UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

AVALIAÇÃO DE ROEDORES SILVESTRES COMO FONTE DE INFECÇÃO DE *Hepatozoon canis* A CÃES

LARISSA DE CASTRO DEMONER

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração em Biologia de Parasitas e Micro-organismos.

Profa. Adjunta Lucia Helena O'Dwyer de Oliveira

BOTUCATU – SP

2016

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Palavras-chave: Cães; Hepatozoon; Hospedeiros paratênicos; Roedores silvestres; Transmissão.

Dedicatória

À minha família, em especial minha vó Lezinha

Ao meu marido João Marcelo

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"Só existem dois dias no ano que nada pode ser feito. Um se chama ontem e o outro se chama amanhã, portanto hoje é o dia certo para amar, acreditar, fazer e principalmente viver."

(Dalai Lama)

SUMÁRIO

RESUMO

Espécies do gênero *Hepatozoon* incluem um grupo diverso de parasitas com mais de 340 espécies descritas em anfíbios, répteis, aves e mamíferos. A hepatozoonose canina é uma hemoparasitose transmitida por carrapatos relatada em diversas regiões do mundo e pode estar relacionada às espécies *Hepatozoon canis* e *Hepatozoon americanum.* Em nosso país, diferentes estudos demonstraram que *H. canis* é a espécie envolvida na infecção dos cães e os relatos são comuns, principalmente, em áreas rurais. Trabalhos previamente conduzidos mostram que o vetor de *H. canis* em áreas rurais do Brasil permanece desconhecido, o que pode sugerir a existência de outros mecanismos de transmissão, como a predação de hospedeiros paratênicos. Assim, este trabalho teve como objetivo investigar a presença de *Hepatozoon* spp. em pequenos roedores silvestres capturados em fragmentos florestais nos arredores de propriedades rurais do município de Botucatu, São Paulo, Brasil, onde o parasita foi também estudado em cães domésticos. Foram analisadas 158 amostras sanguíneas de cães, pela reação em cadeia da polimerase (PCR) e pela técnica de extensão de sangue periférico. Amostras de sangue e tecidos de 67 roedores foram coletadas para pesquisa do parasita pela técnica de extensão sanguínea, histopatologia e também por diagnóstico molecular. Os resultados do presente estudo mostraram uma prevalência elevada de *H. canis* em cães de áreas rurais do município de Botucatu (66,45%). Além disso, as espécies de roedores silvestres capturadas em fragmentos de florestas nas proximidades dessas áreas rurais são infectadas por *Hepatozoon* spp., porém, diferentes de *H. canis*. Adicionalmente, a observação de cistos monozoicos nos tecidos dos roedores examinados indicam que, além de atuarem como hospedeiros intermediários, pequenos roedores silvestres podem atuar também como hospedeiros paratênicos de *Hepatozoon* spp., já que albergam nos tecidos formas infectantes para hospedeiros intermediários predadores.

Palavras-chave: *Hepatozoon,* transmissão, hospedeiros paratênicos, roedores silvestres, cães.

ABSTRACT

Hepatozoon spp. belong to a diverse group of parasites with more than 340 species described in amphibians, reptiles, birds and mammals. Canine hepatozoonosis is a tick-borne disease reported throughout the world and can be related to *Hepatozoon canis* and *Hepatozoon americanum*. In our country, previously studies have shown that *H. canis* is the species involved in the infection of dogs and reports are common, especially in rural areas. *Hepatozoon canis* vectors in rural areas of Brazil remain unknown, which might suggest the existence of other transmission mechanisms, such as predation of paratenic hosts. Thus, this study aimed to investigate the presence of *Hepatozoon* spp. in wild rodents trapped in forest fragments surrounding rural areas of Botucatu, São Paulo, Brazil, where the prevalence of the parasite was also studied in domestic dogs. In dogs, the study was conducted in 158 blood samples using polymerase chain reaction (PCR) and peripheral blood extension technique. Blood samples and tissues from 67 rodents were collected for parasite blood extension technique, histopathology and for molecular diagnosis. The results showed a high prevalence of *H. canis* in dogs from rural areas of Botucatu (66.45%). In addition, species of Brazilian wild rodents are infected with *Hepatozoon* spp., other than *H. canis*. Additionally, the finding of monozoics cysts in the examined rodents may indicate that, in addition to intermediate hosts, small wild rodents may also act as paratenic hosts of *Hepatozoon* spp., because they harbor infective stages for intermediate predators hosts.

Keywords: *Hepatozoon,* transmission, paratenic hosts, wild rodents, dogs.

1. INTRODUÇÃO

Espécies do gênero *Hepatozoon* são protozoários do Filo Apicomplexa e de ciclo heteroxeno obrigatório. Uma variedade de vertebrados atuam como hospedeiros intermediários incluindo anfíbios, répteis, aves e mamíferos (Smith, 1996; Baneth et al., 2003).

O gênero exibe um ciclo de vida complexo, com desenvolvimento de grandes oocistos poliesporocísticos no interior dos hospedeiros definitivos (piolhos, pulgas, carrapatos, ácaros mesostigmatas, triatomíneos e mosquitos) (Desser, 1990; Smith, 1996; Baneth et al., 2007) e transmissão para o hospedeiro intermediário vertebrado geralmente ocorrendo pela ingestão do hospedeiro invertebrado infectado (Smith, 1996). Após a ingestão, os esporozoítos liberados dos oocistos invadem os tecidos do hospedeiro vertebrado, onde formam merontes e/ou estágios císticos (Johnson et al., 2008a). Após ciclos merogônicos, os merozoítos formados invadem as células sanguíneas (leucócitos ou eritrócitos, dependendo da espécie de parasita envolvida), onde se desenvolvem em gamontes (Figura 1) (Smith, 1996; Baneth et al., 2007; O'Dwyer, 2011).

A hepatozoonose canina é uma enfermidade transmitida por carrapatos que tem sido relatada em diversas regiões do mundo (Baneth, 2011; Vargas-Hernandez et al., 2012; Giannelli et al., 2013; Aktas et al., 2015). Atualmente, existem duas espécies que infectam cães, *Hepatozoon canis* e *Hepatozon americanum.* Uma doença benigna é a forma mais comum de apresentação na infecção por *H. canis* e frequentemente está associada a baixas parasitemias. Em contraste, a infecção por *H. americanum* é debilitante e geralmente fatal (Baneth, 2011). A caracterização genética que vem sendo conduzida no Brasil demonstra que *H. canis* é o agente etiológico da hepatozoonose canina em nosso país (Rubini et al., 2008; O'Dwyer, 2011; de Miranda et al., 2014).

O carrapato marrom do cão, *Rhipicephalus sanguineus* sensu lato (s.l.), é considerado o vetor biológico da enfermidade e seu papel na transmissão do parasita foi evidenciado em diferentes estudos (Baneth et al., 2007; Giannelli et al., 2013). No entanto, estudos morfológicos e filogenéticos demonstraram evidências da existência de um complexo chamado "*R. sanguineus*", cujo número de espécies ainda é incerto (Moraes-Filho et al., 2011; Dantas-Torres e Otranto, 2015).

