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# *Hepatozoon* spp. infections in wild rodents in an area of endemic canine hepatozoonosis in southeastern Brazil

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ABSTRACT

*Hepatozoon canis* is a tick-borne parasite that occurs worldwide. In rural areas of Brazil, *H. canis* vectors remain unknown, which has led to speculation about alternative routes of transmission. Small rodents can play a role in the transmission (via predation) of *Hepatozoon americanum*, which led us to question whether predation might be an alternative mode of transmission for *H. canis*. Thus, this study investigated whether *Hepatozoon* spp. are present in wild small rodents in forest fragments that surround rural areas in Botucatu County, São Paulo, Brazil, where canine hepatozoonosis is endemic. The study included blood samples from 158 dogs, which were screened by microscopy and molecular analysis. Blood samples and tissues from 67 rodents were obtained for histopathology and molecular detection. The prevalence of *H. canis* was high (66.45%) in dogs from rural areas of Botucatu, São Paulo, Brazil. The molecular analysis showed that wild rodent species in Brazil were infected with *Hepatozoon* spp. other than *H. canis*. Therefore, although the hypothesis that sylvatic rodents act as reservoirs for *H. canis* was not supported, the presence of monozoic cysts in the rodents suggests that, in addition to intermediate hosts, wild small rodents in Brazil might act as paratenic hosts of *Hepatozoon* spp. because they harbor infective stages for intermediate host predators.



## 1. Introduction

*Hepatozoon* species (phylum Apicomplexa) are blood parasites that use a wide range of vertebrates as intermediate hosts, including amphibians, reptiles, birds, and domestic and wild mammals (Smith, 1996; Baneth et al., 2003). Members of this genus exhibit a complex and obligate heteroxenous life cycle in which oocysts form in invertebrate definitive hosts (Smith et al., 1999; Baneth et al., 2007) and transmission to vertebrate intermediate hosts commonly occurs through the ingestion of infected invertebrate hosts (various blood-sucking arthropods) (Smith, 1996).

Hepatozoonosis caused by *Hepatozoon canis* is a canine tick-borne disease that occurs worldwide (Baneth, 2011; Aktas et al., 2015). The brown dog tick, *Rhipicephalus sanguineus* sensu lato (s.l.), is the main vector of the disease (Baneth et al., 2007; Giannelli et al., 2013); however, studies indicate the existence of a "*R. sanguineus* group", although the number of species in the group is uncertain (Dantas-Torres and Otranto, 2015; Nava et al., 2015), which suggests that the vector competence of this tick species for *H. canis* might differ among geographical regions (Dantas-Torres and Otranto, 2015). In Hungary, for example, Hornok et al. (2013) found a high prevalence of canine hepatozoonosis in regions where *R. sanguineus* s.l. is not considered to be endemic. In Brazil, apparently, the tropical lineage of *R. sanguineus* s.l. is not associated with the transmission of the protozoan (Rubini et al., 2009; Demoner et al., 2013).

In addition to infection through the ingestion of ticks, non-vector transmission of *H. canis* has been demonstrated (e.g., vertical transmission) (Murata et al., 1993). Several studies have strongly suggested that *Hepatozoon americanum* (the causative agent of canine hepatozoonosis in the USA) can be transmitted by predation (ingestion of cystic forms of the parasite in laboratory rodents and rabbits) (Johnson et al., 2008a; Johnson et al., 2008b; Johnson et al., 2009a). Furthermore, in the southeastern USA, case reports have

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linked *H. americanum* infection to a high proportion of dogs that had access to natural prey (Johnson et al., 2009b). Epidemiological data have indicated a high prevalence of *H. canis* infections among dogs in rural areas of Brazil (O'Dwyer, 2011). In rural areas, dogs often roam the forests and, probably, engage in predation (Rubini et al., 2008; de Miranda et al., 2014).

In Brazil, *H. canis* DNA has been detected in *Amblyomma cajennese* sensu lato; however, this group of ticks might not play a role as vectors (Melo et al., 2016). Additionally, although *H. canis* oocysts have been identified in *Rhipicephalus microplus* (formerly, *Boophilus microplus*) and *Amblyomma ovale* ticks (Forlano et al., 2005; de Miranda et al., 2011), the vector competence of these ticks is unknown.

*Hepatozoon* species have been identified in wild small rodents in Europe (Laakkonen et al., 2001; Criado-Fornelio et al., 2006), Africa (Maia et al., 2014), North America (Johnson et al., 2007) and South America (Wolf et al., 2016). Although they are important reservoirs for various vector-borne pathogens and have an important trophic position, which have implications for predator-prey transmission, information about *Hepatozoon* spp. infecting wild rodent populations is lacking. The vector competence of tick species in the transmission of *H. canis* in Brazil is not well understood; therefore, there might be unknown epidemiological aspect(s) (e.g., ingestion of cystozoite forms in paratenic hosts). Within that context, this study investigated whether *Hepatozoon* spp. are present in wild rodents in forest fragments near rural areas in Botucatu County, São Paulo, southeastern Brazil, where canine hepatozoonosis is endemic.

## 2. Material and methods

### 2.1. Dog sampling

Between January and June 2013, 158 apparently asymptomatic dogs were randomly sampled from 40 rural households in the municipality of Botucatu, São Paulo, southeastern Brazil. EDTA blood samples were obtained from cephalic or jugular veins for use in the isolation of DNA. Thin blood smears were taken from the marginal veins of the ear, fixed with metanol, stained with Giemsa, and screened for *Hepatozoon* gametocytes.

