

Molecular characterization of *Hepatozoon* sp. from Brazilian dogs and its phylogenetic relationship with other *Hepatozoon* spp.

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Abstract

To characterize phylogenetically the species which causes canine hepatozoonosis at two rural areas of Rio de Janeiro State, Brazil, we used universal or *Hepatozoon* spp. primer sets for the 18S SSU rRNA coding region. DNA extracts were obtained from blood samples of thirteen dogs naturally infected, from four experimentally infected, and from five puppies infected by vertical transmission from a dam, that was experimentally infected. DNA of sporozoites of *Hepatozoon americanum* was used as positive control. The amplification of DNA extracts from blood of dogs infected with sporozoites of *Hepatozoon* spp. was observed in the presence of primers to 18S SSU rRNA gene of *Hepatozoon* spp., whereas DNA of *H. americanum* sporozoites was amplified in the presence of either universal or *Hepatozoon* spp.-specific primer sets; the amplified products were approximately 600 bp in size. Cloned PCR products obtained from DNA extracts of blood from two dogs experimentally infected with *Hepatozoon* sp. were sequenced. The consensus sequence, derived from six sequence data sets, were blasted against sequences of 18S SSU rRNA of *Hepatozoon* spp. available at GenBank and aligned to homologous sequences to perform the phylogenetic analysis. This analysis clearly showed that our sequence clustered, independently of *H. americanum* sequences, within a group comprising other *Hepatozoon canis* sequences. Our results confirmed the hypothesis that the agent causing hepatozoonosis in the areas studied in Brazil is *H. canis*, supporting previous reports that were based on morphological and morphometric analyses.

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1. Introduction

Species of the genus *Hepatozoon*, previously classified as a member of family Haemogregarinidae, have been transferred to the family named Hepatozoidae. The transfer is based upon several considerations, including morphometric, morphological, and host specificity factors; and most recently upon molecular characterization (Barta, 1989; Smith, 1996; Mathew et al., 2000). The genus *Hepatozoon* comprises more than 300 species; 46 of them are known to infect mammals, 120 infect reptiles and the others are found as parasites of birds and amphibians (Smith, 1996). Smith and Desser (1997) suggested that the genus *Hepatozoon* should be partitioned into at least two genera owing to the high variability of transmission mechanisms, life cycle and morphology. However, these authors also concluded that the number of species described might not represent the real phylogenetic identity of all, since differences observed only in morphological traits could be a reflection of their reproductive isolation or of definitive host specificity. Most of the information used to reconstruct the evolutionary history of *Hepatozoon* species suggests that they are representative of a paraphyletic genus when compared to other protozoa species (Barta, 1989; Mathew et al., 2000).

Hepatozoon canis was originally described in India as the agent of canine hepatozoonosis (James, 1905), the vertebrate and invertebrate host ranges and the life cycle in definitive invertebrate hosts have been studied over the years (Wenyon, 1926; Barta, 1989). Two of 46 species associated with hepatozoonosis in mammals are known to cause disease in dogs (*Canis familiaris*). The species *H. canis* (James, 1905) is the one commonly found associated with hepatozoonosis in dogs from Europe, Asia, Africa and Latin America. In general, hepatozoonosis caused by *H. canis* is marked by a chronic and mild disease affecting spleen, lymph nodes and bone marrow, resulting in anemia and lethargy (Baneth and Weigler, 1997). In North America, *Hepatozoon americanum* has been identified as the causative agent of a severe emergent disease affecting dogs that can lead to death (Vincent-Johnson et al., 1997; Baneth et al., 2003).

Transmission of *Hepatozoon* to dogs is mediated by ingestion of infected ticks, the definitive invertebrate host, where the parasite develops the sexual phase of its life cycle. During the cycle in arthropod vectors, sporozoites develop within sporocysts which are transmitted when infected ticks are ingested by intermediate vertebrate hosts. Several tick species have been incriminated as potential vectors. In the United States, *Amblyomma maculatum* is known to transmit *H.*

americanum, and its role as an active vector was confirmed by experimental transmission studies (Mathew et al., 1998; Panciera et al., 1999; Ewing et al., 2002). In contrast, *H. canis* is known to be transmitted by the ixodid *Rhipicephalus sanguineus* (Christophers, 1907; Craig et al., 1978; Baneth et al., 1998). On the other hand, *Haemophysalis* spp. is suspected to be the vector for *H. canis* (former *Hepatozoon* sp. Fukuoka) found in dogs in Japan (Murata et al., 1995; Oyamada et al., 2005).

