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Prevalence and molecular characterization of *Hepatozoon canis* in dogs from urban and rural areas in Southeast Brazil

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ABSTRACT

The objective of this survey was to investigate the prevalence of *Hepatozoon* infection in dogs in the rural and urban areas of Uberlândia, Brazil by PCR and molecular characterization. DNA was obtained from blood samples collected from 346 local dogs from both genders and various ages. Seventeen PCR products from positive blood samples of urban dogs and 13 from the rural dogs were sequenced. Partial sequences of the 18S rRNA gene indicated that all 30 dogs were infected with *Hepatozoon canis* similar in sequence to *H. canis* from southern Europe. Four local dog sequences were submitted to GenBank (accessions JN835188; KF692038; KF692039; KF692040). This study indicates that *H. canis* is the cause of canine hepatozoonosis in Uberlândia and that infection is similarly widespread in rural and urban dogs.

Canine hepatozoonosis is a tick-borne disease caused by protozoan parasites from the genus *Hepatozoon* which includes two species that infect dogs: *Hepatozoon canis* and *Hepatozoon americanum* (Baneth et al., 2003). The prevalence of *H. canis* infection in dogs has been reported from several regions and ranges from 0.9% (Criado-Fornelio et al., 2009) to 71% (Vojta et al., 2009).

Reports of *Hepatozoon* sp. in dogs from Brazil indicated that *H. canis* is responsible for the occurrence of canine hepatozoonosis. However, the occurrence of isolated variants in certain geographic regions is possible (Rubini et al., 2005). Most of the diagnosed cases in Minas Gerais, Brazil were identified previously on occasionally performed laboratory tests and not by a systematic survey (Mundim et al., 1992). No studies which characterize the *Hepatozoon* sp., or its variants, affecting dogs in the rural and urban areas of Uberlândia have been published; therefore, this study investigated the prevalence of *Hepatozoon* sp. infection in dogs in the rural and urban areas of Uberlândia by PCR and molecular characterization of parasite.

Blood samples from urban dogs were collected from dogs living in 147 houses in the city of Uberlândia by surveying houses door to door. Only dogs that had no access to rural environments were selected. Additional blood samples were collected from dogs living in 69 farms, where animals move freely on the property. One or two dogs were sampled per household or farm.

Two milliliters of blood were obtained by jugular vein puncture and stored in tubes containing EDTA. These samples were collected from 346 apparently healthy dogs of varied breeds, 166 (48%) males and 180 (52%) females. One hundred and thirty-four samples (38.7%) were collected from dogs in rural households and 212 (61.3%) in urban households in the municipality of Uberlândia. These dogs included 92 (26.6%) puppies (from 1 month to 1 year) and 254 (73.4%) adults (>1 year old), based on their dentition pattern or information from their owners. The samples were stored at –70 °C until processed and analyzed.

Ticks from infested dogs were collected, placed in jars labeled with each animal's number, and the genera and/or species were identified based on the dichotomous key (Barros-Battesti et al., 2006). The entire body of each dog was inspected in order to observe the presence of ticks. These were collected manually and independent of gender or developmental stage. All ticks were collected from dogs that had one to four ticks, and a maximum of 10 ticks were collected from dogs that were infested with more than four ticks.

The multiple logistic regression test (at 5% level of significance) was used to assess the possible influence of the variables area, age and gender on dog infection with *Hepatozoon*. The chi-square and binomial tests (at 5% level of significance) were used in order to assess the possible relationship between area and gender and between area and age variables using the BIO ESTAT 4.0 software (Ayres et al., 2005).

DNA extraction was performed using a QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany). A fragment of the 18S rRNA gene was

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Table 1
Comparison of *Hepatozoon canis* positivity as indicated by PCR in naturally infected dogs from urban and rural areas of Uberlândia, MG, Brazil, in relation to gender.

Area	Number of PCR + dogs	Positive for *Hepatozoon* sp.		p value*
		Male (n = 166) n (%)	Female (n = 180) n (%)	
Rural	113 (41.2%)	64 (56.6)^{Aa}	49 (43.4)^{Bb}	0.0337*
Urban	161 (58.8%)	69 (42.8)^{Ba}	92 (57.2)^{Aa}	
TOTAL	274	133 (80.1)^{A}	141 (78.3)^{A}	

Chi-square test (*p < 0.05).
Binomial test: Different lowercase letters in the column indicate statistically different values (p < 0.05). Different uppercase letters in the row indicate statistically different values (p < 0.05).

amplified using primers Hep F (5′-ATA-CAT-GAG-CAA-AAT-CTC-AAC-3′) and Hep R (5′-CTT-ATT-ATT-CCA-TGC-TGC-AG-3′), which amplify a conserved *Hepatozoon* region (Inokuma et al., 2002). The amplification reaction was carried out as previously described by Sasanelli et al. (2010). DNA from a *Hepatozoon* sp. isolate obtained from a dog in Brazil by Rubini et al. (2005) was used as a positive control. The PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were evaluated using the ChromasPro software version 1.33 (http://chromas.software.informer.com/) and were compared to other sequences available at the GenBank using the BLAST 2.2.9 software (www.ncbi.nlm.nih.gov/BLAST/). Sequences were further analyzed using the MEGA version 5.05 software (www.megasoftware.net) and a phylogenetic tree was constructed by the Minimum-Evolution algorithm in agreement with the Neighbor-Joining and the Maximum-Likelihood algorithms using the Kimura 2-parameter model. Bootstrap replicates were performed to estimate the node reliability, and values were obtained from 1000 randomly selected samples of the aligned sequence data.

