Raccoon, n=4 (OK)	Gray fox (GA)	White-footed mouse (OK)	Cotton mouse (GA)	Woodrat 1 (TX)	Woodrat 2 (TX)	Woodrat 1 (CA)	Woodrat 2 (CA)	Cotton rat (OK)	Grey squirrel (GA)	Woodchuck (MO)	Snake (OK)	*H. americanum* AY864676	*H. americanum* OSU-2	*H. americanum* OSU-1	*H. canis* AY461378	*Hepatozoon* spp. AY620232	*H. ayorgbor* EF157822	*Adelina* AF494058
Opossum	100	92.5	95.0	90.3	93.7	90.3	90.5	93.5	90.9	95.0	95.0	94.1	93.2	95.0	94.8	94.4	94.6	94.8	95.2	94.1	94.6	93.7	94.3	90.1	93.7	93.5	93.6	95.0	94.8	87.4
Bobcat 1 (GA)		100	94.9	91.7	93.8	91.7	91.9	93.6	91.9	94.2	94.6	93.1	92.3	94.0	94.0	93.4	93.6	93.8	94.2	93.1	93.8	92.7	92.5	91.5	93.8	93.6	93.1	94.6	94.0	86.3
Bobcat 2 (GA)			100	92.4	95.9	92.4	92.6	95.7	93.0	96.3	96.7	95.2	95.6	95.0	95.0	94.4	94.6	94.8	95.2	94.3	94.6	93.9	94.3	92.2	95.9	95.7	96.1	97.8	95.0	87.8
Domestic cat (OK)				100	96.2	99.6	99.8	96.4	99.4	93.0	93.4	90.9	91.1	91.9	91.9	91.4	91.5	91.7	92.2	91.1	91.9	90.9	90.9	99.4	96.2	96.0	91.7	92.4	91.9	84.2
Coyote 1 (TX)					100	96.2	96.4	99.8	96.8	96.7	97.2	94.4	94.6	94.4	94.4	93.8	94.0	94.2	94.6	93.5	94.4	92.9	93.7	96.0	100	99.8	95.2	95.9	94.4	87.3
Coyote 2-1 (OK)						100	99.8	96.4	99.4	93.0	93.4	90.9	91.2	92.0	92.0	91.4	91.5	91.8	92.2	91.1	92.0	90.9	90.9	99.8	96.2	96.0	91.8	92.4	92.0	84.2
Coyote 2-2 (OK)							100	96.6	99.6	93.2	93.6	91.1	91.4	92.2	92.2	91.6	91.7	91.9	92.4	91.3	92.2	91.1	91.1	99.6	96.4	96.2	91.9	92.6	92.2	84.4
Coyote 3-1 (OK)								100	97.0	96.5	97.0	94.2	94.4	94.2	94.2	93.5	93.7	94.0	94.4	93.3	94.2	92.7	93.5	96.2	99.8	99.6	95.0	95.7	94.2	87.0
Coyote 3-2 (OK)									100	93.6	94.1	91.5	91.8	92.4	92.4	91.8	91.9	92.1	92.6	91.5	92.4	91.3	91.5	99.2	96.8	96.6	92.4	93.0	92.4	84.4
Swamp rabbit (OK)										100	99.1	94.8	94.3	95.0	95.0	94.4	94.6	94.8	95.2	94.1	94.6	93.5	94.3	92.8	96.7	96.5	95.2	96.3	95.0	87.1
Cottontail rabbit (OK)											100	95.2	94.8	95.4	95.4	94.8	95.0	95.2	95.6	94.6	95.0	93.9	94.8	93.2	97.2	97.0	95.6	96.7	95.4	87.6
Raccoon, n=4 (OK)												100	94.1	93.7	93.7	93.1	93.3	93.5	93.9	93.3	93.5	92.4	92.8	90.7	94.4	94.2	95.0	95.8	93.7	86.9
Gray fox (GA)													100	93.1	93.1	92.4	92.6	92.8	93.3	92.4	92.6	92.0	92.4	90.9	94.6	94.4	98.0	95.6	93.1	87.0
White-footed mouse (OK)														100	99.3	98.9	99.1	99.3	99.8	98.7	99.1	98.0	97.2	91.8	94.4	94.2	93.9	95.0	99.1	88.1
Cotton mouse (GA)															100	98.7	98.9	99.1	99.6	98.5	98.9	97.8	96.9	91.8	94.4	94.2	93.9	95.0	99.6	87.9
Woodrat 1 (TX)																100	99.3	99.1	98.0	98.5	97.4	96.5	96.5	91.2	93.8	93.5	93.3	94.8	98.7	87.7
Woodrat 2 (TX)																	100	99.3	99.3	98.3	98.7	97.6	96.7	91.3	93.7	93.5	93.5	95.0	98.9	87.9
Woodrat 1 (CA)																		100	99.6	98.5	98.9	97.8	96.9	91.5	94.0	93.7	93.7	95.2	99.1	87.9
Woodrat 2 (CA)																			100	98.9	99.3	98.3	97.4	92.0	94.6	94.4	94.1	95.2	99.6	87.9
Cotton rat (OK)																				100	98.3	97.2	96.3	90.9	93.5	93.3	93.0	94.1	98.5	87.4
Grey squirrel (GA)																					100	97.6	96.7	91.8	94.4	94.2	93.5	95.0	98.9	87.7
Woodchuck (MO)																						100	95.9	90.7	92.9	92.7	93.2	93.7	97.8	86.6
Snake (OK)																							100	90.7	93.7	93.5	93.5	94.3	96.9	86.8
H. americanum AY864676																								100	96.0	95.8	91.5	92.2	91.8	84.0
H. americanum OSU-2																									100	99.8	95.2	95.9	94.4	87.3
H. americanum OSU-1																										100	95.0	95.7	94.2	87.0
H. canis AY461378																											100	93.1	93.9	87.8
Hepatozoon spp. AY620232																												100	95.0	87.8
H. ayorgbor EF157822																													100	87.9
Adelina AF494058																														100