Each dog was inspected for ectoparasites. Fleas and ticks were removed and stored in 70% ethanol. Ectoparasites were identified using a stereomicroscope and taxonomic keys (Barros-Battesti et al., 2006; Martins et al., 2010; Linardi and Santos, 2012). To investigate the presence of *Hepatozoon* oocysts in the hemocoel, ticks were dissected following the procedure of Demoner et al. (2013).

### 2.2. Trapping of wild rodents and sample collection

Between September 2013 and June 2014, 67 rodents belonging to five species were live-trapped in forest fragments that surrounded the rural communities in which the domestic dogs were examined. Rodents were collected along terrestrial linear transects using pitfalls traps (each pitfall line consisted of six 60-l buckets spaced 10 m apart) and aluminum Sherman traps (31 × 8 × 9 cm; each transect consisted of 20 Sherman traps spaced at 2 m intervals). Trapping occurred on seven consecutive days, and traps were baited with a mixture of peanut butter, canned sardines, cornmeal, and oatmeal, which has been considered an effective attractant for wild rodents (Peres et al., 2013).

For tissue collection and necropsy, each captured rodent was anesthetized by isoflurane inhalation and weighed, and for DNA isolation, peripheral blood samples were obtained by cardiac puncture. Rodents were euthanized by increasing the anesthetic dose and their organs were removed. Tissue samples were collected for PCR assays and histopathological analysis. For histological analyses, tissues were formalin-fixed, paraffin-embedded, sliced at a 5 μm thickness, and stained with hematoxylin and eosin (H&E). To prevent, for example, Hantavirus and Arenavirus infections, the rodents were trapped and handled following biosafety guidelines (Mills et al., 1995).

### 2.3. DNA isolation, amplification and sequencing

Genomic DNA was extracted from the dog blood samples (approximately 200 μl) and from the rodent samples (100–200 μl of blood and 20–50 mg of skeletal muscle, spleen, and liver) using the Illustra Blood genomicPrep Mini Spin Kit® and the Illustra Tissue Mini Spin Kit® (GE Healthcare, Buckinghamshire, UK), following the protocols provided by the manufacturer. PCR reactions were performed in a total volume of 25 μl containing 12.5 μl of GoTaq® Colorless Master Mix (Promega Corporation, WI, USA), 1 μl of each primer, 5 μl of DNA and 5.5 μl of ultra-pure sterile water. A negative (distilled sterile water) and a positive (*H. canis*-positive dog) control were used in each reaction.

For the PCR screening of the canine blood samples, the primer pair HepF (5′-ATA-CAT-GAG-CAA-AAT-CTC-AAC-3′) and HepR (5′-CTT-ATT-ATT-CCA-TGC-TGC-AG-3′) was used to amplify a 666-bp fragment of the 18S ribosomal RNA gene of *Hepatozoon* spp. (Inokuma et al., 2002). The PCR parameters were 94 °C for 3 min, followed by 37 cycles at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 1 min, and at 72 °C for 7 min.

Detection of *Hepatozoon* species in rodent blood and tissues was performed by conventional PCR using the primers HepF300 (5′-GTT-TCT-GAC-CTA-TCA-GCT-TTC-GAC-G-3′) and Hep900 (5′-CAA-ATC-TAA-GAA-TTT-CAC-CTC-TGA-C-3′), which target a 600-bp fragment of the 18S rRNA gene of *Hepatozoon* spp. (Ujvari et al., 2004). The reactions were performed using the following parameters: 94 °C for 3 min, 35 cycles at 94 °C for 45 s, 56 °C for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 7 min.

For additional phylogenetic analysis, a second PCR protocol targeted a larger segment (approximately 1120 bp) of the 18S rRNA gene in some of the dog samples and all of the rodent samples that tested positive for *Hepatozoon* species in the first PCR. The primer pair 4558 (5-′GCT-AAT-ACA-TGA-GCA-AAA-TCT-CAA-3′) and 2733 (5′-CGG-AAT-TAA-CCA-GAC-AAA-T-3′) was used (Mathew et al., 2000). The following amplification conditions were used for that reaction: 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, annealing at 56 °C for 1 min, 72 °C for 90 s and, following the cycles, a final extension of 72 °C for 7 min.

The resulting amplicons were viewed under ultraviolet light on 1% agarose gel (80 V, 60 min) stained with GelRed™ (Biotium, Hayward, USA), and were measured by comparison with 1Kb plus DNA Ladder (Invitrogen, Carlsbad, EUA) as a molecular marker. The PCR products were purified using Illustra ExoProStar 1-Step (GE Healthcare, Buckinghamshire, UK), then sequenced directly using the Taq DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, USA) in an automated sequencer (ABI-PRISM 377, Applied Biosystems). 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).

### 2.4. Phylogenetic analysis

Phylogenetic reconstructions were based on the sequences obtained in this study (the two *Hepatozoon* spp. sequences from the rodents and a *H. canis* sequence from a domestic dog) and 12 additional *Hepatozoon* spp. sequences retrieved from GenBank. Multiple alignment analysis was performed using the Clustal X version 2.0 (Larkin et al., 2007), and a phylogenetic tree was constructed based on the Neighbor-Joining algorithm and the Kimura

two-parameter model for generating the distance matrix, as implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al., 2013). To estimate the confidence of the branching patterns in the Neighbor-Joining tree, we used the Bootstrap Test with 1000 replications (Felsenstein, 1985).

### 2.5. Data analysis

Cross Tabulation and a Chi-Square Test were used to verify the correlation between two types of categorical variables. In all analyses, the alpha level was set at 0.05. Statistical tests were performed using IBM SPSS Statistics version 22.