The project was approved by the Ethics Committee for the Use of Animals of the Federal University of Uberlândia (Protocol 033/09).

Of 212 dogs from urban and 134 dogs from rural areas, 161 (75.9%) and 113 (84.3%), respectively, were positive for *Hepatozoon* sp. by PCR, totaling 274 (79.2%) positive dogs. No significant differences (p > 0.05) were observed between areas.

The differences in prevalence between genders were statistically significant (p < 0.05) in each studied area. In rural settings there were more positive males than females, unlike in the urban area, wherein the proportion of positive females was higher (Table 1). Furthermore, a higher proportion of infected adults than puppies were observed in both areas (Table 2).

Of the 212 dogs from the urban area, 148 (69.8%) were infested with ticks, all of them were identified as *Rhipicephalus sanguineus*. Of the 134 dogs from the rural area, 18 (13.4%) had tick infestation, of which 14 (77.8%) were infested with *R. sanguineus*, a known vector of *H. canis* (Baneth et al., 2001), three (16.7%) with *Rhipicephalus (Boophilus) microplus*, suspected as a potential *H. canis* vector (de Miranda et al., 2011), and one (5.5%) with *Amblyomma cajennense*, not known to be a vector of *H. canis*. Of those dogs infested with *R. sanguineus*, one dog was infested also with *A. cajennense* and another dog with *R. (B.) microplus*.

Table 2
Comparison of *Hepatozoon canis* positivity as indicated by PCR in naturally infected dogs from urban and rural areas of Uberlândia, MG, Brazil, in relation to age.

Area	Number of PCR + dogs	Positive for *Hepatozoon* sp.		p value*
		Puppy (n = 92) n (%)	Adult (n = 254) n (%)	
Rural	113 (41.2%)	44 (38.9)^{Ba}	69 (61.1)^{Ab}	0.0021*
Urban	161 (58.8%)	34 (21.1)^{Ba}	127 (78.9)^{Aa}	
TOTAL	274	78 (84.7)^{A}	196 (77.1)^{A}	

Chi-square test (*p < 0.05).
Binomial test: Different lowercase letters in the column indicate statistically different values (p < 0.05). Different uppercase letters in the row indicate statistically different values (p < 0.05).

Seventeen PCR products from 161 PCR positive blood samples of urban dogs were sequenced. The BLAST analyses of an approximately 630 bases fragment of the 18S rRNA gene indicated that 14 dog sequences were 99% identical to *H. canis* GenBank accession AY150067 (a fox from Spain) which was the closest match by BLAST, and three sequences were 99% identical to GenBank accession JX027010 (from a *R. sanguineus* tick taken off a dog in Nigeria). Thirteen PCR positive blood samples from the rural dogs were sequenced. The BLAST analyses of sequences from 11 rural dogs were 99% identical to GenBank accession KF034778 (a *R. sanguineus* tick from Turkey) while sequences from two dogs were 99% identical to GenBank accession GU371448 (a red fox from Italy).

Two *H. canis* sequences from urban dogs from Uberlândia were deposited in Genbank as accessions KF692039 and KF692040 and two sequences from rural dogs were deposited in GenBank as accessions JN835188 and KF692038. Phylogenetic analyses of 585 bases of the 18S rRNA gene using three different algorithms were similar and showed that accessions JN835188, KF692038 and KF692040 clustered with *H. canis* sequences from other locations and countries. KF692039 clustered with *H. canis* from Nigeria and separately from the three other Uberlândian *H. canis* sequences. *H. canis* from rural dogs in Uberlândia did not cluster separately from urban dogs' *H. canis* sequences. All *H. canis* sequences clustered seperately from *H. americanum* and *H. felis* (Fig. 1).

Several studies have focused on the phylogenetic characterization of *Hepatozoon* spp. isolated from different regions of Brazil, in neighboring countries and from hosts other than dogs (De Bortoli et al., 2011; Rubini et al., 2005; Vargas-Hernandez et al., 2012). In the present study, *H. canis* was shown to be the cause of canine hepatozoonosis in Uberlândia and was found to be closely related to the causative agent of canine hepatozoonosis in southern Europe. Despite the fact that there was a variation in the 18S rRNA sequence among dogs in both urban and rural locations in Uberlândia, these sequences still differed from those of other *Hepatozoon* species such as *H. americanum* and *H. felis*. The detection of *H. canis* in Uberlândia is consistent with the findings of Paludo et al. (2005) and Rubini et al. (2005) in other geographic regions of Brazil.

The infection rates observed in this study were higher than the rates reported by Rubini et al. (2005) and Spolidorio et al. (2009) that observed infection rates between 25% and 67% in dogs from rural and urban areas using PCR. The prevalence rate of *Hepatozoon* sp. infection can vary considerably and depends on the design of the study, sampling methodology, characteristics of the canine population, distribution of the vector, and the diagnostic methods employed.

Other studies have found that infection rates were higher in rural than in urban areas (O'Dwyer et al., 2001; Spolidorio et al., 2009). In this study there was no statistical difference between the studied areas; however, the highest proportion of positive animals came from the rural area. Dogs in rural areas may have free access to the forest and other environments where different species of wild and domestic animals are present. Under these conditions, rural dogs may become infested with different species of ticks which primarily infest wildlife animals (Labruna and Campos Pereira, 2001).

Regarding the higher *Hepatozoon* sp. infection rate of females in urban areas, and conversely, the higher rate of male infection in rural dogs, the results of this survey were different from the observations reported by Gomes et al. (2010). Male dogs from the rural area can transit more frequently between different rural properties chasing females on heat or fighting other dogs, and are thus more