VITA

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        The research presented in this dissertation was conducted to better understand the
Hepatozoon species present in vertebrates in the United States. In Chapter 3, *Hepatozoon*
spp. infecting domestic dogs in Oklahoma were genetically characterized using a
hypervariable region of the 18S rRNA gene. A surprising degree of diversity was
revealed in the amplicon sequences compared, suggesting multiple strains of *Hepatozoon
americanum* or species of *Hepatozoon* not yet genetically characterized are cycling in
dogs in the United States. In this chapter, *Hepatozoon* spp. genetic sequence data most
identical to those of *Hepatozoon canis* are reported in dogs for the first time in North
America. The second study (Chapter 4) was a pilot trial conducted to evaluate the
efficacy of ponazuril (toltrazuril sulfone) treatment in a dog experimentally infected with
H. americanum to assess parasite clearance and clinical improvement of the infected dog.
Although possibly responsible for the dog's clinical improvement, ponazuril treatment
did not clear the animal of *H. americanum* as evidenced by PCR and microscopic
detection of parasite in muscle tissue samples harvested at necropsy. Chapter 5 of this
dissertation presents a study conducted to genetically characterize *Hepatozoon* spp.
infecting various vertebrate host species, known and previously unrecognized, in the
southern United States. *Hepatozoon* spp. 18S rDNA was amplified by PCR from whole
blood or tissue samples collected from 16 different vertebrate species from Oklahoma,
Missouri, Georgia, Texas, or the southern portion of California. *Hepatozoon* spp.
sequence comparisons revealed phylogenetic relationships indicative of predator/prey
patterns, with possible transmission cycles apparent between canids and rabbits and
snakes and rodents. Another interesting finding from this study was that a sequence from
a gray fox in Georgia was most identical to a sequence previously reported as *H. canis*
from a domestic dog in Spain. Lastly, the research presented in Chapter 6 describes an
experiment designed to assess the occurrence of transplacental transmission of *H.
americanum* in a chronically infected dam. Seven pups whelped from a dam
experimentally infected with *H. americanum* in a chronic phase of disease were
monitored for circulating parasite by PCR and xenodiagnosis. Pups remained free of
clinical signs throughout the study, were negative by PCR at all bleed dates, and did not
infect *Amblyomma maculatum* in the xenodiagnostic experiment conducted 12 weeks
post whelping.

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