### 2.6. Ethical approval

All animal procedures were conducted following approval by the Ethical Committee for Animal Research at the Instituto de Biociências/UNESP (CEUA—Comissão de Ética no Uso de Animais), protocol 431, and in accordance with Brazilian laws under a permanent scientific collection license approved by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) through the System Authorization and Information on Biodiversity (SISBIO 36283-3).

## 3. Results

### 3.1. H. canis *in dogs*

Thirty-nine (24.6%) of 158 dogs presented circulating *Hepatozoon* gametocytes in stained thin blood smears. PCR assays indicated that 66.45% (105/158) of the dogs were infected with *Hepatozoon* sp. The sequences obtained from 20 positive samples were 100% identical. Sequencing yielded sequences that were 99–100% identical to various *H. canis* genotypes that have been deposited in GenBank. A *H. canis* sequence from the dogs examined in our study has been deposited in the Genbank (accession number KU569168 ).

### 3.2. *Ectoparasites on* H. canis-*infected dogs*

Of the 105 dogs that were positive for *H. canis* based on PCR analyses, 52.38% had at least one species of ectoparasite. The only flea species found, *Ctenocephalides felis felis*, was the most prevalent and abundant ectoparasite among the infected dogs (86.4% of 229 ectoparasites recovered). Thirty-four (61.81%) of the dogs had infestations of that flea species. Ten (18.1%) of the infected dogs were infested by ixodid ticks (13.5% of all ectoparasites), which were of four species: *R. sanguineus* s.l. (n = 18), *A. ovale* (n = 6), *Amblyomma sculptum* (n = 6), and *R. microplus* (n = 1). The remaining 11 dogs (20%) harbored mixed infestations by fleas and ticks. Neither the presence of ticks ($\chi^2 = 0.04$, df = 1) nor the presence of fleas ($\chi^2 = 0.08$, df = 1) were significantly ($p > 0.05$) correlated with the presence of *H. canis* infections.

To recover *H. canis* oocysts, ticks from infected dogs were dissected. All but one (1.9%) tested negative for the presence of parasitic forms. A single semi-engorged female *R. microplus* tick had 132 typical sporulated *H. canis* oocysts ($200.0 \pm 14.7 \times 158.9 \pm 6.0$ μm).

### 3.3. Hepatozoon *spp. infections in wild rodents*

Thirty-seven (55.2%) of the 67 rodents examined showed evidence of *Hepatozoon* spp. infection based on DNA in at least one type of tissue examined (blood, liver, spleen, and skeletal muscle). All of the rodent species captured were susceptible to infection (Table 1).

Thirty-seven partial *Hepatozoon* sp. 18S rRNA sequences were produced using the 4558 and 2733 primer set. The multiple alignment identified two distinct *Hepatozoon* genotypes, designated *Hepatozoon* sp. genotype Rodent SP-1 and *Hepatozoon* sp. genotype Rodent SP-2, which had 13 nucleotide differences (1.3%, 973/986). Most (33) of the sequences, which were identical, were from *Hepatozoon* sp. genotype Rodent SP-1 and were derived from the blood or tissue of 11 *Akodon* sp. and one *O. nigripes.* The remaining four sequences, which were identical, belonged to *Hepatozoon* sp. genotype Rodent SP-2 and were obtained from the blood, liver, and spleen of two *O. flavescens* specimens. The two *Hepatozoon* sequences identified in the present study have been deposited in GenBank as follows: *Hepatozoon* sp. genotype Rodent SP-1 (KU667308) and *Hepatozoon* sp. genotype Rodent SP-2 (KU667309).

**Table 1**
Prevalence of *Hepatozoon* infections in rodents collected in the municipality of Botucatu, São Paulo, Brazil.

| Rodent species | Number of specimens | *Hepatozoon* PCR positive (prevalence%) |
|---|---|---|
| *Oligoryzomys nigripes* | 40 | 32.5% |
| *Oligoryzomys flavescens* | 3 | 100% |
| *Akodon* sp. | 19 | 89.5% |
| *Necromys lasiurus* | 4 | 75% |
| *Sooretamys angouya* | 1 | 100% |
| Total | 67 | 55.2% |

The *Hepatozoon* genotypes were 99% identical to the following *Hepatozoon* sp. sequences available in GenBank: AB181504 (*Hepatozoon* sp. in a giant rat from Thailand), FJ719815, FJ719816, FJ719817, FJ719818, FJ719819 (*Hepatozoon* sp. in wild rodents from Chile), AY600625, AY600626, JX644997 (*Hepatozoon* sp. detected in sylvatic rodents from Europe), and EF157822 (a *Hepatozoon ayorgbor* sequence detected in the African snake *Python regius*). In addition, *Hepatozoon* sp. genotype Rodent SP-1 and *Hepatozoon* sp. genotype Rodent SP-2 were, respectively, 99% and 98% identical to *Hepatozoon* sp. Rodent MT (accession number KP757838) detected in wild rodents from the Brazilian Pantanal. Genetic similarities between the *Hepatozoon* isolates from rodents from São Paulo, Brazil, and the *H. canis* sequences from the dogs in the present study and *H. canis* sequences that have been deposited in the GenBank database [DQ43950 (Venezuela) and AY150067 (Spain)] were lower (95%).

Histopathological examination of tissues revealed cystozoites within monozoic cysts (Fig. 1) in the spleen, lungs, and kidneys of 16.2% (n = 37) of the *Hepatozoon*-infected rodents (*Akodon* sp.).