In Brazil, canine hepatozoonosis is mainly reported in rural areas where ticks of several species are found infesting dogs, whereas *R. sanguineus* has been associated with its transmission in urban areas (Freire, 1972; Massard et al., 1981; Ribeiro et al., 1997; O'Dwyer et al., 2001). During a study of hepatozoonosis in two rural areas of Rio de Janeiro State, we found that most infected dogs were infested with *Amblyomma cajennense*, *A. aureolatum*, *Amblyomma ovale* and *R. sanguineus* (Forlano et al., 2005). The presence of circulating gametocytes in dogs experimentally infected by administration of tick-derived sporozoites led to the conclusion that *A. ovale* is a potential vector for *Hepatozoon* spp. (Forlano et al., 2005). Besides, detection of gametocytes in circulating blood of newborn puppies from one experimentally infected dam indicated that *Hepatozoon* sp. also could be transmitted vertically (unpublished data). The causative agent of hepatozoonosis in Brazilian dogs has been identified as *H. canis* (Gupta et al., 1994; Alencar et al., 1997; O'Dwyer et al., 2001). In most cases, authors have reported the isolates found in Brazil as *Hepatozoon* sp. or *H. canis* (O'Dwyer et al., 2001; Paludo et al., 2003). With the recent availability of molecular tools and the increase in *Hepatozoon* sequences at GenBank, a few reports have focused in the phylogenetic characterization of *Hepatozoon* isolates in Brazil (Rubini et al., 2005; Paludo et al., 2003; Criado-Fornelio et al., 2006).

The main objective of the present study was to characterize by molecular tools, namely, PCR and sequencing of 18S SSU rRNA, the phylogenetic identity of *Hepatozoon* spp. isolates recovered from naturally and experimentally infected dogs, including five puppies to which the disease was vertically transmitted from an experimentally infected dam.

2. Materials and methods

2.1. Sample characterization

Thirteen dogs naturally infected and nine experimentally infected, between ages of 6 months and 7 years

and from no defined breed, were selected after positive identification through light microscopy of *Hepatozoon* sp. in their peripheral blood (Forlano et al., 2005). These 22 animals included five puppies that had acquired infection by vertical transmission from one experimentally infected dam. A susceptible dog that was not exposed was used as negative control, whereas *H. americanum* sporozoites isolated from *A. maculatum* (from Oklahoma, USA), were used only as positive controls for PCR reactions. The sequence used for comparative study of phylogenetic relationships was obtained from GenBank.

2.2. Total DNA extraction from blood samples of dogs and *H. americanum* sporozoites isolates

Blood samples were collected by venous puncture and stored in vacutainer tubes containing 10% solution of sodium citrate as anticoagulant, kept on ice for transportation and stored at -20°C . An aliquot of 300 μL of blood was used for total DNA extraction using the Easy-DNATM Kit (Genomic Isolation-Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. *H. americanum* sporozoites collected from experimentally infected *A. maculatum* and stored in ethanol were concentrated by centrifugation at 8 k rpm microcentrifuge (Eppendorf AG, Model 5415 C, Hamburg, Germany) and processed for total DNA extraction using the same kit as previously described. The DNA concentration obtained for each sample was estimated by spectrophotometer (Lambda 11, UV/vis, Perkin-Elmer, Wellesley, MA, USA) at 260 nm UV wavelength.

2.3. Polymerase chain reaction (PCR) using primers for 18S SSU rRNA gene coding region

Two sets of primers were used to amplify DNA extracted from blood sample of dogs and from *H. americanum* sporozoites. The set of universal primers for the region coding for the 18S SSU rRNA were 2867F (5'-AACCTGGTTGATCCTGCCAG-3') and 2868R (5'-TGATCCTTCTGCAGGTTACCTAC-3') (Mathew et al., 2000). We also used a set of primers designed to amplify partially the 18S SSU rRNA gene sequence of *Hepatozoon* spp. HepF (5'-ATACATGAGCAAATCTCAAC-3') and HepR (5'-CTTATTATTCCATGCTGCAG-3') (Inokuma et al., 2002). Each reaction contained 100 ng/ μL of DNA extract from blood samples or from sporozoites. At the final concentration, each reaction contained: 2.5 μL of 10 \times reaction buffer (10 mM of Tris-HCl pH 8.3; 50 mM of KCl,) 3.0 mM

