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Short communication

First molecular characterization of canine hepatozoonosis in Argentina: evaluation of asymptomatic *Hepatozoon canis* infection in dogs from Buenos Aires

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Abstract

Canine hepatozoonosis is an expanding tick-borne disease in Argentina. Hepatozoonosis was studied during 1 year in six dogs from the same household in Buenos Aires. Blood parasitemia with *Hepatozoon* gamonts was found in five dogs and all six were positive by PCR for *Hepatozoon* sp. Although the levels of parasitemia fluctuated during the year, no clinical signs of disease were detected during the follow up period. Amplification and sequencing of a 650 bases fragment of the 18S rRNA gene from all six dogs yielded fragments that were 99% identical to *H. canis*. The results of the partial 18S rRNA genotyping with the sub-clinical course of infection and lack of severe hematological abnormalities are compatible with clinical and molecular descriptions of *Hepatozoon canis* infection from other areas of the world. This is the first molecular characterization of *Hepatozoon* from Argentina.

Keywords: *Hepatozoon canis*; Ticks; Parasitemia; PCR; Argentina

1. Introduction

Canine hepatozoonosis is a disease transmitted by the ingestion of ticks infected with apicomplexan protozoa of the genus *Hepatozoon*. *Hepatozoon canis* (James, 1905), described in Asia, Europe, Africa and Brazil, is transmitted by the tick *Rhipicephalus sanguineus* (Baneth et al., 2001). A second *Hepatozoon* species that infects dogs is *Hepatozoon americanum* (Vincent-Johnson et al., 1997). It is found in the southeastern United States, transmitted by the Gulf coast tick *Amblyomma maculatum* and causes severe myositis and lameness (Mathew et al., 1998).

H. canis infects leukocytes and hemolymphatic tissues. Infection has been reported to be associated with a variety of clinical signs ranging from sub-clinical

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infection to a severe life threatening disease with distinct hematological, biochemical and histopathological alterations (Baneth and Weigler, 1997; Gavazza et al., 2003). It has been suggested that the presence of clinical disease might be related to predisposing factors such as immunosuppressive conditions or therapy, genetic defect in neutrophilic function, associated infectious agents such as canine distemper virus (CDV) or immature immune system in animals younger than 4–6 months (Baneth et al., 1997). The most frequent clinical signs observed are fever, anorexia, severe weight loss and lethargy (Baneth and Weigler, 1997). Non-regenerative anemia and neutrophilic leukocytosis were the most frequent hematological alterations found in a preliminary study of canine hepatozoonosis carried out by the authors in Argentina (unpublished data).

Canine hepatozoonosis in Buenos Aires is an expanding infection. It was first described in 1999 (Silva et al., 1999) and subsequently, seven cases were reported in the western region of Greater Buenos Aires (Esarte et al., 1999). However, no species confirmation by molecular methods was carried out in either study.

The aim of the present study was to identify the *Hepatozoon* species found in dogs in Buenos Aires by molecular testing and to describe naturally occurring infection in six dogs that inhabit the same house in a southern suburb of Buenos Aires. Epidemiological and hematological data on these dogs were collected during nearly a 1-year follow up.

2. Materials and methods

Six mixed-breed dogs, 1–12 years old, that live in the same household in Lomas de Zamora in southern Greater Buenos Aires, Argentina, were studied between April 2005 and February 2006. Circulating *Hepatozoon* gamonts had been detected in two of these dogs before the study began (dogs 1 and 3). Blood samples were obtained during four different dates (April, May and July 2005 and February 2006) and evaluated in the DIAP Laboratory. Clinical evaluation of the dogs including tick investigation was done.

Blood was collected in EDTA tubes by puncture of the cephalic vein. The hematological parameters that were evaluated included: packed cell volume (PCV), total plasma proteins by refractometry, leukocyte count and absolute leukocyte numbers. Thin blood smears stained with May Grünwald-Giemsa were examined by light microscopy. Parasitemia was determined by observing 100 microscopic fields of blood smears and buffy coat smears at 1000× magnification. Absolute parasitemia was calculated by multiplying the percentage of parasitized neutrophils by the total number of neutrophils/μl.

Drops of EDTA-anticoagulated blood from each sample collected in April 2005 were spotted on sterile filter papers, dried at room temperature and sent to the Hebrew University of Jerusalem for molecular testing. DNA was extracted from the filter papers using the phenol chloroform method and PCR was performed by amplification of a fragment of the 18S rRNA gene using the HepR and HepF primers as described by Inokuma et al. (2002). The PCR DNA products were sequenced using BigDye Terminator v 3.1 Cycle Sequencing Kit (Perkin-Elmer, Applied Biosystems Divisions, Foster City, CA) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Divisions, Foster City, CA) at the Hylabs Laboratories (Rehovot, Israel) according to the recommendations of the manufacturer. Obtained sequences were evaluated with the ChromasPro software version 1.33 and compared to sequence data available from GenBank using the BLAST 2.2.9 program (http://www.ncbi.nlm.nih.gov/BLAST/).