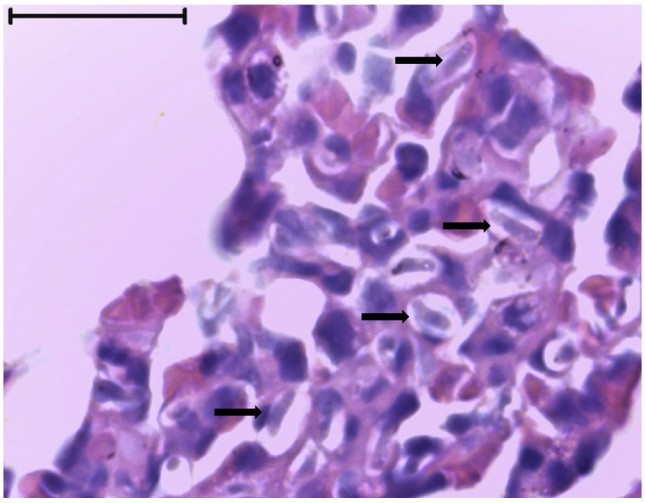

**Fig. 1.** Monozoic cysts containing cystozoites (arrows) in the lung of a naturally *Hepatozoon*-infected *Akodon* sp. from São Paulo, Brazil. Hematoxylin & Eosin stain, Bar = 10 μm.

On average, parasites measured $4.5 \pm 1.2 \times 1.6 \pm 0.5$ μm. *Hepatozoon* sp. 18S rRNA sequences obtained from the tissues containing monozoic cysts were 100% identical to the sequence *Hepatozoon* sp. genotype Rodent SP-1.

### 3.4. Phylogenetic analysis

The Neighbor-Joining phylogenetic tree based on a 504-bp fragment of the 18S rRNA gene (Fig. 2) indicated that the *Hepatozoon* species detected in sylvatic rodents were distinct from the *H. canis* sequences detected in dogs from Brazil and other countries (bootstrap values ranged from 65 to 99). Although the sequences from rodents were grouped in a distinct cluster from an *H. americanum* sequence (with bootstrap support of 84), they were closer to *H. americanum* than they were to *H. canis* isolates.

*Hepatozoon* sp. genotype Rodent SP-1 and *Hepatozoon* sp. genotype Rodent SP-2 grouped with *Hepatozoon* sequences from wild rodents from various countries. In addition, the two genotypes were positioned within a *Hepatozoon* spp. group detected in rodents from South America (FJ719816 and KP757838) and distinct from a *Hepatozoon* spp. group obtained from rodents in Europe (AY600626, KF418366, JX644997, AY600625). *Hepatozoon* sp. genotype Rodent SP-1 clustered close to *H. ayorgbor,* from an African ball python.

## 4. Discussion

More than a century ago, Christophers (1907) first identified the role of *R. sanguineus* s.l. ticks in the transmission of canine hepatozoonosis caused by *H. canis.* That tick species is the main vector of the disease in many regions of the world (Baneth et al., 2007; Giannelli et al., 2013). In Brazil, however, the *R. sanguineus* group does not appear to be involved in the transmission of *H. canis*, and efforts to identify tick species that might serve as competent vectors have not been successful (Forlano et al., 2005; Demoner et al., 2013). Small rodents can play a role through predation in the transmission of *H. americanum* (Johnson et al., 2008b), which led us to suppose that predation might be a transmission route for *H. canis.* To our knowledge, our study is the first to investigate the relationships among *Hepatozoon* species that infect domestic canids and wild rodents in Brazil.

Molecular analyses revealed a high (66.45%) prevalence of *H. canis* among apparently asymptomatic dogs from rural areas in Brazil, which was not unexpected because it appears to be a common parasite in rural areas of Brazil. Rubini et al. (2008) reported a 53.3% prevalence of canine hepatozoonosis in rural communities in São Paulo, Brazil. In rural areas of Minas Gerais, Brazil, de Miranda et al. (2014) found a high (84.3%) prevalence of *H. canis* in domestic dogs. Moreover, *H. canis* infection was also very prevalent (75.9%) in dogs from urban areas, which does not corroborate previous studies that found a prevalence ranging from 0.48% to 7.6% in dogs from urban areas in Brazil (Gomes et al., 2010; Ramos et al., 2010; Ramos et al., 2015).

In our study, although ectoparasite infestation and *H. canis* infections were not correlated, most (61.8%) of the infected dogs were infested by *C. felis felis* fleas. Canine hepatozoonosis is well- known to be transmitted by ticks (Baneth, 2011); however, some *Hepatozoon* species can be transmitted by other blood-sucking arthropods such as fleas, mosquitoes, mesostigmatid mites, and sandflies (Smith, 1996; Laison et al., 2003). In addition, fleas are invertebrate hosts of *Hepatozoon* spp. that infect mammals (Watkins et al., 2006). The role of ticks in the transmission of *H. canis* in Brazil is unclear and a high proportion of *Hepatozoon*-positive dogs were infested by fleas; therefore, there is value in assessing whether these ectoparasites have an important role as vectors of the disease in rural areas of Brazil, which might lead to new approaches for investigating epidemiological aspects of *H. canis.*

The proportion of the naturally *H. canis*-infected dogs that had tick infestations was low (18%). In addition, only one (a *R. microplus* tick) of 31 ticks was infected with typical *Hepatozoon* sp. oocysts. de Miranda et al. (2011) found *H. canis* oocysts in a female *R. microplus* collected from a naturally infected dog. *R. microplus* is a one-host tick (Gonzales, 1974) and, probably, this ixodid does not play an important role in the transmission of the protozoan, primarily because transovarial transmission does not occur in *Hepatozoon* species (Baneth et al., 2007). Furthermore, dogs are not the preferred hosts of *R. microplus* (Franque et al., 2007), and its role in the epidemiology of *H. canis* remains questionable.