of MgCl_2 , 200 μM of each dNTP, 10 pmol of each primer, 1.25 U of *Taq* DNA polymerase (Cenbiot[®], 5 U/ μL , Porto Alegre, RS, Brazil) and DNase-RNase free water were used to adjust the final volume of 25 μL (Invitrogen, Carlsbad, CA, USA). For the amplification of both sets of primers a block gradient thermocycler (Eppendorf AG, Mastercycler Gradient, Hamburg, Germany) was used to test five different annealing temperatures. The standard PCR conditions utilized were those suggested by Inokuma et al. (2001): 94°C for 1 min; followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 2 min. A final extension step at 72°C for 5 min was performed prior to stopping the reaction at 4°C . Water was used as negative control to check the reagents and DNA extracted from blood of susceptible dogs was used as negative control for background reaction. The products of amplification reaction were visualized after electrophoresis on 1% agarose gels using TAE 1 \times as running buffer at horizontal apparatus (Loccus LCH, 7 \times 8 or 12 \times 14, Loccus Biotecnologia, São Paulo, SP, Brazil). The DNA marker used was 1 Kb Ladder plus (100–12000 pb, Gibco BRL, Life Technology, Carlsbad, CA, USA). The results were recorded and the image analyzed by 1D Image Software using Gel Logic 100 equipment (Kodak, San Francisco, CA, USA).

2.4. Cloning of PCR products

The partial amplification products of 18S SSU rRNA gene were cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). To each reaction were added 2.5 μL of PCR products, 1.0 μL of 10 \times reaction buffer, 2.0 μL of pCR2.1 vector, 1 μL of T4 DNA ligase and Milli-Q deionized water qsq to final volume of 10 μL . The reactions were incubated for 4 h at 14°C . After ligation, 100 μL of *Escherichia coli* JM 109 competent cells were mixed with 5 μL of ligation products and transformed by heat-shock treatment as described in Sambrook et al. (1989). Colonies of transformed cells were selected in plates containing Luria-Bertani media (LB) and 100 $\mu\text{g}/\text{mL}$ of Ampiciline and 50 $\mu\text{g}/\text{mL}$ of Kanamicine, after overnight incubation at 37°C .

2.5. Screening of inserts and plasmid extraction for sequencing

Selected colonies were picked up from plates and suspended in 100 μL of ultra-pure Milli-Q deionized water. The cell suspension was washed (3 \times) and the supernatant discharged at each step after centrifugation at 8 k rpm for 3 min in microcentrifuge (Eppendorf AG,

Model 5415 C, Hamburg, Germany). An aliquot of 4 μ L was used for PCR in the presence of HepF and HepR primers as described previously by Inokuma et al. (2002). After insert detection, the recombinant plasmids of interest were extracted from each transformed cell by the method described by Birboim and Doly (1979) and submitted either for direct sequencing or for a further PCR reaction when necessary.

2.6. Sequencing

Extracted recombinant plasmid or PCR products were purified prior to sequencing reaction. A volume of 32 μ L of DNA was mixed with 8 μ L of 5 M NaCl and 40 μ L of 22% PEG 8000 solution. After homogenization the samples were incubated overnight at 4 °C. The purified plasmid DNA or PCR products were collected by centrifugation at 14 k rpm (Eppendorf AG, Model 5415 C, Hamburg, Germany). The pellet was washed with 50 μ L of 70% ethanol, dried at room temperature and suspended in 20 μ L of DNase–RNAase free water. The sequencing reactions were performed with 500 ng of DNA, 5 pmol of primer (HepF or HepR) and 4 μ L of DYEnamic™ ET Dye terminator Kit, MegaBace (GE Healthcare, former Amersham®), Freiburg, Germany). After the labeling reaction, products were precipitated by the addition of 1 μ L of 7.5 M NH₄Ac to each well of the plate. After homogenization the plate was centrifuged at 4 k rpm for 1 s in a microcentrifuge (Eppendorf AG, Model 5810 R, Hamburg, Germany). The labeled DNA was precipitated by the addition of 27.5 μ L of ethanol, homogenized in vortex and incubated overnight at –20 °C. The precipitated labeled DNA was collected by centrifugation at 4 k rpm for 30 min. After draining off the supernatant, the pellet was washed with 150 μ L of 70% ethanol and excess was removed by instantaneous centrifuging of the plate at inverted position. The sequencing products were suspended in 7.5 μ L of loading buffer (GE Healthcare, former Amersham®), Freiburg, Germany). The plate containing the labeled DNA sequencing product was then analyzed at the MegaBace 1000 at loading conditions of 2 kV for 60 s and electrophoresis was performed at 9 kV for 100 min.