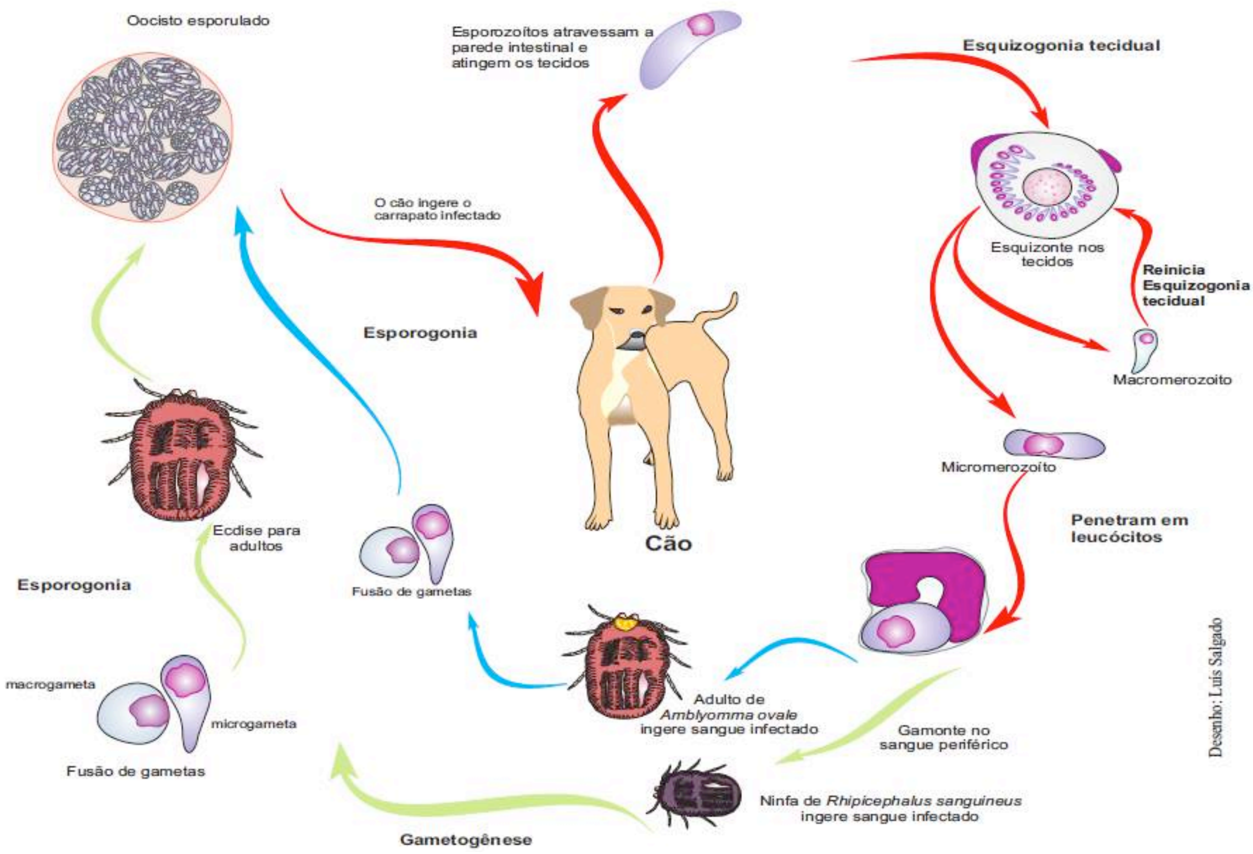

Figura 1. Ciclo biológico de *Hepatozoon canis* (Fonte: O'Dwyer, 2011).

Esse fato pode explicar as diferenças existentes quanto a competência vetorial de *H. canis* entre populações de *R. sanguineus* s.l. Na Itália e em Israel, por exemplo, esse grupo de carrapatos é reconhecido vetor da hepatozoonose canina (Baneth et al., 2007; Giannelli et al., 2013). Por outro lado, na Hungria, alta prevalência de *H. canis* tem sido reportada em regiões livres de *R. sanguineus* s.l. (Hornok et al., 2013). Semelhantemente no Brasil, é possível que as espécies do grupo *R. sanguineus* s.l. existentes não sejam vetoras de *H. canis* (Forlano et al., 2005; Gomes et al., 2010; Demoner et al., 2013).

Em nosso país, os estudos epidemiológicos têm demonstrado que as infecções por *H. canis* são prevalentes em áreas rurais (O´Dwyer et al., 2001; Rubini et al., 2008; de Miranda et al., 2014), onde os cães são infestados por diferentes espécies de ixodídeos, incluindo *R. sanguineus* s.l., *Rhipicephalus microplus* e espécies do gênero *Amblyomma* (Labruna e Campos Pereira, 2001; Ferreira et al., 2013).

No Japão, oocistos de *H. canis* foi relatado em carrapatos *Haemaphysalis* sp. (Murata et al., 1995). Adicionalmente, no Brasil, *R. microplus* e *Amblyomma ovale* têm sido considerados como potenciais vetores do hemoparasita (Rubini et al., 2009; de Miranda et al., 2011). No entanto, *R. microplus* é um carrapato monoxeno e infesta preferencialmente bovinos (Gonzales, 1974; Machado et al., 2014). Além do mais, normalmente, somente o estágio adulto de *A. ovale* é encontrado parasitando cães, e assim, a infecção e a transmissão de *H. canis* parecem ocorrer apenas na fase adulta (Forlano et al., 2005; Labruna et al., 2005; Rubini et al., 2009). Sabe-se que transmissão transovariana não ocorre em *Hepatozoon* spp. e, portanto, é possível que estas espécies de carrapato tenham pouca relevância na transmissão do parasita, pois fêmeas após o ingurgitamento não voltam a se alimentar (Demoner et al., 2013).

Apesar da ingestão de oocistos esporulados ser a principal forma de transmissão de *Hepatozoon* spp. para o hospedeiro intermediário vertebrado, o ciclo biológico alternativo de algumas espécies podem incluir mais de um hospedeiro intermediário, o qual atua também como hospedeiro paratênico, que demonstra nos tecidos uma forma infectante quiescente, os cistozoítos. Estas formas císticas são comumente relatadas no ciclo biológico de espécies de *Hepatozoon* que infectam anuros, lagartos e serpentes. Nesses casos, o hospedeiro intermediário vertebrado se torna infectado ingerindo os cistozoítos presentes no hospedeiro paratênico (Figura 2) (Smith, 1996; Viana et al., 2012; Pereira et al., 2013). A observação de cistos de *Hepatozoon* spp. em mamíferos vem sendo relatada (Desser, 1990; O'Dwyer et al., 2004; Baneth et al., 2007), o que sugere cistozoítos como importante modo de transmissão também neste grupo de animais.

No Canadá, Desser (1990) observou em esquilos (*Sciurus carolinensis*) infectados com *Hepatozoon griseisciuri*, além dos merontes, formas teciduais císticas contendo cistozoítos. Assim, o autor sugeriu que este parasita poderia ser transmitido tanto pela ingestão de artrópode infectado como por predação.

