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Short communication

# *Didelphis albiventris* naturally infected with *Hepatozoon canis* in southeastern Brazil

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ABSTRACT

*Hepatozoon* species are vector-borne pathogens that infect domestic and wild animals. Marsupials of the species *Didelphis albiventris* are adapted to urban and peri-urban areas and act as reservoir hosts for several parasites. The present study evaluated the occurrence of infection by *Hepatozoon* species in synantropic *D. albiventris* from Botucatu, São Paulo, Brazil. Blood samples and ectoparasites from 19 *D. albiventris* were collected from urban and peri-urban areas. *Hepatozoon* spp. detection was performed by microscopy and molecular analysis. One opossum was positive for *Hepatozoon* spp. in microscopy analysis and PCR, while another animal was positive only in PCR. The obtained sequences were 100% identical to *Hepatozoon canis*. Six species of ticks and two species of fleas were detected on *D. albiventris*. This is the first report of *H. canis* in synantropic *D. albiventris*. In Brazil, *H. canis* transmission among dog populations is not well established, which highlights the importance of investigating the role that opossums might play in the epidemiology of this protozoan.

## 1. Introduction

*Hepatozoon* species (Adeleorina: Hepatozoidae) are vector-borne parasites that infect domestic and wild animals. More than 300 species have been described infecting leukocytes or erythrocytes of mammals, reptiles, amphibians and birds. The life cycle is heteroxenous and involves an intermediate vertebrate host and a definitive invertebrate host such as ticks, lice, fleas, flies, and leeches (Smith, 1996).

Canine hepatozoonosis is caused by *Hepatozoon americanum* or *Hepatozoon canis,* and ixodid ticks have been reported as vectors (Baneth et al., 2003). In the southeastern region of Brazil, molecular characterization has shown that *H. canis* is the species involved in canine hepatozoonosis (Rubini et al., 2005; Demoner et al., 2016). Studies have revealed the potential of *H. canis* to infect a wide range of wildlife, such as foxes and golden jackals (Criado-Fornelio et al., 2003; Duscher et al., 2013; Farkas et al., 2014).

The opossum *Didelphis albiventris* is omnivore, well adapted to several environments, exhibit high synanthropy and their contact with domestic animals and humans in urban areas is increasingly frequent (Muller et al., 2005). Due to these characteristics, *Didelphis* spp. are considered disseminators of pathogens. In addition, opossums may act as reservoir host or definitive host of protozoa, helminths and arthropods (Muller et al., 2005).

In Colombia, Ayala et al. (1973) observed *Didelphis marsupialis* infected by two distinct haemogregarines and one of them was identified as *Hepatozoon didelphydis*. In French Guyana, 25% and 17% of *D. marsupialis* and *D. albiventris,* respectively, were parasitized by *Hepatozoon* spp. *Hepatozoon didelphydis* was identified in *D. marsupialis* and *Hepatozoon* sp. that was found in *D. albiventris* is probably a new species (Thoisy et al., 2000). *Hepatozoon* species have never been detected in marsupials in Midwestern and Southern regions of Brazil (Criado-Fornelio et al., 2006; Wolf et al., 2016). As for other regions of the country, there is no data available neither on the presence nor the absence of *Hepatozoon* infecting marsupials.

Considering the reports of *Hepatozoon* spp. infection in marsupials (Ayala et al., 1973; Thoisy et al., 2000; Merino et al., 2009; Allen et al., 2011) and the role of *D. albiventris* as reservoirs for various vector-borne pathogens, our study evaluated the occurrence of *Hepatozoon* spp. infecting the synantropic *D. albiventris* and report for the first time *H. canis* infecting these animals.

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## 2. Materials and methods

### 2.1. Samples collection

A total of 19 blood samples of *D. albiventris,* from urban and *peri*-urban areas in Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W), São Paulo, Brazil were collected between August and October 2013. The captured animals, apparently healthy, were sent to the Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS) and posteriorly were anesthetized with a ketamine and midazolam combination. Blood samples were taken from the tail vein and were kept in EDTA tubes at −20 °C until DNA isolation. After blood collection, thin blood smears were prepared, fixed with methanol and stained with Giemsa for microscopic examination.