2.7. Phylogenetic analysis

Sequence chromatograms were evaluated and edited in the Consed program (Gordon et al., 1998). The sequences were initially aligned using Clustal W (<http://www.ebi.ac.uk/clustalw>) and the alignment was corrected manually using the MEGA3 program (Kumar

et al., 2004) in order to obtain the consensus sequence. This consensus sequence was Blasted (<http://www.ncbi.nlm.nih.gov>) to find homologous sequences of 18S SSU rRNA coding region available at the GenBank database. Fifty-one sequences of *Hepatozoon* species were blasted. Even though several species were not identified, their vertebrate (or invertebrate) hosts were almost always mentioned in GenBank. Thus, in order to avoid unnecessary redundancies through the inclusion several individuals of the same species, we performed a preliminary analysis and whenever two or more unidentified or identified species that parasitize the same host fell together in the same cluster, we included just one or two of them. The single exception was the inclusion of several sequences of *H. canis*, which is reported to infect *C. familiaris* from several different countries and other vertebrate hosts, including domestic cat and other sylvatic carnivorous. Three different methods were applied to build the phylogenetic trees using the 18S SSU rRNA sequences: maximum likelihood (ML) (Felsenstein, 1981), neighbor joining (NJ) (Saitou and Nei, 1987) and maximum parsimony (MP). All trees were calculated with the program Paup* 4.0 (Swofford, 1998). In ML phylogenies, a careful selection of the evolutionary model is necessary to ensure the reliability of the tree. Therefore, we used the ModelTest 3.07 program (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998) to establish the model of DNA evolution that best fit our data. The model of choice was the HKY + G with non-variable sites (Hasegawa et al., 1985), considering a value of gamma shape parameter of 0.3628. This model was also used to infer the NJ tree. To find the maximum likelihood tree, the heuristic search algorithm used an initial NJ tree built with default options (Saitou and Nei, 1987). Then, a TBR (tree-bisection-reconnection) heuristic algorithm was used to search for the ML tree under the maximum likelihood framework. This algorithm is known to generate the most accurate results by computer simulations (Takahashi and Nei, 2000). The MP tree was inferred with a branch and bound algorithm. To estimate the robustness of each tree, we used the non-parametric bootstrap test (PB) (Efron et al., 1996; Felsenstein, 1985), whose values were inferred through a fast search approach, with 1000 replications. These analyses were also done with PAUP* program (Swofford, 1998).

3. Results and discussion

The total DNA extracted from blood samples of dogs, naturally and/or experimentally infected with *Hepatozoon* spp., was used for PCR reaction in the

presence of universal or genus-specific primer. The universal primers 2867F and 2868R were designed according to sequences of generic representatives of the same family, and the genus-specific primers (HepF and HepR) were designed by Inokuma et al. (2002) based on *Hepatozoon* spp. sequences. Five different annealing temperatures (51.9, 53.1, 54.4, 57.1 and 60.1 °C) were tested to achieve the optimal PCR conditions for both sets of primers. The amplification patterns observed showed that the annealing temperature for both primers was optimal at 57 °C, using *H. americanum* sporozoites as positive control, since improvement in efficiency and decrease of background was observed in comparison to other temperatures tested (data not shown). Amplification products of approximately 1650 bp were observed when the DNA extracted from *H. americanum* sporozoites was used as target in presence of the universal primers (Fig. 1). The presence of a faint band at the same position was also observed for the DNA

extracted from the blood of a naturally infected dog but the specificity of this primer set was inadequate to efficiently amplify our target. Using this set of primers Mathew et al. (2000) were able to get amplification products from DNA of their isolate of *H. americanum* and *H. catesbiana*, but not to all species of *Hepatozoon* tested, including *H. canis*. In the present study, when the set of primers specific to *Hepatozoon* spp. was used, amplification products were observed to DNA from blood of dogs naturally and/or experimentally infected and to DNA from sporozoites of *H. americanum* used as positive control (Fig. 2).

To check the efficiency of those primer sets to detect *Hepatozoon* in experimentally infected dogs or newborn puppies infected by vertical transmission from an experimentally infected dam, we used as template the DNA extracted from blood of these dogs (Fig. 3). No amplification was observed when the universal primer set was used to amplify total DNA from blood of all puppies infected by vertical transmission (Fig. 3). On the other hand, amplification products were observed when HepF and HepR were used as the primer set in the reaction to all five puppies and four experimentally infected dogs. The amplified products were approximately 600 bp in size, and comprised a region previously used for phylogenetic