In the present study, none of 18 *R. sanguineus* s.l. ticks collected from naturally infected dogs were infected with stages of *Hepatozoon* sp. Demoner et al. (2013) were unable to experimentally transmit *H. canis* to *R. sanguineus* s.l. ticks. Additionally, Melo et al. (2016) performed PCR for the detection of *H. canis* in *R. sanguineus* s.l. ticks (n = 320) collected from domestic dogs and none of the ticks tested positive. Collectively, these findings strongly indicate that the *R. sanguineus* group in Brazil does not act as a vector of *H. canis*.

Overall prevalence of *Hepatozoon* infection was high (55%) among wild rodents in southeastern Brazil. Merino et al. (2009) detected unidentified *Hepatozoon* species in 47% of 17 rodents (*Abrotrix olivaceus* and *Abrotrix sanborni*). Maia et al. (2014) found a 41% prevalence of *Hepatozoon* parasites in wild rodents from North Africa. In North America, *Hepatozoon* spp. are highly prevalent in wild rodents. For example, in the USA, Johnson et al. (2007) detected *Hepatozoon* sp. in 58% of cotton rats and 33.3% of white-footed mice. Wolf et al. (2016) were the first to detect *Hepatozoon* sp. in sylvatic rodents in Brazil, reported a low (7%) prevalence in *Calomys callosus.* Thus, apparently, *Hepatozoon* might be a common parasite of wild rodents worldwide. Differences between studies in the infection rates in Brazil might have been due to the geographical distribution of the parasite and the availability of suitable definitive hosts.

Analysis of the 18S rRNA gene sequences of *Hepatozoon* and the phylogenetic analysis clearly showed that the two *Hepatozoon* genotypes detected in wild rodents in southeastern Brazil are genetically distinct and phylogenetically distant from *Hepatozoon* species that infect dogs. In the USA, Johnson et al. (2007) obtained *Hepatozoon* sequences from wild rodents trapped in endemic areas for American canine hepatozoonosis that were genetically distinct from *H. canis* and *H. americanum*. Maia et al. (2014) performed phylogenetic analyses of *Hepatozoon* sequences from various predators (wild canids and snakes) and their prey (rodents and lizards) in North Africa and concluded that transmission by predation may occur less frequently between prey and canid predators than it does in predator-prey systems involving snakes and lizards. In addition, the *Hepatozoon* sequences obtained from wild rodents were not genetically related to *H. canis* isolates. Those results encourage us to suggest that wild rodents are not involved in the transmission of *H. canis* to domestic dogs; therefore, they might not have a role in the transmission of canine hepatozoonosis in rural areas in southeastern Brazil.

The *Hepatozoon* sequences obtained from Brazilian rodents were genetically similar (99%) to a *H. ayorgbor* sequence found in an African ball python, *P. regius* (Sloboda et al., 2008). In addition, Sloboda et al. (2008) transmitted *H. ayorgbor* to a snake by feeding it tissues from experimentally infected rodents, which suggests that there are three hosts (mosquito-rodent-serpent) in the life cycle of *H. ayorgbor*. Allen et al. (2011) identified a *Hepatozoon* sp. sequence from the snake *Boa constrictor imperator* that was very similar to rodent sequences. The transmission of *Hepatozoon* spp. from prey to snakes usually involves frogs or lizards as a paratenic host (Smith, 1996; Smith et al., 1999); however, given those findings, we sus-