Serum was extracted from whole blood for a biochemical panel in April 2005 and for protein fractions in February 2006. Blood clots separated from serum in February of 2006 were tested for CDV by RT-PCR in the Kambiotec Laboratory in Argentina. The PCR product was analyzed by electrophoresis in ethidium bromide stained gels and an inhibition control was included in the assay as previously described (Shin et al., 1995; Frisk et al., 1999; Saito et al., 2006).

Dogs included in the study did not receive any immunosuppressive drugs or treatment against *Hepatozoon* infection during the follow up period.

3. Results

The dogs included in the study lived in a yard between a leather workshop and the owner's home. The environment included vegetation and was suitable for the development of ticks, however, none were found on the animals. According to the owner they had been parasitized by ticks during the previous year. Dog 1 was the mother of dogs 3 and 6; dogs 2, 4 and 5 were not related to each other.

The dogs showed no evidence of clinical signs of disease during the study period. However, five of the six dogs had detectable parasitemia during some state of the study (Table 1). In April 2005, dogs 1, 3 and 6 had 223, 84 and 80 gamonts/μl blood, respectively. In the following month, no gamonts were observed in dog 3, but dog 5 showed a parasitemia of 58 gamonts/μl blood. In July no

Table 1
Longitudinal follow up of the main hematological findings from six dogs with hepatozoonosis living in the same household in Buenos Aires, Argentina

Dog	Parameter	April 2005	May 2005	July 2005	February 2006
1 (female, 12 years)	Parasitemia*	223	165	71	†
	PCV (%)	50	46	41	
	PP (g/dl)	8	7.2	7.4	
	WBC (/ml)	10250	10600	11050	
	Albumin (g/dl)	2	–	–	
2 (male, 1 year)	Parasitemia*	0	0	0	82
	PCV (%)	47	42	44	45
	PP (g/dl)	7.2	6.4	6.8	6.8
	WBC (/ml)	12850	16000	11100	9400
	Albumin (g/dl)	2.65	–	–	–
3 (female, 3 years)	Parasitemia*	84	0	0	1646
	PCV (%)	43	42	45	41
	PP (g/dl)	6.4	6	5.6	7.6
	WBC (/ml)	11650	12050	7900	8800
	Albumin (g/dl)	2.3	–	–	–
4 (female, 7 years)	Parasitemia*	0	0	0	0
	PCV (%)	51	51	52	46
	PP (g/dl)	7.2	7	6.6	7.2
	WBC (/ml)	12000	12050	13500	10000
	Albumin (g/dl)	3.1	–	–	–
5 (male, 1 year)	Parasitemia*	0	58	0	75
	PCV (%)	48	45	49	52
	PP (g/dl)	6.8	6.8	6.8	7.6
	WBC (/ml)	11250	8850	12350	9200
	Albumin (g/dl)	2.3	–	–	–
6 (male, 3 years)	Parasitemia*	80	96	0	378
	PCV (%)	40	46	46	42
	PP (g/dl)	6	6	6.6	7
	WBC (/ml)	11600	17200	11300	7600
	Albumin (g/dl)	2	–	–	–

* Expressed as gamonts/μl.
† Animal died in second half of 2005. PCV: packed cell volume (normal values 37–55%). PP: plasma proteins (normal values 6–7.9 g/dl.). WBC: white blood cells (normal values 6000–18,000/μl). Albumin (normal values 2.4–3.6 g/dl.).

dog had circulating gamonts, except for dog 1, whose parasitemia was of 71 gamonts/μl blood. This animal suddenly died in the second half of 2005 without earlier clinical signs, according to the owner's explanation. In February 2006, all the surviving animals were parasitemic except for number 4, reaching parasitemias as high as 1646 gamonts/μl blood (dog 3). Dog 4 remained non-parasitemic as detected by microscopy throughout the study period. In no opportunity were gamonts detected in the buffy coat smears of the animals negative by conventional blood smears.

Amplification and sequencing of a 650 bases fragment of the 18S rRNA gene from all six dogs yielded fragments that were 99% identical to *H. canis* (GenBank accession no. AF176835) by BLAST analysis. Dog 4 who was negative for *H. canis* by blood smear evaluation was positive for this parasite by PCR.

In December 2003, prior to the beginning of the study, circulating gamonts were detected in dog 1. Since then and until July 2005 the PCV increased from 28 to 41% and the parasitemia decreased from 19,530 to 71 gamonts/μl blood without any treatment. An additional decrease in *Hepatozoon* parasitemia was noted in dog 3 who in March 2005 had a parasitemia of 94 gamonts/μl and no gamonts were detected in the blood of this dog 2 months later.

Eosinophilia was found in all six dogs in April, May and July 2005 and was the only leukocyte absolute count alteration encountered.