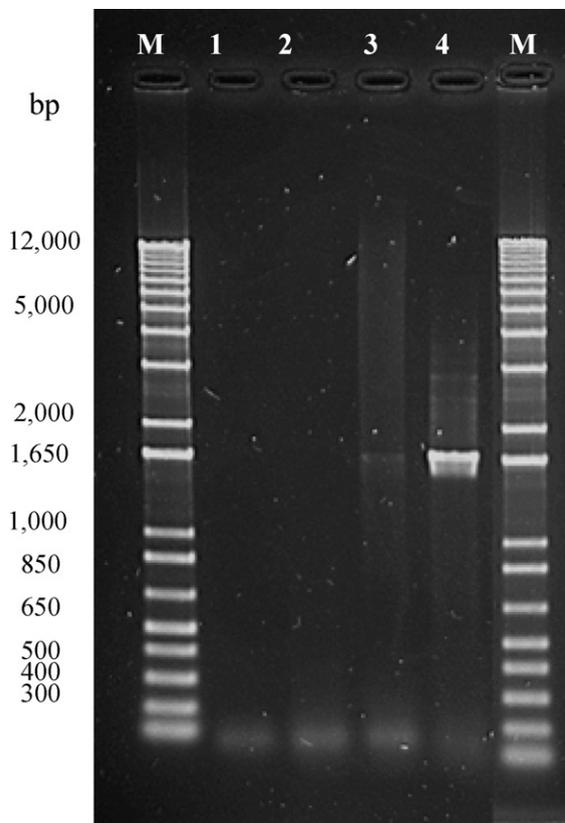


Fig. 1. *Hepatozoon* spp. 18S SSU rRNA partial gene amplification using universal primers (2867F and 2868R). Symbols: (M) marker 1 Kb plus DNA ladder; (1) negative control; (2) total DNA extract from blood of susceptible dog (not infected); (3) total DNA extract from blood of dog naturally infected with *Hepatozoon* spp.; (4) DNA extract of *H. americanum* sporozoites.

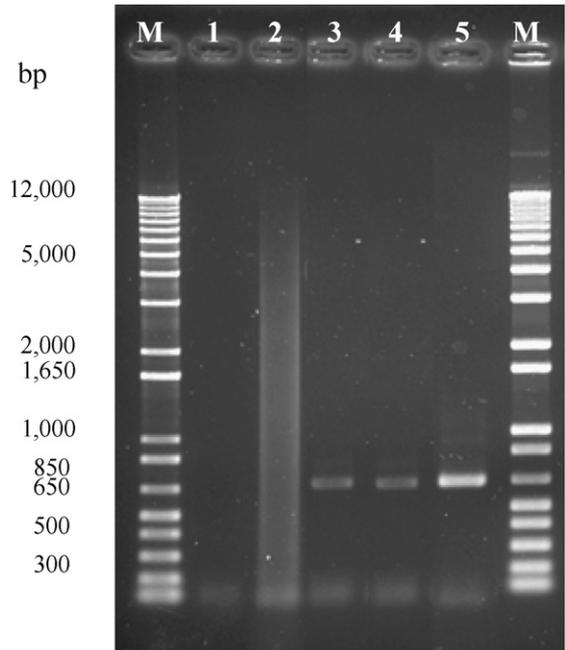


Fig. 2. Use of 18S SSU rRNA genus-specific (HepF and HepR) primer set for detection of *Hepatozoon* spp. in blood DNA extracts of dogs naturally or experimentally infected. Symbols: (M) 1 Kb plus DNA ladder; (1) negative control; (2) susceptible dog (not infected); (3) naturally infected dog; (4) experimentally infected dog; (5) positive control DNA of sporozoites of *H. americanum*.