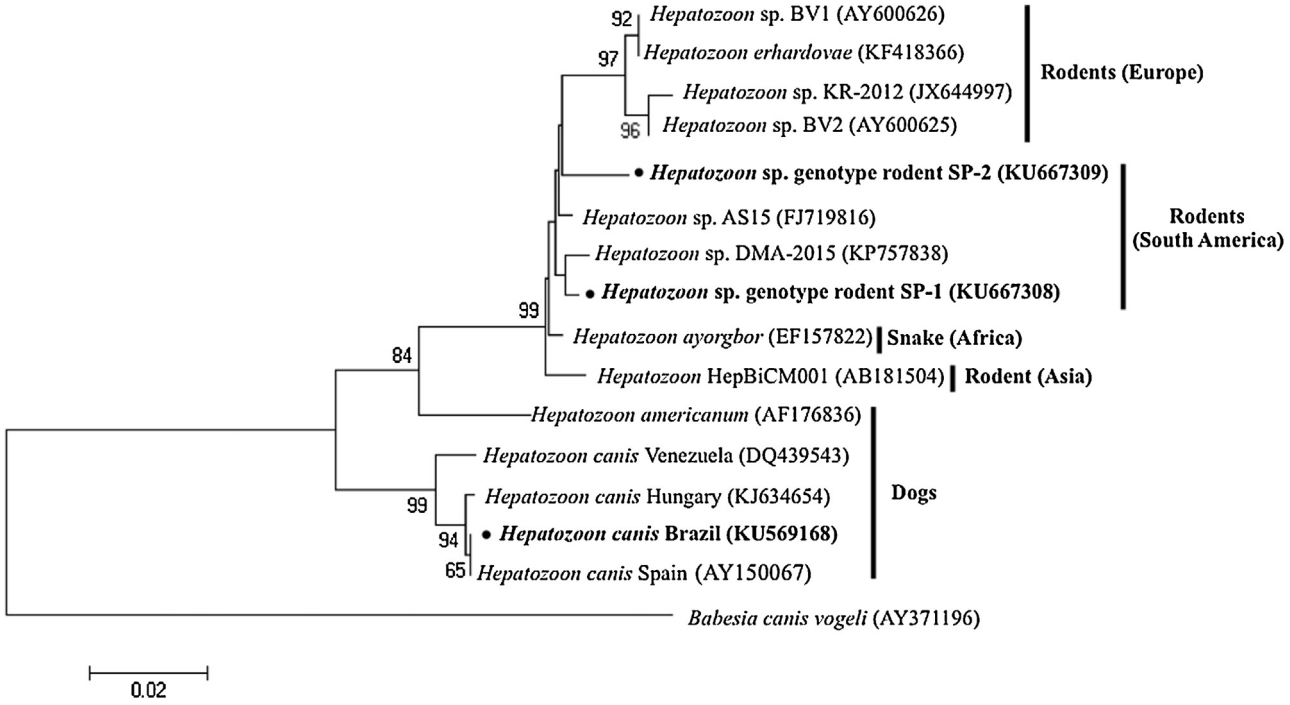


**Fig. 2.** Phylogenetic relationships among *Hepatozoon* species based on the partial sequence of the 18S rRNA gene. Neighbor-joining tree associated with the sequences obtained from domestic dogs and wild rodents in southeastern Brazil and sequences available in the GenBank database. Numbers on the nodes indicate bootstrap values after 1000 replications. Bootstrap values < 50 are not shown. Scale indicates an evolutionary distance of 0.02 nucleotides per position in the sequence. *Babesia canis vogeli* was used as an outgroup. New sequences identified in the study are indicated in bold.

pect that rodents have an important role in the transmission of *Hepatazoon* species that infect snakes.

Monozoic cysts that contained cystozoites were detected in some of the *Hepatozoon*-infected rodents, which is unlike previous studies that did not identify cysts in tissues from naturally infected rodents (Laakkonen et al., 2001; Johnson et al., 2007). Tissue cyst formation has been documented in *Hepatozoon* spp. that infect reptiles and anurans (Smith et al., 1994). Those cyst forms might be infective to intermediate host predators (Johnson et al., 2008a). Our study has shown that wild rodents in Brazil can be an intermediate host or a paratenic host of *Hepatozoon* spp., which underscores the need to understand the role that rodents might play in the epidemiology of *Hepatozoon* spp.

In conclusion, we identified two novel *Hepatozoon* genotypes that infect wild small rodents in Southeastern Brazil, which are not closely related to *H. canis.* Therefore, the question concerning how dogs in Brazil become infected remains without answers; however, because sylvatic rodents harbor infective tissue stages, they might be a source of infection for wild predators.

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## References

Aktas, M., Özübek, S., Altay, K., Balkaya, I., Utuk, A.E., Kırbase, A., Simsek, S., Dumanlı, N., 2015. A molecular and parasitological survey of *Hepatozoon canis* in domestic dogs in Turkey. Vet. Parasitol. 209, 264–267.

Allen, K.E., Yabsley, M.J., Johnson, E.M., Reichard, M.V., Panciera, R.J., Ewing, S.A., Little, S.E., 2011. Novel *Hepatozoon* in vertebrates from the southern United States. J. Parasitol. 94, 648–653.

Baneth, G., Mathew, J.S., Shkap, V., Mancitire, D.K., Barta, J.R., Ewing, S.A., 2003. Canine hepatozoonosis: two disease syndromes caused by separate *Hepatozoon* spp. Trends Parasitol. 19, 27–31.

Baneth, G., Samish, M., Shkap, V., 2007. Life cycle of *Hepatozoon canis* (Apicomplexa: adeleorina: hepatozoidae) in the tick *Rhipicephalus sanguineus* and domestic dog (*Canis familiaris*). J. Parasitol. 93, 283–299.

Baneth, G., 2011. Perspectives on canine and feline hepatozoonosis. Vet. Parasitol. 181, 3–11.

Barros-Battesti, D.M., Arzua, M., Bechara, G.H., 2006. Carrapatos de importância médico-veterinária da região Neotropical—Um guia ilustrado para identificação de espécies. São Paulo: Vox/International Consortium on Ticks and Tick-borne Diseases/Butantan, 223 p.

Christophers, S.R., 1907. The sexual cycle of *Leucocytozoon canis* in the tick: scient. Mem. Off. Med. Sanit. Dept. Gov. India 28, 1–14.

Criado-Fornelio, A., Ruas, J.L., Casado, N., Farias, N.A.R., Soares, M.P., Müller, G., Brum, J.G.W., Berne, M.E.A., Buling-Saraña, A., Barba-Carretero, J.C., 2006. New molecular data on mammalian *Hepatozoon* species (Apicomplexa: adeleorina) from Brazil and Spain. J. Parasitol. 92, 93–99.

Dantas-Torres, F., Otranto, D., 2015. Further thoughts on the taxonomy and vector role of *Rhipicephalus sanguineus* group ticks. Vet. Parasitol. 208, 9–13.

Demoner, L.C., Rubini, A.S., Paduan, K.S., Metzger, B., Antunes, J.M.A.P., Martins, T.F., Mathias, M.I.C., O'Dwyer, L.H., 2013. Investigation of tick vectors of *Hepatozoon canis* in Brazil. Ticks Tick Borne Dis. 4, 542–546.

Felsenstein, J., 1985. Confidence limits of phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Forlano, M., Scofield, A., Elisei, C., Fernandes, K.R., Ewing, S.A., Massard, C.L., 2005. Diagnosis of *Hepatozoon* spp. in *Amblyomma ovale* and its experimental transmission in domestic dogs in Brazil. Vet. Parasitol. 134, 1–7.

Franque, M.P., Santos, H.A., da Silva, G.V.O., Tajiri, J.T., Massard, C.L., 2007. Biological characteristics of *Boophilus microplus* (Acari: ixodidae) on dog under experimental infestation. Braz. J. Vet. Parasitol. 16, 238–242.