Johnson et al. (2008a) conseguiram transmitir experimentalmente *H. americanum* a pequenos roedores (*Sigmodon hispidus, Mus musculus* e *Rattus novergicus*), a partir de oocistos recuperados de *Amblyomma maculatum.* Através de histopatologia, foi possível observar cistozoítos em diferentes tecidos. Os tecidos desses animais foram oferecidos a um cão suscetível, e este apresentou gamontes circulantes 42 dias após a exposição (Johnson et al., 2008b). Estes resultados sugerem que roedores poderiam atuar como hospedeiros paratênicos de *H. americanum* e ser potencial fonte de infecção a cães domésticos.

Allen et al. (2011), ao realizarem estudo filogenético com *Hepatozoon* spp. de diferentes espécies animais, observaram que as sequências analisadas sugeriram a existência de um ciclo presa-predador, uma vez que, por exemplo, a sequência de *Hepatozoon* sp. de serpentes era muito semelhante a de roedores e as sequências de coelhos eram muito próximas as de carnívoros.

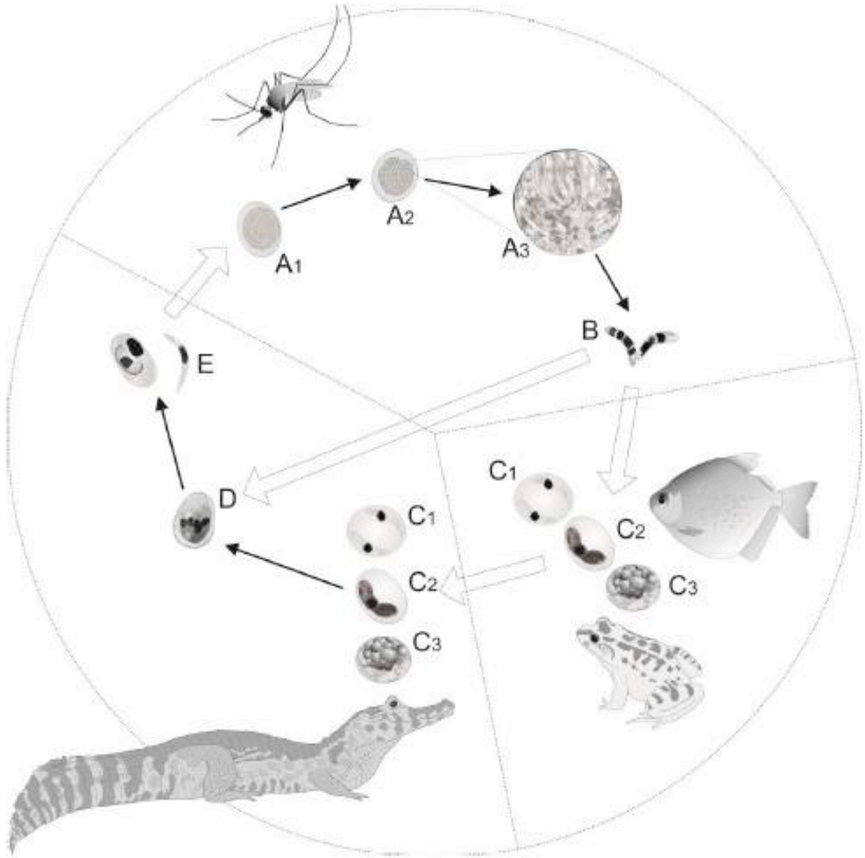

Figura 2. Ciclo biológico de *Hepatozoon caimani* (dos Santos, 2014. Ilustração: Fábio Ricardo da Rosa). O desenvolvimento esporogônico de *H. caimani* ocorre em mosquitos *Culex* sp., nos quais o oocisto (**A1**) se desenvolve e inicia esporulação (**A2**), dando origem ao oocisto maduro (esporulado) (**A3**), contendo esporozoítos infectantes (**B**) para os hospdeiros intermediários ou paratênicos. O hospedeiro intermediário (jacaré) se infecta ingerindo mosquitos infectados com oocistos esporulados, ocorrendo liberação dos esporozoítos no trato digestivo, que em seguida atingem diversos órgãos (rim e fígado, por exemplo) e se diferenciam em merontes (**D**). Os merozoítos liberados dos merontes invadem os eritrócitos, onde se desenvolvem em gamontes (intra e/ou extraeritrocíticos) (**E**), que são as formas infectantes para o artrópode hematófago. Uma forma alternativa de transmissão ocorre quando o hospedeiro intermediário se alimenta de hospedeiros paratênicos infectados com formas císticas (**C1, C2, C3**) de *H. caimani.*

Relatos de *H. canis* em espécies de canídeos selvagens têm sido frequentes (Duscher et al., 2013; Farkas et al., 2014). Sequências genéticas semelhantes às de *H. canis* foram obtidas de raposas vermelhas (*Vulpes vulpes)* na Itália e Croácia (Dezdek et al., 2010; Gabrielli et al., 2010). Não se sabe como ocorre a infecção, mas acredita-se que a predação seja um possível mecanismo de transmissão para estes animais.

Conforme o exposto, nota-se a necessidade de estudar caminhos alternativos de infecção por *H. canis* em cães de áreas rurais do Brasil, já que o modo convencional de transmissão, ingestão de carrapato vetor, não está claramente elucidado. Ainda, cães de áreas rurais têm acesso livre às áreas de matas e florestas e por esta razão podem, eventualmente, estar envolvidos na predação. Pesquisas com *H. americanum* demonstraram, experimentalmente, a importância da predação de hospedeiros paratênicos (pequenos roedores) como fonte de infecção para cães (Johnson et al., 2008 a, b), e pode ser que seja um mecanismo de transmissão também para *H. canis.* Assim, investigamos *Hepatozoon* spp. em pequenos roedores silvestres capturados nos arredores de áreas rurais de Botucatu/SP. Essa pesquisa resultou em dois artigos científicos, os quais compõem os Capítulos 1 e 2 da presente Tese.

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3. OBJETIVOS

O presente estudo teve como objetivo geral investigar o potencial de pequenos roedores silvestres como possíveis reservatórios de *H. canis* para cães em áreas rurais de Botucatu/SP. Para isso, objetivou-se especificamente:

- Realizar a detecção direta do parasita em esfregaços sanguíneos de cães e roedores, e em lâminas histológicas de amostras teciduais de roedores.
- Realizar a detecção molecular em amostras sanguíneas de cães e roedores, e em amostras teciduais de roedores.
- Verificar a prevalência de *Hepatozoon* spp. nas duas categorias (roedores silvestres e cães domésticos).
- Comparar as sequências de *Hepatozoon* obtidas com sequências disponíveis no GenBank, buscando a identidade das espécies envolvidas.

4. CAPÍTULO 1[2]

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

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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 ($200.0 \pm 14.7 \times 158.9 \pm 6.0\,\mu$m).

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.).