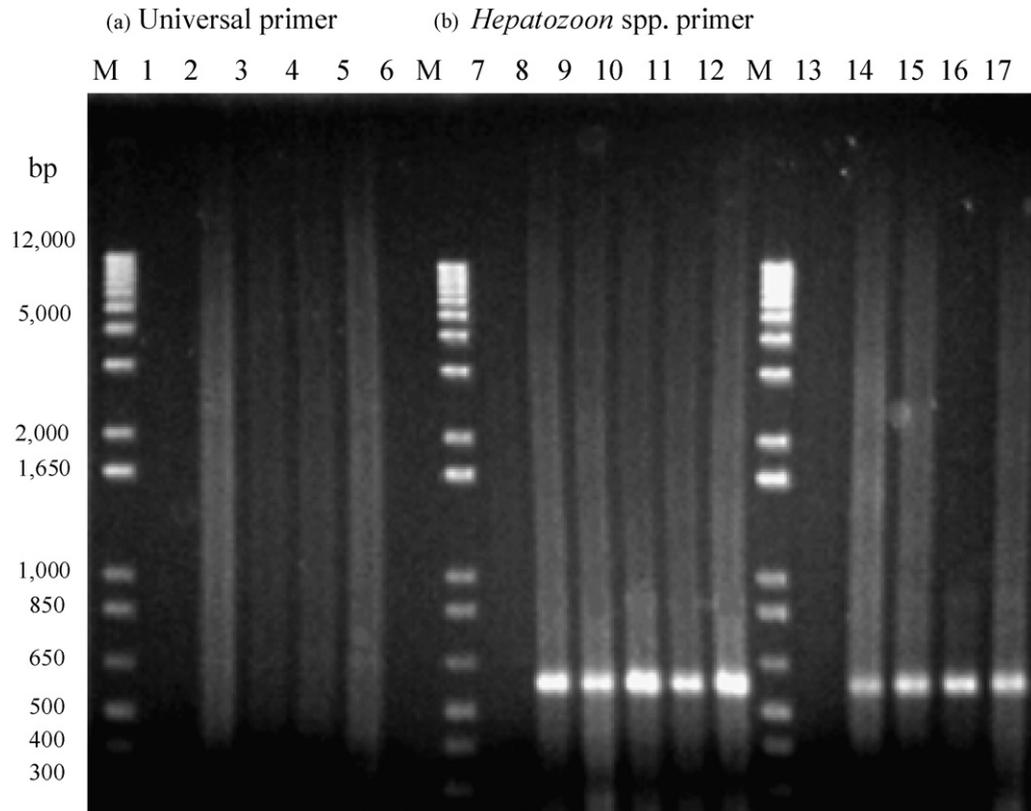


Fig. 3. Evidence of experimental and vertical transmission of *Hepatozoon* spp. detected by amplification of 18S SSU rRNA gene from total DNA extracted from blood samples of dogs. (a) Universal primers (2867F and 2868R): (M) 1 Kb DNA ladder; (1) negative control primers; (2–6) newborn puppies infected by vertical transmission, not amplified by these primers (b) *Hepatozoon* spp. primers (HepF and HepR): (M) 1 Kb DNA ladder; (7 and 13) negative control; (8–12) newborn puppies infected by vertical transmission; (14–17) dogs experimentally infected.

analysis (Inokuma et al., 2002). The inconsistency associated with the identity of the causative agent reflects the lack of molecular data available from other studies of canine hepatozoonosis. The PCR technique, associated with sequencing when necessary, is an alternative to the time-consuming microscopic observation of blood films for the parasite. These data support the potential use of this genus-specific primer set to detect *Hepatozoon* spp. in animals suspected to have hepatozoonosis. In addition, this primer set might be particularly valuable in distribution surveys and ecological studies. Indeed, the improvements in primer development is contributing to the adoption of this procedure, which has recently been used to screen for *Babesia* spp. and *H. canis* infections of dogs in a village of eastern Sudan (Oyamada et al., 2005).

In Brazil, the causative agent of canine hepatozoonosis has been diagnosed either as *Hepatozoon* spp. or as *H. canis*. These diagnoses are based mostly on the morphology of gamonts in circulating leukocyte and on the mild symptoms commonly observed in affected dogs (Gupta et al., 1994; Alencar et al., 1997; O'Dwyer et al.,

2001; Paludo et al., 2003). The *Hepatozoon* species found in naturally infected dogs in rural areas of Rio de Janeiro State (which was also experimentally transmitted) was efficiently detected only when the genus-specific primer set was used. These data, considered in association with symptoms observed in affected animals, and morphometric analyses previously published (Forlano et al., 2005), suggest that our Brazilian isolate is not *H. americanum*, which is responsible for American canine hepatozoonosis reported in the United States.

Recently, a few reports have focused on the phylogenetic characterization of *Hepatozoon* species isolated from different regions of Brazil and from hosts other than dogs (Paludo et al., 2003; Rubini et al., 2005, 2006; Criado-Fornelio et al., 2006). In our previous study, we reported that morphologic and morphometric analyses of the oocysts, sporocysts and sporozoites of *Hepatozoon* spp. found in *A. ovale* suggest that they are very similar to those observed/reported for *H. canis* recovered from *R. sanguineus* (Forlano et al., 2005). However, to confirm the identity of our isolates and to