Giannelli, A., Ramos, R.A., Di Paola, G., Mencke, N., Dantas-Torres, F., Baneth, G., Otranto, D., 2013. Transtadial transmission of *Hepatozoon canis* from larvae to nymphs of *Rhipicephalus sanguineus*. Vet. Parasitol. 196, 1–5.

Gomes, P.V., Mundim, M.J.S., Mundim, A.V., de Ávila, D.F., Guimarães, E.C., Cury, M.C., 2010. Occurrence of *Hepatozoon* sp: in dogs in the urban area originating from a municipality in southeastern Brazil. Vet. Parasitol. 175, 155–161.

Gonzales, J.C., 1974. O carrapato do boi: vida, resistência e controle. Mestre Jou, São Paulo, 101 p.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hornok, S., Tánczos, B., Fernández de Mera, I.G., de la Fuente, J., Hofmann-Lehmann, R., Farkas, R., 2013. High prevalence of

*Hepatozoon*-infection among shepherd dogs in a region considered to be free of *Rhipicephalus sanguineus*. Vet. Parasitol. 196, 189–193.
Inokuma, H., Okuda, M., Ohno, K., Shimoda, K., Onishi, T., 2002. Analysis of the 18S rRNA gene sequence of a *Hepatozoon* detected in two Japonese dogs. Vet. Parasitol. 106, 265–271.
Johnson, E.M., Allen, K.E., Panciera, R.J., Ewing, S.A., Little, S.E., Reichard, M.V., 2007. Field survey of rodents for *Hepatozoon* infections in an endemic focus of American canine hepatozoonosis. Vet. Parasitol. 150, 27–32.
Johnson, E.M., Allen, K.E., Breshears, M.A., Panciera, R.J., Little, S.E., Ewing, S.A., 2008a. Experimental transmission of *Hepatozoon americanum* to rodents. Vet. Parasitol. 151, 164–169.
Johnson, E.M., Allen, K.E., Panciera, R.J., Little, S.E., Ewing, S.A., 2008b. Infectivity of *Hepatozoon americanum* cystozoites for a dog. Vet. Parasitol. 154, 148–150.
Johnson, E.M., Allen, K.E., Panciera, R.J., Ewing, S.A., Little, S.E., 2009a. Experimental transmission of *Hepatozoon americanum* to New Zealand White rabbits (*Oryctolagus cuniculus*) and infectivity of cystozoites for a dog. Vet. Parasitol. 164, 162–166.
Johnson, E.M., Panciera, R.J., Allen, K.E., Sheets, M.E., Beal, J.D., Ewing, S.A., Little, S.E., 2009b. Alternate pathway of infection with *Hepatozoon americanum* and the epidemiologic importance of predation. J. Vet. Intern. Med. 23, 1315–1318.
Laakkonen, J., Sukura, A., Oksanen, A., Henttonen, H., Soveri, T., 2001. Haemogregarines of the genus *Hepatozoon* (Apicomplexa: adeleina) in rodents from northern Europe. Folia Parasitol. 48, 263–267.
Laison, R., Paperna, I., Naiff, R.D., 2003. Development of *Hepatozoon caimani* (Carini 1909) Pessôa, De Biasi & De Souza, 1972 in the caiman *Caiman c. crocodilus*, the frog *Rana catesbeiana* and the mosquito *Culex fatigans*. Mem. Inst. Oswaldo Cruz. 98, 103–113.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and clustal X version 2.0. Bioinformatics 23, 2947–2948.
Linardi, P.M., Santos, J.L.C., 2012. *Ctenocephalides felis felis* vs. *Ctenocephalides canis* (Siphonaptera: Pulicidae): some issues in correctly identify these species. Braz. J. Vet. Parasitol. 21, 345–354.
Maia, J.P., Alvares, F., Boratynski, Z., Brito, J.C., Leite, J.V., Harris, D.J., 2014. Molecular assessment of *Hepatozoon* (Apicomplexa: adeleorina) infections in wild canids and rodents from North Africa, with implications for transmission dynamics across taxonomic groups. J. Wildl. Dis. 50, 837–848.
Martins, T.F., Onofrio, V.C., Barros-Battesti, D.M., Labruna, M.B., 2010. Nymphs of the genus *Amblyomma* (Acari Ixodidae) of Brazil: descriptions, redescriptions, and identification key. Ticks Tick Borne Dis. 1, 75–99.
Mathew, J.S., Van Den Bussche, R.A., Ewing, S.A., Malayer, J.R., Latha, B.R., Panciera, R.J., 2000. Phylogenetic relationships of *Hepatozoon* (Apicomplexa: adeleorina) based on molecular, morphologic, and life cycle characters. J. Parasitol. 86, 366–372.
Melo, A.L.T., Witter, R., Martins, T.F., Pacheco, T.A., Alves, A.S., Chitarra, C.S., Dutra, V., Nakazato, L., Pacheco, R.C., Labruna, M.B., Aguiar, D.M., 2016. A survey of tick-borne pathogens in dogs and their ticks in the Pantanal biome. Brazil. Med. Vet. Entomol. 30, 112–116.
Merino, S., Vásquez, R.A., Martínez, J., Celis-Diez, J.L., Gutiérrez-Jiménez, L., Ippi, S., Sánchez-Monsalvez, I., de la Puente, J.M., 2009. Molecular characterization of an ancient *Hepatozoon* species parasitizing the 'living fossil' marsupial 'Monito del Monte' *Dromiciops gliroides* from Chile. Biol. J. Linn. Soc. 98, 568–576.