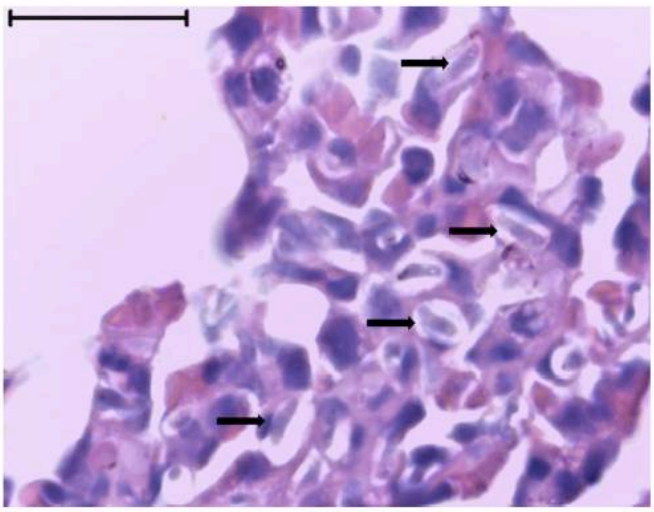

Fig. 1. Monozoic cysts containing cystozoites (arrows) in the lung of a naturally *Hepatozoon*-infected *Akodon* sp. from São Paulo, Brazil. Hematoxylin & Eosin stain, Bar = 10 μm.

On average, parasites measured $4.5 \pm 1.2 \times 1.6 \pm 0.5\,\mu m$. *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-

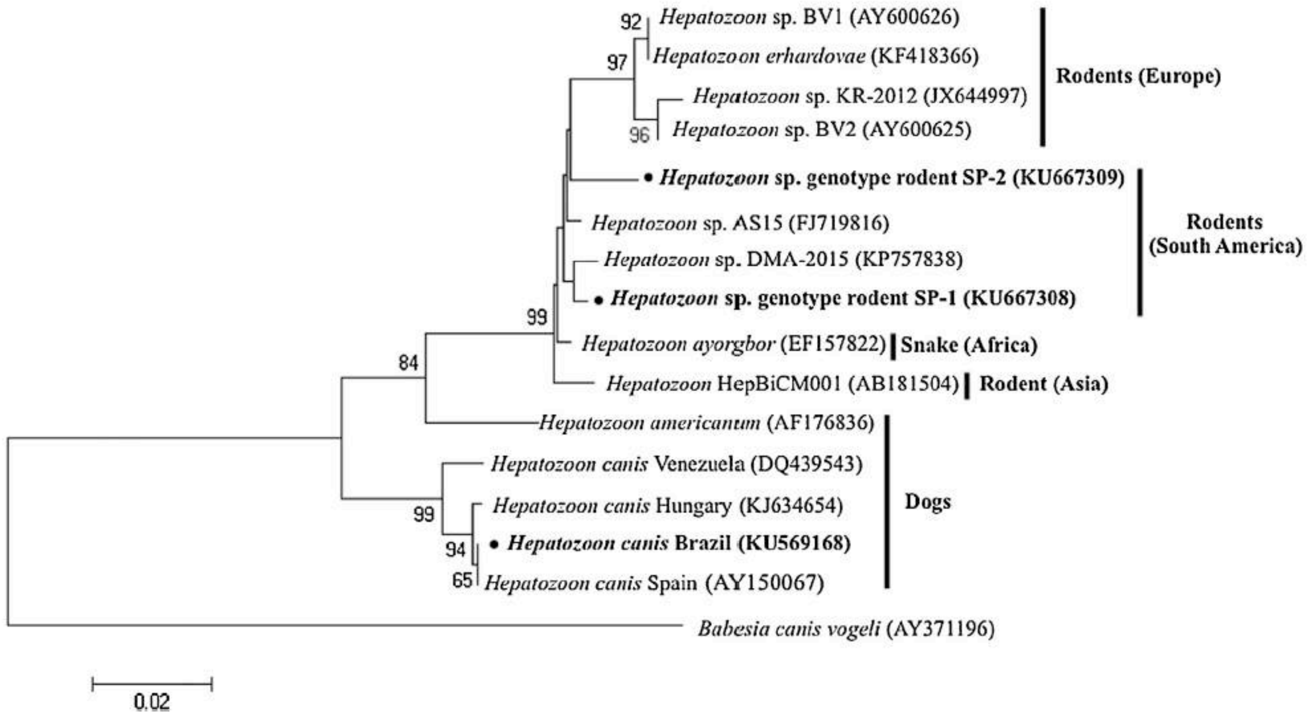

Fig. 2. Phylogenetic relationships among *Hepatozoon* species based on the partial sequence of the 18S rRNA gene. Neighbor-joining tree associated with the sequences obtained from domestic dogs and wild rodents in southeastern Brazil and sequences available in the GenBank database. Numbers on the nodes 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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5. CAPÍTULO 2[3]

ARTIGO CIENTÍFICO:

A new *Hepatozoon* species (Adeleorina: Hepatozoidae) of *Akodon* cf. *montensis* (Rodentia: Cricetidae: Sigmodontinae) from southeastern Brazil

[3] O artigo apresentado a seguir será submetido ao periódico Experimental Parasitology.

A new *Hepatozoon* species (Adeleorina: Hepatozoidae) of *Akodon* cf. *montensis* (Rodentia: Cricetidae: Sigmodontinae) from southeastern Brazil

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Abstract

Members of the genus *Hepatozoon* (Miller, 1908) are blood parasites found in a wide range of host species, including wild rodents; however, information about life cycle, distribution and *Hepatozoon* species diversity infecting these mammals are lacking. Here, we studied the parasite stages and DNA sequences of *Hepatozoon* spp. of seven naturally infected *Akodon* cf *montensis* Thomas, 1913. Thin blood smears, tissue samples and whole blood were obtained for morphology, morphometry and for molecular analysis. Biological and morphological features of the parasite, such as tissue tropism, gamonts and meronts size and morphology as well as the DNA sequence comparison and the phylogenetic analysis indicate that the *Hepatozoon* spp. detected in this study is distinct from those previously reported in small rodents. Thus, we proposed a new species, named *Hepatozoon milleri* sp. nov. Based on morphological and molecular characteristics, this is the first description of a new species of *Hepatozoon* from wild small rodents (*A.* cf. *montensis*) in Brazil.

Keywords: *Hepatozoon*, parasite stages, molecular characterization, *Akodon* cf. *montensis*, Brazil.

1. Introduction

Hepatozoon species are apicomplexan blood protozoan parasites with a worldwide distribution and adapted to a wide variety of vertebrates (Smith, 1996). The complex life cycle is heteroxenous and involves blood-sucking vectors (ticks, mesostigmatid mites, fleas, mosquitoes and lice), in which sexual stages are formed (multisporocystic oocysts containing infective sporozoites). Transmission to vertebrate intermediate hosts occurs through the consumption of infected arthropods. After ingestion, the released sporozoites undergo merogony in a variety of sites (lungs, spleen, liver, kidney and skeletal muscle), depending on the vertebrate hosts or the parasite species. The merogony is followed by the gamontogony, with the development of gamonts in leukocytes or erythrocytes (Smith et al., 1994; Ewing and Panciera, 2003; Laison et al., 2003; Watkins et al., 2006; Baneth et al., 2007).

Alternative life cycles of some *Hepatozoon* spp. can include more than one intermediate host, which acts as paratenic host, harboring in the tissues an infective quiescent form, the cystozoites (Smith, 1996). Those cyst forms have been demonstrated in the life cycle of *Hepatozoon* species that infect anurans, reptiles and mammals (Desser, 1990; Smith et al., 1994; Laison et al., 2003; Viana et al., 2012). In this predator-prey transmission, the vertebrate intermediate hosts become infected through the ingestion of prey containing cystozoites stages of the parasite (Smith, 1996; Johnson et al., 2008; Viana et al., 2012).