*Didelphis albiventris* were inspected for the presence of ectoparasites. Arthropods were stored in 70% ethanol. Morphological identification of ectoparasites was performed using taxonomic keys (Barros-Battesti et al., 2006; Martins et al., 2010; Linardi and Santos, 2012).

### 2.2. DNA extraction, amplification and sequencing

Total DNA of each sample was isolated from 200 µl of blood using the Illustra Blood genomic Prep Mini Spin Kit® (GE Healthcare, Buckinghamshire,UK), following the manufacturer's instructions. The detection of *Hepatozoon* species was performed by nested PCR using the 4558/2733 (Mathew et al., 2000) and Hep300/Hep900 (Ujvari et al., 2004) primers pairs, targeting the 18S rRNA region. In each PCR assay, a negative (distilled water) and a positive (*H. canis* DNA isolated from a dog naturally infected) control were used.

The amplified DNA segment was purified using Illustra ExoProStar 1-Step (GE Healthcare, Buckinghamshire, UK) in agreement with the manufacturer's recommendations, sequenced using BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an automated Applied Biosystems ABI 3500 DNA genetic analyzer.

### 2.3. Phylogenetic analysis

The obtained DNA sequences were edited using BioEdit software, version 7.2.5 (Hall, 1999) and compared for similarity to the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Obtained sequences in this study were aligned with sequences available in GenBank by the MUSCLE algorithm, in the software GENEIOUS v.7.1.3 (Biomatters, http://www.geneious.com). The jModelTest v.2.1.10 (Darriba et al., 2012) was used to identify the best evolutionary model for maximum likelihood analysis. The best model chosen based on the Akaike Information Criterion (AIC) was TPM2uf + G. A phylogenetic tree was inferred by the maximum likelihood methods using PhyML v.3.0 (Guindon et al., 2010), with 1000 bootstrap replicates. For displayed phylogenetic tree FigTree v.1.4.3 was used (http://tree.bio.ed.ac.uk/software/figtree/).

### 2.4. Ethical approval

This study was approved by the local ethics committee on animal use (protocol 10/2012/CEUA/FMVZ) and by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) through the System of Authorization and Information on Biodiversity (SISBIO 33162-2).

## 3. Results

The microscopic examination of thin blood smears revealed one *D. albiventris* infected with *Hepatozoon* sp. An ellipsoidal *Hepatozoon* gamont was detected surrounded by a capsule near a leucocyte (Fig. 1). The gamont measured 8.88 × 5.17 µm and the nucleus 3.84 × 3.25 µm.

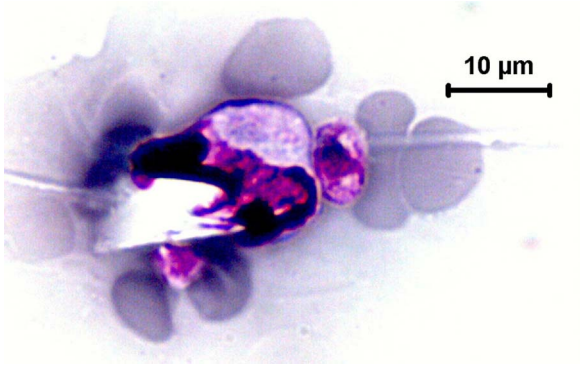


**Fig. 1.** *Hepatozoon canis* gamont near a leucocyte of a naturally infected *Didelphis albiventris* from São Paulo, Brazil.