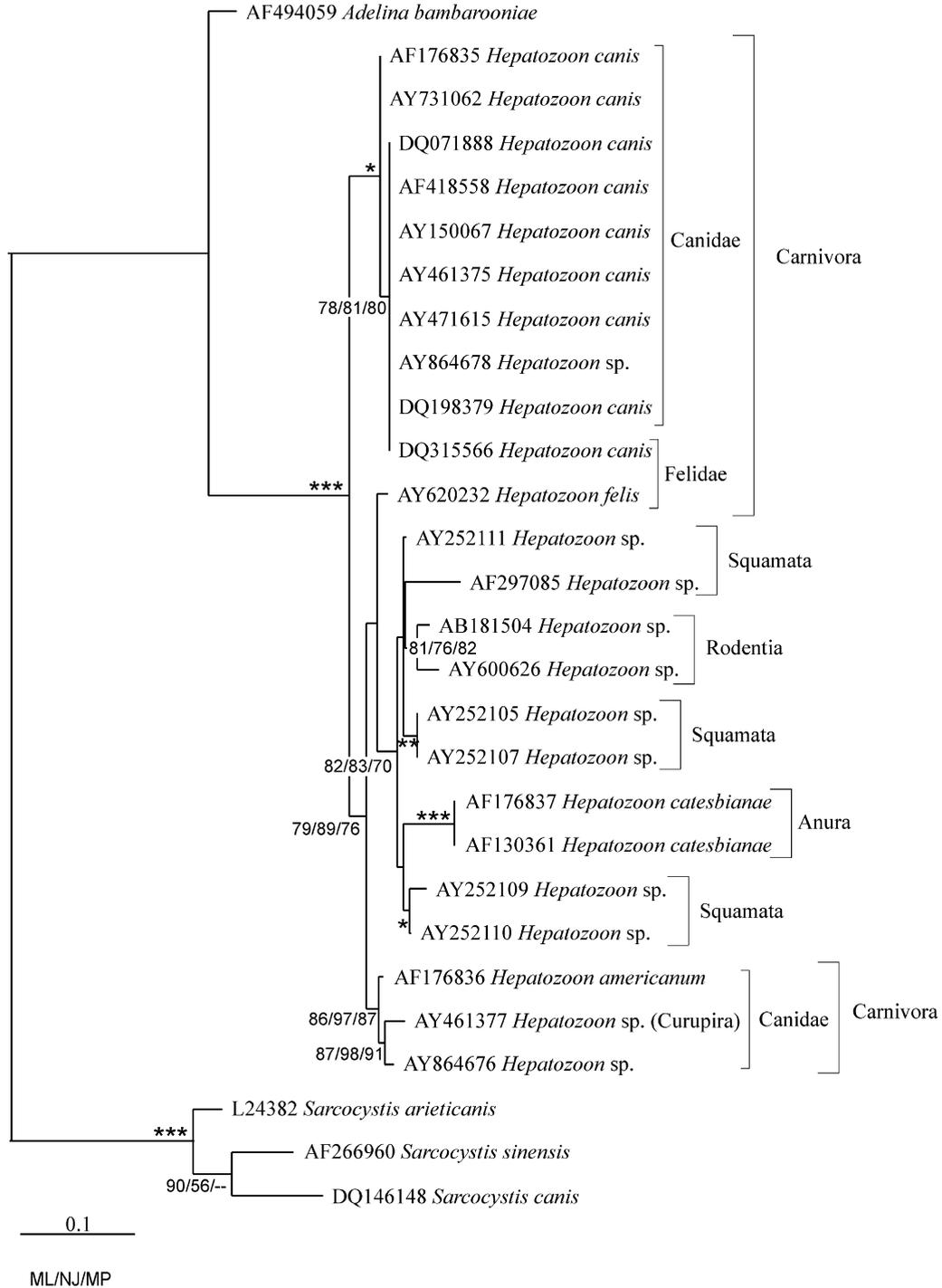


Fig. 4. Maximum likelihood tree constructed using the 18S SSU rRNA gene sequences of 21 *Hepatozoon* specimens and four out-group species (*S. arieticanis*, *S. canis*, *S. sinensis* and *A. bambarooniae*). The bootstrap values of the three phylogenetic methods used here (ML, NJ, MP) are shown alongside each interior branch. The interior branches marked with one (*), two (**) or three asterisks (***) are those which all bootstrap values were higher than 90%, 95% and 99%, respectively. The *Hepatozoon* specimen isolated from dogs experimentally infected with sporozoites obtained from naturally infected *A. ovale* collected from rural area of Rio de Janeiro is identified as DQ071888.

ascertain their relationship to species in the genus *Hepatozoon*, these PCR products were cloned and sequencing and phylogenetic analyses were performed. The consensus sequence of *Hepatozoon* spp. (partial 18S SSU rRNA coding region) derived from six aligned sequences was used to search for homologous sequences at GenBank. Our sequence was very similar to the sequences of *H. canis*, *Hepatozoon* sp. and to a lesser extent to sequences of *H. americanum* or of other *Hepatozoon* species associated with reptiles and amphibians, also available at GenBank.