Hepatozoonosis has been detected in a wide range of wildlife species, including wild rodents (Criado-Fornelio et al., 2006; Metzger et al., 2008; André et al., 2010; Farkas et al., 2014); however, most of the descriptions of *Hepatozoon* infections in wild rodents are based primarily on molecular features, without incorporating morphological information of the protozoan (Maia et al., 2014; Wolf et al., 2016). Therefore, unidentified *Hepatozoon* species have been reported in wild rodents, which emphasizes the importance of including description of life cycle stages to designate newly recognized *Hepatozoon* organisms.

Wild rodents harbor numerous vector-borne pathogens, including those with zoonotic importance (Silaghi et al., 2012; Duscher et al., 2014); however, the knowledge about *Hepatozoon* species diversity, distribution, vectors and life cycle in wild rodent populations remains quite limited. Herein, we describe a new species of *Hepatozoon* detected in wild small rodents, *A.* cf. *montensis*, in southeastern Brazil, by combining genetic and morphological data.

2. Material and Methods

This study comprised blood and tissue samples from seven *A.* cf. *montensis,* which were found to be infected with *Hepatozoon* sp. by microscopy and PCR. Rodents were live-trapped in three forest fragments in the municipality of Botucatu, São Paulo State, southeastern Brazil, between November 2013 to June 2014, as part of a study on canine hepatozoonosis*,* in order to evaluate the relationships among *Hepatozoon* spp. that infect wild rodents and domestic dogs (Demoner et al., 2016).

Rodents were collected along terrestrial transects, using Sherman traps and Pitfalls traps, as previously described (Demoner et al., 2016). Captured animals were anesthetized with inhalation of isoflurane and blood collection was performed by cardiac puncture, for use in the DNA isolation and for preparation of blood smears. A thin blood smear was prepared of each rodent, fixed with methanol, stained with Wright-Giemsa, and screened for *Hepatozoon* gamonts. Euthanasia was performed by deep anesthesia with isoflurane, followed by organs removal (heart, lung, kidney, spleen, liver, and skeletal muscle). Collected tissues were preserved either for molecular identification of *Hepatozoon* sp. and for histological examination. For histological evaluation, formalin-fixed tissues samples were routinely processed and stained with hematoxylin and eosin (H&E). Parasites morphology and morphometry were analyzed in a computerized image analysis system and measurements were obtained using the software Qwin Lite 2.5 (Leica).

DNA from rodent blood and tissue samples (approximately 100-200 µl of whole blood and 20–50 mg of skeletal muscle, spleen, liver, lung and kidney) was isolated using the Illustra Blood genomicPrep Mini Spin Kit® and the Illustra Tissue Mini Spin Kit® (GE Healthcare, Buckinghamshire, UK), according to manufacturer's instructions.

Amplification of the 18S ribosomal RNA gene (a 666-bp fragment) of *Hepatozoon* spp. was performed from the rodent samples using the primer pair 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') (Ujvari et al., 2004). In order to amplify a larger segment of the 18S rRNA gene of *Hepatozoon* spp. (approximately 1120 bp), a second PCR assay was carried out using the primers 4558 (5-'GCT-AAT-ACA-TGA-GCA-AAA-TCT-CAA-3') and 2733 (5'-CGG-AAT-TAA-CCA-GAC-AAA-T-3') (Mathew et al., 2000), for additional phylogenetic analysis. PCR reactions conditions and sequencing were determined as before (Demoner et al., 2016).

By comparing for similarity to the sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences obtained in the present study were identified as those of *Hepatozoon* spp. and were submitted to multiple alignment analysis (with additional *Hepatozoon* spp. sequences retrieved from Genbank) using the Clustal X version 2.0 (Larkin et al., 2007). For phylogenetic reconstruction, the Neighbor-Joining algorithm and the Kimura two-parameter method were used to create the phylogenetic tree, as implemented in MEGA version 6 (Tamura et al., 2013) and bootstrap analysis on 1000 replicates (Felsenstein, 1985).

All animals procedures were conducted in accordance with the guidelines of the Ethical Committee for Animal Research at the Instituto de Biociências/UNESP (CEUA - Comissão de Ética no Uso de Animais - protocol 431) and under a permanent scientific collection license issued by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO 36283-3).

3. Results

3.1. General observations

By morphological examination, three different parasite stages were found in the infected rodents: gamonts in the peripheral blood (within mononuclear leukocytes or extracellular/free parasites); mature and immature meronts (exclusively in the liver, within hepatocytes); and cystic forms (cystozoites within monozoic cysts) in the lung, spleen and kidney. Out of the seven infected rodents, five were parasitized by all parasite stages, one by gamonts and meronts, and the remaining one rodent was parasitized by meronts and cystozoites.

3.2. Molecular and phylogenetic analysis

Based on the PCR and sequencing, a *Hepatozoon* sp. sequence, with lengths ranging from 1000 to 1050 base pairs (bp), was obtained from the blood and tissues of the infected *A.* cf. *montensis*. Blast analysis of that sequence showed nucleotide sequence similarities (97-99%) with other *Hepatozoon* sequences detected in wild rodents from various parts of the world [Genbank accession numbers: FJ719817 and FJ719819 (*Hepatozoon* sp. in *Abrothrix olivaceus* and *Abrothrix sanborni*, respectively, from Chile), AB181504 (*Hepatozoon* sp. in *Bandicota indica* from Thailand), AY600625 and JX644997 (*Hepatozoon* sp. detected in bank voles from Europe)].

The *Akodon*-derived *Hepatozoon* sp. sequence grouped into a major clade containing *Hepatozoon* spp. sequences detected in wild rodents in several regions of the world, while sequences from carnivora formed a distinct clade (Figure 1). Although those sequences from rodents were relatively closely related, the genetic differences supported their segregation in different clusters. Moreover, the new sequence reported in this study clustered with a *Hepatozoon* sequence previously isolated from wild rodents in the Brazilian Pantanal (accession number KP757838) (Wolf et al., 2016), with <1% genetic distance between each other, which was enough to separate them into distinct organisms.

3.3. Parasite morphology

Gamonts: elongated with an oval shape, measuring 10.96 ± 0.87 μm (9.42-13.09) long by 4.9 ± 0.51 μm (3.88-6.38) wide (n=36). Little condensed and granular nuclei, occupying almost the half of the cytoplasm, measured 5.58 ± 1.02 µm (3.71-7.13) long by 3.8 ± 0.51 µm (2.52-4.75) wide (Figure 2).

Immature meronts: round-to-oval in shape, containing amorphous material enclosed within a thick wall (Figure 3A) and measuring 17 ± 3.49 µm (11.52-25.39) long by 14.43 ± 2.17 µm (9.83-18.83) wide (n=42). More developed meronts contained basophilic stained material at the periphery of the cell, presumed to be the merozoite formation (Figure 3B).

Mature meronts: globular shaped with a thick membrane enclosing the well-defined and elongated merozoites (with a single nucleus) in number of 20-30 (Figure 4), formed around the residual body. Meronts measured 20.17 ± 3.26 µm (15.7-26.94) long by 16.42 ± 2.96 µm (12.88-21.57) wide (n=13) and merozoites were 5.63 ± 1.37 µm (3.11-7.9) long by 1.58 ± 0.27 µm (1.26-2.06) wide (n=14).