The animal positive in the microscopy was also PCR-positive for *Hepatozoon* spp. Another animal was positive only by PCR, so *Hepatozoon* DNA was detected in two *D. albiventris* specimens. The two *Hepatozoon* sequences obtained in this present study were identical and BLAST search revealed 100% of similarity to *H. canis* from a Brazilian dog (KU569168), from a crab-eating fox (AY150067), and from a Spanish fox (AY461375). *Hepatozoon canis* sequences identified in our study were deposited in *GenBank* (KY392884 and KY392885). A phylogenetic tree inferred on a 607 bp fragment of the 18S rRNA gene (Fig. 2) showed that *H. canis* sequence obtained from *D. albiventris* grouped in the same clade with *H. canis* sequence detected in a dog from Botucatu (KU569168) and with *H. canis* sequences from wild animals (AY461375, AY461376 and AY150067). In addition, the *H. canis* sequence obtained from *D. albiventris* was positioned in a distinct clade of *Hepatozoon* spp. sequences from marsupials (FJ719813 and FJ719814).

Eighteen tick specimens were collected on five *D. albiventris*. Ticks were identified as three *Ixodes loricatus* (adults), four *Amblyomma sculptum* (nymphs), two *Amblyomma dubitatum* (nymphs), one *Amblyomma ovale* (nymph), three *Ornithodoros mimon* (larvae) and five *Amblyomma* sp. (larvae). Ten of the opossums examined were infested with fleas. A total of 40 flea specimens were collected. The fleas consisted of 33 *Ctenocephalides felis* and seven *Polygenis* sp. The two *H. canis* infected animals were not infested by ticks or fleas.

## 4. Discussion and conclusion

Our results demonstrated that the synantropic *D. albiventris* is susceptible to infection with *H. canis*. Other *Hepatozoon* species have been reported in marsupials such as *D. marsupialis* in Colombia (Ayala et al., 1973), *D. albiventris* and *D. marsupialis* in French Guyana (Thoisy et al., 2000), *Dromiciops gliroides* in Chile (Merino et al., 2009) and *Didelphis virginianum* in the United States (Allen et al., 2011). However, to our knowledge, this is the first report of *H. canis* infection in *D. albiventris* from Brazil.

*Hepatozoon canis* is considered endemic in Botucatu, São Paulo, Brazil, with prevalence of 66.45% in dogs from rural areas (Demoner et al., 2016) and 67.7% in dogs from urban areas (Rubini et al., 2005), however, vectors, paratenic hosts and reservoirs for *H. canis* in this region remain unknown. In the present study, *D. albiventris* was infected with *H. canis* and their role as *H. canis* reservoir should be evaluated in the future.

*Hepatozoon* spp. have never been detected in *D. albiventris* in previous studies performed in the Brazilian Pantanal and in Southern Brazil (Criado-Fornelio et al., 2006; Wolf et al., 2016). Nevertheless, we detected two (10.52%) *D. albiventris* with *H. canis* infection. Aktas et al. (2015) suggested that factors such as vector density, geographic distribution, and host immune status may play a role in the prevalence of