Mills, J.N., Childs, J.E., Ksiazek, T.G., Peters, M.D., Velleca, W.M., 1995. Methods for Trapping and Sampling Small Mammals for Virologic Testing. US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, Atlanta, 61 p.
Murata, T., Inoue, M., Tateyama, S., Taura, Y., Nakama, S., 1993. Vertical transmission of *Hepatozoon canis* in dogs. J. Vet. Med. Sci. 55, 867–868.
Nava, S., Estrada-Peña, A., Petney, T., Beati, L., Labruna, M.B., Szabó, M.P.J., Venzal, J.M., Mastropaolo, M., Mangold, A.J., Guglielmone, A.A., 2015. The taxonomic status of *Rhipicephalus sanguineus* (Latreille, 1806). Vet. Parasitol. 208, 2–8.
O'Dwyer, L.H., 2011. Brazilian canine hepatozoonosis. Braz. J. Vet. Parasitol. 20, 181–193.
Peres, M.G., Bacchiega, T.S., Appolinário, C.M., Vicente, A.F., Allendorf, S.D., Antunes, J.M.A.P., Moreira, S.A., Legatti, E., Fonseca, C.R., Pituco, E.M.M., Okuda, L.H., Pantoja, J.C.F., Ferreira, F., Megid, J., 2013. Serological study of vaccinia virus reservoirs in areas with and without official reports of outbreaks in cattle and humans in São Paulo. Brazil. Arch. Virol. 158, 2433–2441.
Ramos, R., Ramos, C., Araújo, F., Oliveira, R., Souza, I., Pimentel, D., Galindo, M., Santana, M., Rosas, E., Faustino, M., Alves, L., 2010. Molecular survey and genetic characterization of tick-borne pathogens in dogs in metropolitan Recife (north-eastern Brazil). Parasitol. Res. 107, 1115–1120.
Ramos, C.A.N., Babo-Terra, V.J., Pedroso, T.C., Filho, A.F.S., de Araújo, F.R., Cleveland, H.P.K., 2015. Molecular identification of *Hepatozoon canis* in dogs from Campo Grande, Mato Grosso do Sul, Brazil. Braz. J. Vet. Parasitol. 24, 247–250.
Rubini, A.S., Paduan, K.S., Lopes, V.V.A., O'Dwyer, L.H., 2008. Molecular and parasitological survey of *Hepatozoon canis* (Apicomplexa Hepatozoidae) in dogs from rural area of São Paulo state, Brazil. Parasitol. Res. 102, 895–899.
Rubini, A.S., Paduan, K.S., Martins, T.F., Labruna, M.B., O'Dwyer, L.H., 2009. Acquisition and transmission of *Hepatozoon canis* (Apicomplexa: hepatozoidae) by the tick *Amblyomma ovale* (Acari: ixodidae). Vet. Parasitol. 164, 324–327.
Sloboda, M., Kamler, M., Bulantová, J., Votyípka, J., Modryí, T.G., 2008. Rodents as intermediate hosts of *Hepatozoon ayorgbor* (Apicomplexa: adeleina: hepatozoidae) from the African ball python, *Python regius*? Folia Parasitol. 55, 13–16.
Smith, Desser, S.S., Martin, D.S., 1994. The development of *Hepatozoon sipedon* sp. nov. (Apicomplexa Adeleina: hepatozoidae) in its natural host, the Northern water snake (*Nerodia sipedon sipedon*), in the culicine vectors, *Culex pipiens* and *C. territans*, and in an intermediate host, the Northern leopard frog (*Rana pipiens*). Parasitol. Res. 80, 559–568.
Smith, T.G., Kim, B., Desser, S.S., 1999. Phylogenetic relationships among *Hepatozoon* species from snakes, frogs and mosquitoes of Ontario Canada, determined by ITS-1 nucleotide sequences and life-cycle, morphological and developmetal characteristics. Int. J. Parasitol. 29, 293–304.
Smith, T.G., 1996. The genus *Hepatozoon* (Apicomplexa: adeleina). J. Parasitol. 82, 565–585.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Ujvari, B., Madsen, T., Olsson, M., 2004. High prevalence *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. J. Parasitol. 90, 670–672.
Watkins, R.A., Moshier, S.E., Pinter, A.J., 2006. The Flea, *Megabothris abantis* An invertebrate host of *Hepatozoon* sp. and a likely definitive host in *Hepatozoon* infections of the Montane Vole, *Microtus montanus*. J Wildl. Dis. 42, 386–390.
Wolf, R.W., Aragona, M., Muñoz-Leal, S., Pinto, L.B., Melo, A.L.T., Braga, I.A., Costa, J.S., Martins, T.F., Marcili, A., Pacheco, R.C., Labruna, M.B., Aguiar, D.M., 2016. Novel *Babesia* and *Hepatozoon* agents infecting non-volant small mammals in the Brazilian Pantanal, with the first record of the tick *Ornithodoros guaporensis* in Brazil. Ticks Tick Borne Dis. 7, 449–456.
de Miranda, R.L., Castro, J.R., Olegário, M.M.M., Beletti, M.E., Mundim, A.V., O'Dwyer, L.H., Eyal, O., Talmi-Frank, D., Cury, M.C., Baneth, G., 2011. Oocysts of *Hepatozoon canis* in *Rhipicephalus* (*Boophilus*) *microplus* collected from a naturally infected dog. Vet. Parasitol. 177, 392–396.
de Miranda, R.L., O'Dwyer, L.H., de Castro, J.R., Metzger, B., Rubini, A.S., Mundim, A.V., Eyal, O., Talmi-Frank, D., Cury, M.C., Baneth, G., 2014. Prevalence and molecular characterization of *Hepatozoon canis* in dogs from urban and rural areas in Southeast Brazil. Res. Vet. Sci. 97, 326–329.