Monozoic cysts: ovoid shape with a cytoplasm staining white surrounding a single zoite, which is curved and mononuclear (Figure 5). Cysts measured 5.4 ± 0.82 µm (4-6.93) long by 1.76 ± 0.28 µm (1.18-2.58) wide (n=28).

Considering the morphological and molecular features, we described a new *Hepatozoon* species in *A.* cf. *montensis* from Brazil, named *Hepatozoon milleri* sp. nov.

3.4. Taxonomic summary

Phylum Apicomplexa Levine, 1970.

Family Hepatozoidae Wenyon, 1926.

Genus *Hepatozoon* Miller, 1908.

Type host: Akodon cf. *montensis* (Rodentia: Cricetidae: Sigmodontinae).

Other hosts: unkown.

Vector: unkown.

Type locality: The specimens were captured in forest fragments from municipality of Botucatu, São Paulo State, southeastern Brazil (22º53'09"S, 48º26'42"W).

Material deposited: Hapantotype blood smears and tissue slides will be deposited at the “Coleção Helmintológica do Instituto de Biociências de Botucatu (CHIBB)’’, at the Parasitology Department, IBB, Unesp, Brazil.

Gene sequence: The 18S rRNA gene sequence has been deposited in the Genbank under the accession number KU667308.

Etymology: the name *Hepatozoon milleri* sp. nov. is homage to the Dr. William Whitfield Miller, who first erected the genus *Hepatozoon* to describe intraleukocytic haemogregarines in laboratory rats.

4. Discussion

Hepatozoon spp. infections have been reported in a wide variety of vertebrates and are found in several mammalian hosts in Brazil; however, data of *Hepatozoon* spp. infecting Brazilian wild rodents are still lacking. Morphological features of parasite stages suggested that the *A.* cf. *montensis* specimens in the present study were infected with a new species of *Hepatozoon,* named *Hepatozoon milleri* sp. nov., which was supported phylogenetically, with the *Hepatozoon* sequence from the rodents grouped separately from other *Hepatozoon* isolates detected in wild rats from other parts of the world. Thus, our study is the first to describe a new species of *Hepatozoon* in wild small rodents in Brazil, based on molecular and microscopy characteristics.

By microscopy, we visualized the developmental stages in the peripheral blood (typical *Hepatozoon* gamonts) in mononuclear leukocytes (lymphocytes or monocytes) from *Hepatozoon*-infected *A.* cf. *montensis.* The gamontogony (development of gamonts into blood cells) occurs in leukocytes or erythrocytes of infected animals, depending on the *Hepatozoon* species (Smith, 1996). Balfour (1905) was the first to describe a haemogregarine (*Haemogregarina balfouri,* syn. *Hepatozoon balfouri*) of small rodents. The parasite was found in the red blood cells of the jerboa *Jaculus jaculus*. Few years later, Miller (1908) elucidated the life cycle of *Hepatozoon muris* (syn. *Hepatozoon perniciosum*) in laboratory white rats and in its vector, the mite *Lelaps echidninus,* and observed gamonts formation in lymphocytes of infected rodents. Also, Johnson et al. (2007) reported gamonts of an unidentified *Hepatozoon* species in leukocytes of *Sigmodon hispidus* and *Peromyscus leucopus* in USA. Recently, Rigó et al. (2016) detected gamonts of *Hepatozoon erhardovae,* a well-known hemoparasite of European wild rodents, *Myodes glareolus*, in erythrocytes of infected rodents.

The gamonts reported here resemble those of *H. muris* detected by Miller (1908) in the USA and Carini (1910) in Brazil, in laboratory white rats and *Mus decumanus*, respectively, although the parasites described by Miller (1908) were greater in size (12 x 6 µm) and had more condensed nuclei compared to those in our study, which measured an average range of 10.9 x 4.9 µm and had nuclei with a more granular appearance. Some studies have described *Hepatozoon* gamonts in small rodents that shared morphological similarities to the gamonts described here (Laird, 1951; Johnson et al., 2007). However, most of the recent reports on *Hepatozoon* species in wild rodent populations have focused mainly on molecular methods, without demonstrating the blood parasite forms (Criado-Fornelio et al., 2006; Allen et al., 2011; Maia et al., 2014; Wolf et al., 2016); therefore, gamonts comparison among *Hepatozoon* spp. infecting those mammalian is quite difficult. Extracellular parasites was also detected in the blood smears of the infected rodents in the present study, which was not uncommon because previous studies have demonstrated occasionally free gamonts in infected rodents, probably due to the rupture of host cells (Miller, 1908; Carini, 1910; Laird, 1951; Davidson and Calpin, 1976; Rigó et al., 2016).

Variations in the tissue tropism of *Hepatozoon* spp. within host species are quite common and thereby influence the location of the tissue merogony (Smith, 1996). Histological examination of tissues from *Hepatozoon*-infected *A.* cf. *montensis* revealed *Hepatozoon* meronts only in the liver. Nevertheless, as we worked with only seven specimens, we cannot assure that merogony occurs exclusively in the liver. Johnson et al. (2007) observed that the merogony of *Hepatozoon* sp. in *S. hispidus* and *P. leucopus* from North America is limited to the liver. Additionally, *H. muris* in laboratory white rats (Miller, 1908) developed meronts in the liver and Carini (1910) observed meronts of *H. muris* either in the liver or in the lungs of infected *M. decumanus*. Unlike those descriptions, merogony of *H. erhardovae* reported in *Clethrionomys glareolus* (syn. *M. glareolus*) from Europe occurred exclusively in the lungs (Laakkonen et al., 2001). Also, the predilection site of tissue merogony of *Hepatozoon griseisciuri* infecting the grey squirrel, *Sciurus carolinensis,* in the USA, was the lungs (Davidson and Calpin, 1976).

Meronts in various stage of maturity were observed in this study. The earliest meronts were uniform in size and appearance to immature meronts that have been previously described in small rodents infected with *Hepatozoon* sp. (Miller, 1908; Carini, 1910; Johnson et al., 2007; Breshears et al., 2009). On the other hand, the morphology and size range of mature meronts detected in Brazilian wild rodents are not consistent to previous descriptions of developed meronts in rodents from other regions. For example, Miller (1908) detected larger mature meronts (mean of 25 x 30 µm, some reaching 28 x 35 µm) of *H. muris* in infected laboratory rats, containing a few number of merozoites (12-20), whereas we found meronts, which at maturity measured, on the average, 20 x 16 µm, containing up to 30 merozoites. The developed meronts of *H. muris* described by Carini (1910) were smaller (diameter of 15-30 µm) and enclosed numerous merozoites (25-50). Johnson et al. (2007) and Breshears et al. (2009) studied *Hepatozoon* sp. in *S. hispidus* in the USA. The infected wild rodents harbored mature meronts greater (20 x 30 µm) than those reported in our study and had merozoites radially arranged in number of 50, approximately, in contrast to the meronts reported here, which had laterally or radially arranged merozoites.

The parasites of small rodents detected by Miller (1908) and Carini (1910), by that time known as *H. perniciosum* and *Haemogregarina muris,* respectively, are considered now as synonyms of *H. muris,* However, giving those differences presented above and the fact that the merogony of the protozoan described by Carini (1910) occurred in the liver and in the lungs, we suspected that these two parasites might be distinct from each other and for that reason, may belong to two different species.