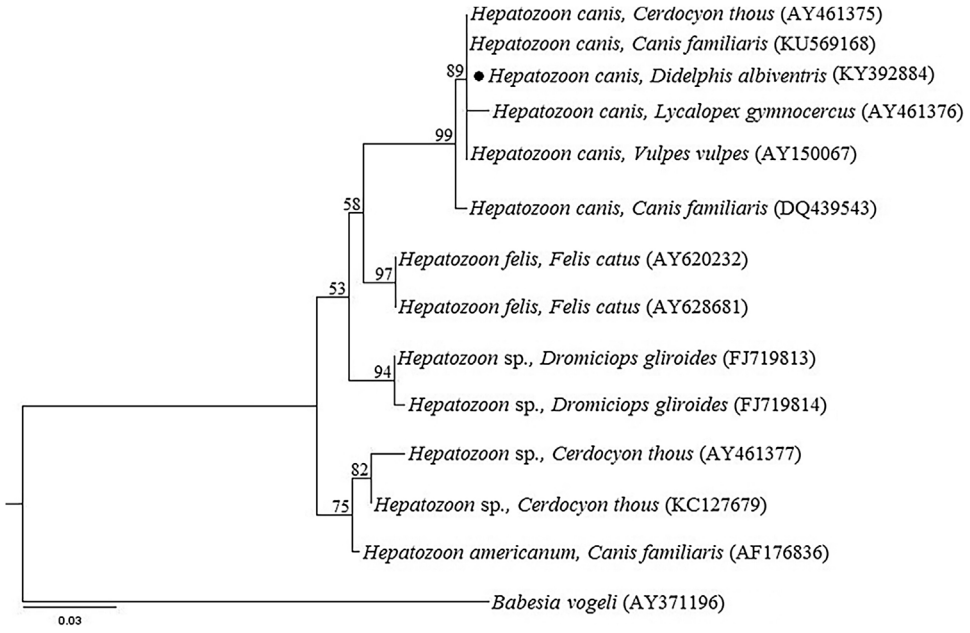


**Fig. 2.** Phylogenetic tree of maximum likelihood analysis based on the partial sequences of the 18S rRNA gene of *Hepatozoon* species. The branch length scale represents 0.03 substitutions per site. *Babesia vogeli* was used as the outgroup. (●) sequence obtained in this study.

*H. canis*. In addition, *H. canis* sequence obtained from *D. albiventris* seems to be from the same lineage of *H. canis* detected in Brazilian dogs from the same region. We collected blood samples of *D. albiventris* from urban and *peri*-urban environments, which might have facilitated cross-species *H. canis* transmission among marsupials and dogs.

In Brazil, the question concerning how dogs become infected with *H. canis* remains without answers (Demoner et al., 2016). Host vertebrate infection with *H. canis* occurs through ingestion of a tick containing mature oocysts. *Rhipicephalus sanguineus* sensu lato (s.l.) is the main vector of *H. canis* (Baneth et al., 2003) and transstadial survival from larvae to nymphs and from nymphs to adults has been shown in *R. sanguineus* s.l. (Baneth et al., 2001; Giannelli et al., 2013). However, in Brazil *R. sanguineus* s.l. seems to not be involved in the transmission of *H. canis*, therefore, other ticks species may transmit the parasite (Forlano et al., 2005; Rubini et al., 2009; Miranda et al., 2011; Demoner et al., 2013). In our study, six tick species (*I. loricatus*, *A. sculptum*, *A. dubitatum*, *A. ovale, Amblyomma* sp. and *O. mimon*) were detected on *D. albiventris. Amblyomma ovale* is considered as potential vector of *H. canis* (Forlano et al., 2005; Rubini et al., 2009; Demoner et al., 2013) and it infests dogs and opossums (Labruna et al., 2005; Blanco et al., 2017). In the present study, only one nymph of *A. ovale* was observed on *D. albiventris.* Conversely, dogs are infested with adult stages (Labruna et al., 2005). This aspect suggests that nymphs might become infected by feeding on *Hepatozoon* positive opossums and when turning into adults, transmit the infection to dogs.

*Ctenocephalides felis* was the most prevalent species infesting opossums in this study. Interestingly, Demoner et al. (2016) have previously reported *C. felis* as the most prevalent and abundant ectoparasite among dogs infected with *H. canis.* Therefore, future studies should be conducted to evaluate the fleas as *Hepatozoon* spp. vectors given that both dogs and opossums are infested by this arthropod. Fleas are invertebrate hosts and, possibly, definitive host of *Hepatozoon* species that infect *Microtus montanus,* a wild rodent from United States (Watkins et al., 2006). As suggested by Demoner et al. (2016), it would be interesting to investigate whether these ectoparasites play a role as vectors of *H. canis*.

In conclusion, this is the first report on *H. canis* infection in synantropic *D. albiventris*. Further studies are necessary to elucidate whether *D. albiventris* have any importance in the eco-epidemiology of *H. canis* in Brazil, since opossums and dogs can share the same habitat and the same ectoparasites.

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