Hypoalbuminemia was found in four animals (Table 1). Another alteration in the serum proteins was hyperglobulinemia (Table 2) due to a high β-globulin fraction value in four of the dogs. The α_1 fraction was lower than the reference values in dog 3

Table 2
Serum protein fractions in six dogs with canine hepatozoonosis living in the same household

	Unit	Dog						Mean	Refernce
		1	2	3	4	5	6		
Total globulins	g/dl	6.38	4.65	4.42	4.05	3.65	4.15	4.55	2.7–4.1
Alfa 1	g/dl	0.24	0.19	0.21	0.19	0.2	0.12	0.19	0.2–0.5
Alfa 2	g/dl	1.22	1.2	1.0	1.09	0.86	0.57	0.99	0.3–1.1
Beta	g/dl	3.53	2.26	2.28	1.91	1.83	2.56	2.40	1.2–2.2
Gamma	g/dl	1.39	1.0	0.92	0.86	0.76	0.9	0.97	0.8–1.8

and the α_2 fraction was high in two animals. Only in one dog did the gamma fragment of the globulins slightly decrease from normal values. The six dogs were negative to the canine distemper virus by RT-PCR.

4. Discussion

This study describes apparently asymptomatic infection in six dogs living in the same household in Buenos Aires. A follow up of the dogs during 1 year revealed fluctuations in the level of parasitemia with no extreme hematological or biochemistry alterations. Sequencing of the 18S rRNA gene indicated that infection was caused by *H. canis*. This is the first genotypic characterization of *Hepatozoon* from Argentina.

Although most of the reports on *H. canis* infection are from Europe, Asia or Africa, canine hepatozoonosis has been described from South America including reports from several regions in Brazil. Infection in one study from Brazil was reported as caused by *H. canis* (O'Dwyer et al., 2001). More recent studies have shown that a *Hepatozoon* sp. closely related or identical to *H. canis* infects dogs in Brazil (Paludo et al., 2005; Forlano et al., 2006) and can be transmitted to dogs by the tick *Amblyomma ovale* (Forlano et al., 2005). Canine hepatozoonosis in Buenos Aires is an emerging infection with a large number of cases detected during the past 5 years (Eiras et al., unpublished data).

The asymptomatic course of hepatozoonosis in this study is more compatible with descriptions of *H. canis* than of *H. americanum* infection. In contrast to the mild disease usually associated with *H. canis*, *H. americanum* causes a more severe and often fatal disease. Dogs present fever, painful myositis, muscle atrophy, weakness and gait abnormalities that range from stiffness to complete recumbency. Infection with *H. americanum* is usually associated with leukocytosis and low gamont parasitemia typically below 0.1% of the leukocytes, whereas higher levels of parasitemia are generally found in *H. canis* infection (Baneth et al., 2003). Comparisons of *H. canis* isolates from different areas of the world have shown that the 18S gene sequences are relatively conserved and share a high similarity level of 97–100% whereas *H. americanum* was found to have a lower degree of similarity to *H. canis* (Inokuma et al., 2002; Karagenc et al., 2006). The parasites sequenced in this study from 6 dogs indeed shared the level of similarity with published sequences of *H. canis* (99%) as would be expected from *H. canis* isolates. However, this comparison is only based on one gene and further studies are warranted to compare other genes and loci.

In general, more dogs were parasitemic during the summer and parasitemia reached its peak during that time. The presence of ticks is related to the season and this appears to be associated with the presence and level of *H. canis* parasitemia. Therefore, the low concentration of circulating blood gamonts found in the autumn and winter months could be explained by the absence of the definitive host tick during those seasons. In February, the summer in Argentina, although the parasitemia increased, no ticks were evident on the six dogs when examined by veterinarians. This was because the owners were cautious of ticks due to their association with hepatozoonosis and eliminated them from the dogs.

The hyperglobulinemia found in some of the animals was due to chronic infection with the production of high levels of globulins as described in canine hepatozoonosis (Baneth and Weigler, 1997). Hypoalbuminemia in hepatozoonosis is due to a lower hepatic albumin synthesis, anorexia or glomerular protein loss (Baneth et al., 1995).

Detection of circulating gamonts in blood smears is the routine diagnostic method for the detection of *H. canis* infection. The absence of parasitemia does not indicate absence of infection, as there can be false negative results in animals with a low parasitemia or a temporary state of no parasitemia despite tissue infection with *Hepatozoon*. The positive *H. canis* PCR in dog 4 with no microscopic detection of parasitemia indicated that infection is more widespread than what is detected by microscopy of blood smears. This is in agreement with a study from Turkey where the prevalence of hepatozoo-

nosis among 349 dogs was 10.6% by blood smear evaluation and 25.8% by the more sensitive technique of blood PCR (Karagenc et al., 2006).

The fact that the evaluated dogs had no clinical signs of disease throughout the year despite the absence of treatment intervention that could have modified the course of the infection is interesting. This is in agreement with the fact that *H. canis* infection is often sub-clinical or may cause a mild disease when there is no immunosupression by other disease agents, cytotoxic drugs or inherited disorders (Craig, 1990).

In conclusion, this study describes the natural evolution of *H. canis* parasitemia in dogs from Buenos Aires which remained asymptomatic during a 1-year follow up. Additional surveys of *H. canis* detection by different diagnostic methods and other clinical and epidemiological characteristics of the infection are currently carried out to investigate the implications of canine hepatozoonosis in Buenos Aires.

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