Cystic stages (a curved zoite within monozoic cysts) were detected in the lungs, spleen and kidneys of some *Hepatozoon*-infected rodents. Laakkonen et al. (2001) detected monozoic and dizoic cysts of *H. erhardovae* in the lungs of *M. glareolus* in Europe and cystic forms of *H. griseisciuri* have been recorded in the lungs of the grey squirrel, *S. carolinensis* (Desser, 1990). Conversely, Johnson et al. (2007) did not observe cysts formation in tissues from *Hepatozoon* naturally infected wild rats from USA. Among *Hepatozoon* spp., the cyst forms differ mainly in size (10-21 µm long by 2-17 µm wide) than it does in morphology, which is typically round-to-oval shaped containing one (monozoic) or two (dizoic) curved mononuclear zoites (Laakkonen et al., 2001; Laison, et al., 2003; Baneth et al., 2007; Johnson et al., 2008). Tissue cysts have been commonly described in the life cycle of *Hepatozoon* spp. that infect reptiles and anurans (Smith et al., 1994; Viana et al., 2012). Those cysts stages might be infective when ingested by predatory species (Smith et al., 1994; Johnson et al., 2008).

Molecularly, we identified a novel sequence of *Hepatozoon* in *A.* cf. *montensis* specimens. Sequences of *Hepatozoon* species have been recorded in wild rodents from Europe (Criado-Fornelio et al., 2006; Rigó, et al., 2016), Africa (Maia et al., 2014), North America (Johnson et al., 2007) and South America (Merino et al., 2009; Demoner et al., 2016; Wolf et al., 2016). Based on phylogenetic analysis and sequence comparison, the sequence reported here is genetic distinct from those previously reported in wild rodents, although closely related.

In conclusion, we described a new *Hepatozoon* species, *Hepatozoon milleri* sp. nov., from wild small rodents in southeastern Brazil. Further investigation should include the identification of possible vectors such as fleas and mesostigmatid mites, the geographical distribution of the protozoan as well as the host range, which might clarify some epidemiological aspects of this new parasite.

5. Acknowledgements

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Figures

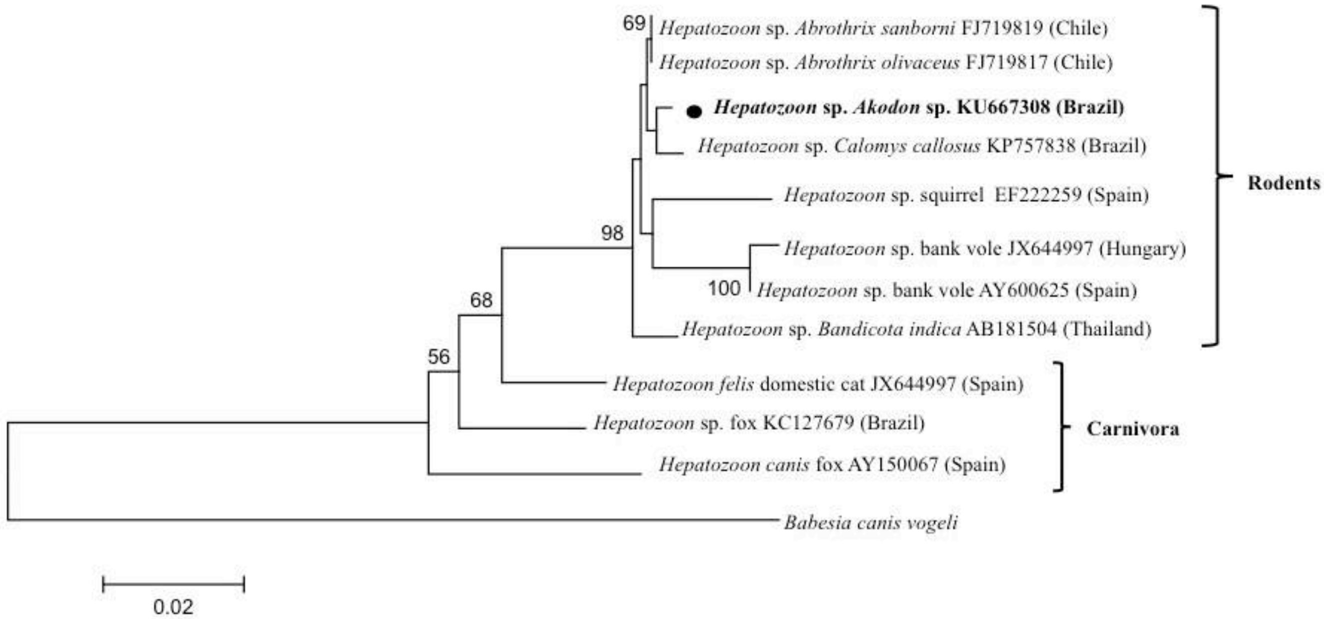

Figure 1. Neighbor-joining tree based on 18S rRNA partial sequences to infer phylogenetic relationships among *Hepatozoon milleri* sp. nov. sequence obtained from *A.* cf. *montensis* in southeastern Brazil and sequences available in the GenBank database. Numbers on the nodes indicate bootstrap values 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 sequence identified in the study is indicated in bold.

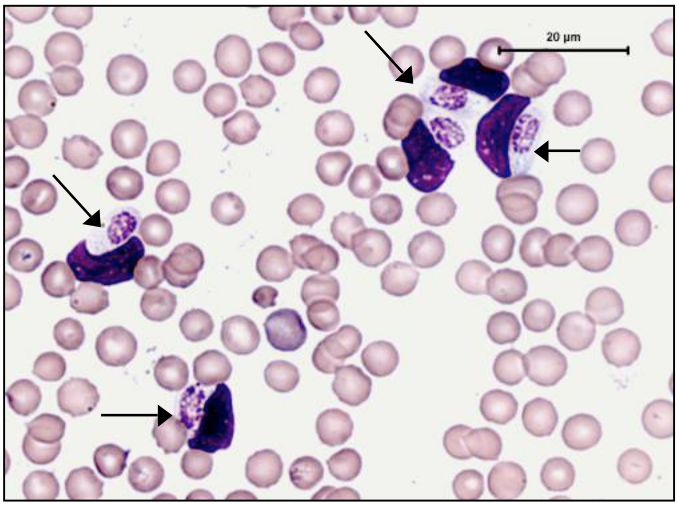

Figure 2. Gamonts of *Hepatozoon milleri* sp. nov. in the peripheral blood of an *Akodon* cf. *montensis*. Giemsa stain.