The 18S SSU rRNA gene partial sequences of *Sarcocystis arieticanis* (L24382), *S. canis* (DQ16148), *S. sinensis* (AF266960) and *Adelina bambarooniae* (AF494059) were used as out-groups to construct the phylogenetic tree, which contains 24 other *Hepatozoon* sequences. Since the topologies of the trees constructed by the three methods used here (ML, MP and NJ) were essentially identical, we have chosen to show the ML topology as well as the bootstrap values found by the three methods (Fig. 4). After separation of the out-groups, *Hepatozoon* spp. splits into two discrete groups. The first group (bootstrap values ML = 92%, NJ = 93%, MP = 95%) comprises our sequence (Accession Number DQ071888) and most *Hepatozoon* sequences associated with *C. familiaris*, especially those isolates from Israel (AF176835) and Japan (AF418558); and one from domestic cat and others associated with related sylvatic species (for example, *Pseudalopex gymnocercus*, *Vulpes vulpes* and *Dusicyon thous*) (Fig. 4). The second group (ML = 79%, NJ = 89%, MP = 76%) branched into two main subclusters, one comprised *H. americanum* and two other related sequences (ML = 86%, NJ = 97%, MP = 87%), while *Hepatozoon* species associated with representatives of the orders Rodentia, Squamata and Anura clustered in the second subcluster (ML, NJ, MP <50%). The separation of *Hepatozoon* spp. associated with mammals into distinct groups was used by Mathew et al. (2000) to reinforce the description of *H. americanum* as a new species, which may have evolved from an unknown vertebrate host as a result of an ancient divergence event (Baneth et al., 2003). Interestingly, two sequences of *Hepatozoon* sp. isolates (AY864676 and AY461377) obtained from different hosts in Brazil and Spain, available at GenBank, are grouped together with *H. americanum* in our analysis. This fact suggests that other species of *Hepatozoon*, beyond those previously reported, may occur in Brazil.

More studies of hepatozoonosis and further characterization of the parasite in different vectors or hosts are needed (Paludo et al., 2003). Recent studies using molecular tools have increased the number of sequences

available at GenBank and description of *Hepatozoon felis*, for example, was facilitated by these data (Rubini et al., 2006). Patton (1908) described *H. felis* as the species responsible for cat infection, but Wenyon (1926) considered the parasite indistinguishable from *H. canis*. Advances using molecular approaches to study the taxonomy and to reconstruct the evolution of *Hepatozoon* spp. and other species complement morphological, morphometric and etiological information about any organism under investigation. Nevertheless, the cluster including *Hepatozoon* spp. from Brazil and Spain with *H. americanum* isolated from dogs in the USA (Oklahoma) should be analyzed with caution. In other words, the occurrence of *H. americanum* in Brazil and Spain should not be inferred using exclusively sequence homology data. Rather, one must use other sources of data, such as studies of the disease it causes in hosts and its life cycle in both intermediate and definitive hosts.

4. Conclusion

Hepatozoonosis in Brazilian dogs is a mild disease with symptoms commonly limited to lethargy and loss of weight in dogs in rural and urban areas, suggesting that the causative parasite is *H. canis*. The sequences analyzed here derive from isolates obtained from patients with naturally occurring disease associated with *A. ovale* as vector and from experimental animals, including pups vertically infected (from an experimentally infected dam). Phylogenetic analysis showed that the sequence of our isolates clustered with all *H. canis* species available at the GenBank, branching separately from other *Hepatozoon* spp., including *H. americanum*. The transmission of *H. canis* in different regions of Brazil has been associated with *R. sanguineus* as vector (O'Dwyer et al., 2001). However, our data indicate that *A. ovale* is also a suitable vector for *H. canis* and that vertical transmission from dams experimentally infected can occur to puppies.

One cannot assume that *H. canis* is the only species responsible for canine hepatozoonosis in Brazil. More detailed studies are necessary to characterize the disease produced in dogs and to confirm occurrence of other *Hepatozoon* species. Such investigations would reinforce evidence derived from molecular studies. Recent reviews suggest that sylvatic rodents, especially the hispid cotton rat (*Sigmodon hispidus*), should be studied as possible hosts for *H. americanum* (Ewing and Panciera, 2003). There are undoubtedly other potential vertebrate hosts that should also be examined. In Brazil, various rodents and other sylvatic species (<http://www.natureserve.org/infonatura>) should be investigated as potential reservoirs

for *Hepatozoon* spp. and other parasites. Such exploratory studies could contribute to understanding the distribution, transmission and endemic cycle of *Hepatozoon* spp. In addition, more work is needed to determine which acarine species are suitable vectors for *Hepatozoon* in Brazil.

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