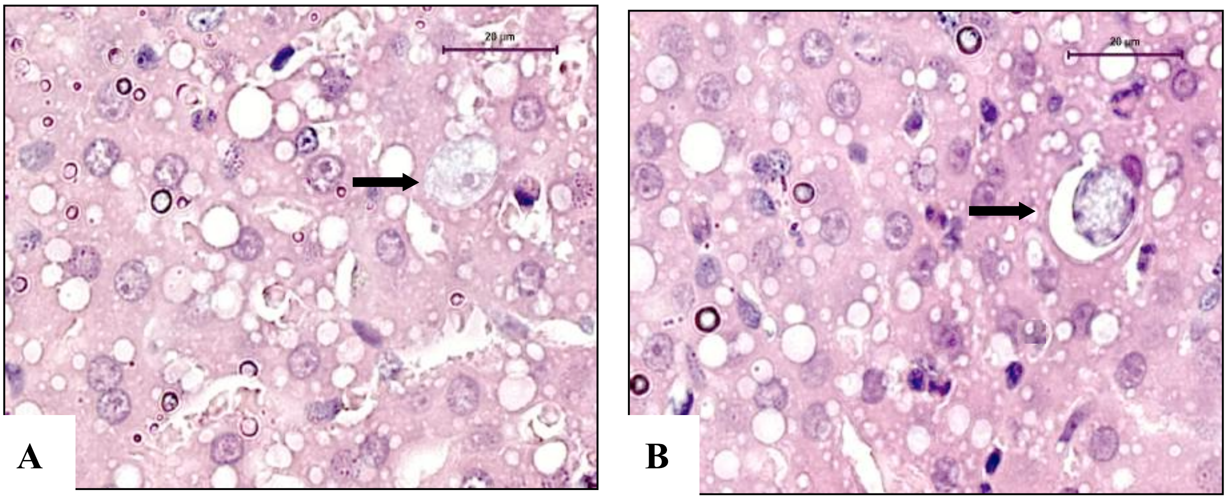

Figure 3. Histologic detection of *Hepatozoon milleri* sp. nov. meronts in the liver of an infected *Akodon* cf. *montensis.* (A) early meront stage containig amorphous material. (B) more developed meront characterized by basophilic stained material at the periphery of the cell, which probably gives rise the merozoites. Hematoxylin & Eosin stain, Bar = 20 µm.

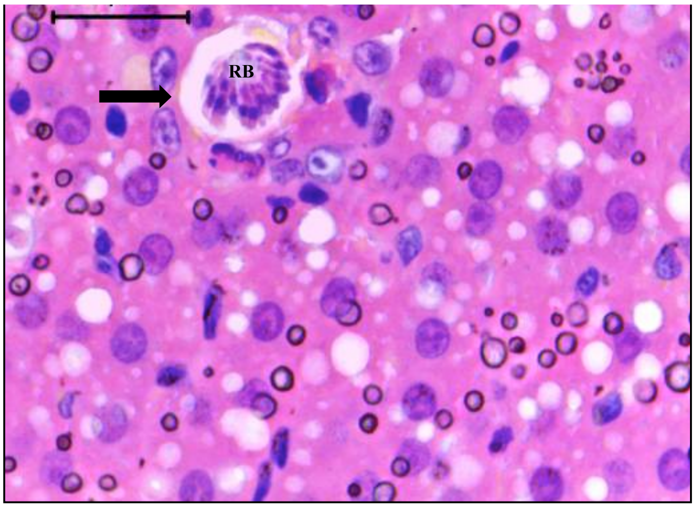

Figure 4. Mature meront of *Hepatozoon milleri* sp. nov. in the liver of an *Akodon* cf. *montensis*. Note numerous well-defined merozoites arranged around the residual body (RB). Hematoxylin & Eosin stain, Bar = 20 µm.

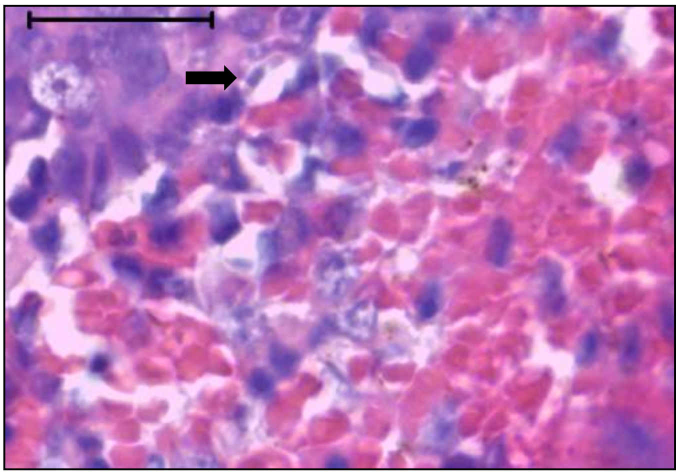

Figure 5. Cystozoite of *Hepatozoon milleri* sp. nov. within a monozoic cyst in the spleen of an infected *Akodon* cf. *montensis*. Hematoxylin & Eosin stain, Bar = 20 µm.

6. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

A hepatozoonose canina causada por *H. canis* é uma enfermidade que tem sido relatada em diversas regiões do nosso país, e muitas vezes com caráter endêmico, principalmente em áreas rurais. Apesar de ser um parasita cuja patogenia é desconhecida, ainda assim não deve ser negligenciado, já que há relatos de doença cliníca em cães associada às infecções por *H. canis.* Desta forma, deve-se buscar os mecanismos que envolvem a transmissão do parasita, objetivando-se desta maneira, preveni-lo.

Apesar de pequenos roedores silvestres terem sido considerados como potenciais reservatórios de *H. americanum* nos EUA, os resultados do nosso trabalho sugerem que roedores silvestres no Brasil não atuam como reservatórios de *H. canis*. Obviamente, outros trabalhos deverão ser conduzidos a fim de elucidar esse aspecto da epidemiologia do parasita no país, pois não se sabe se a transmissão vetorial por carrapatos (mecanismo primário de transmissão de *H. canis*) tem relevância na manutenção do protozoário. No entanto, cabe questionar se a transmissão vertical (no caso de *H. canis,* a transmissão transplacentária), um reconhecido modo de infecção de *H. canis,* estaria colaborando na propagação da infecção entre as populações de cães. Ainda, observamos que grande parte dos cães infectados estavam infestados por pulgas *Ctenocephalides felis felis.* Sabe-se que pulgas são comuns nas infestações dos cães, tanto em ambiente rurais quanto urbanos, e por essa razão devem ser investigadas como possíveis vetores, para talvez futuramente esclarecer essa questão epidemiológica.

Existem relatos muito antigos de espécies de *Hepatozoon* em pequenos roedores e a maioria deles baseados unicamente em caracteres morfológicos do parasita. O advento da biologia molecular facilitou o diagnóstico e caracterização de *Hepatozoon* spp. em diversas espécies animais, incluindo roedores silvestres. Entretanto, os relatos mais recentes de infecção nesses roedores têm se restringido à caracterização molecular, o que certamente auxilia na segregação de diferentes organismos, no entanto, muitas vezes não justifica a nomeação em espécies novas, por falta de dados biológicos e epidemiológicos. A partir dos achados morfológicos e moleculares, identificamos e nomeamos uma nova espécie de *Hepatozoon* em pequenos roedores silvestres do Brasil. No entanto, é necessário que se investigue diversos outros aspectos desse novo parasita, como por exemplo os vetores, possíveis danos ao hospedeiro, bem como a ocorrência em outras regiões do Brasil. Além disso, o encontro de formas císticas de *Hepatozoon* nos roedores infectados sugere que possam atuar como hospedeiros paratênicos do parasita, o que levanta a possibilidade de servirem como fonte de infecção para os predadores naturais.