Research Note

Braz. J. Vet. Parasitol., Jaboticabal, v. 24, n. 2, p. 247-250, abr.-jun. 2015
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612015019

Molecular identification of *Hepatozoon canis* in dogs from Campo Grande, Mato Grosso do Sul, Brazil

Identificação molecular de *Hepatozoon canis* em cães de Campo Grande, Mato Grosso do Sul, Brasil

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Received December 3, 2014
Accepted February 4, 2015

Abstract

The aim of this study was to investigate the occurrence of *Hepatozoon* species infecting dogs in the municipality of Campo Grande, Mato Grosso do Sul (MS), Brazil, using blood samples (n = 165) drawn from dogs. The species *Hepatozoon canis* was identified in 3.63% of the tested animals using molecular tools. Further studies are needed to determine the clinical relevance of this infection and the main arthropod vectors involved in its transmission.

Keywords: Dogs, epidemiology, *Hepatozoon canis*, midwestern Brazil, PCR.

Resumo

O objetivo deste estudo foi identificar a frequência e espécies de *Hepatozoon* infectando cães no município de Campo Grande, Mato Grosso do Sul, Brasil. Uma amostragem de 165 animais foi utilizada e, por meio do uso de ferramentas moleculares, a espécie *Hepatozoon canis* foi identificada em 3,63% dos animais. Mais estudos são necessários para identificar a relevância clínica e os principais vetores envolvidos na transmissão desse protozoário na região.

Palavras-chave: Cães, epidemiologia, *Hepatozoon canis*, centro-oeste do Brasil, PCR.

Introduction

The genus *Hepatozoon* comprises protozoa that can affect a wide range of vertebrate hosts throughout the world. These protozoa are heteroxenous, exhibiting a life cycle with sexual and sporulation phase in the definitive hosts, represented by hematophagous invertebrates such as mosquitoes, mites and ticks (SMITH, 1996). Animals become infected through the ingestion of the definite hosts containing mature oocysts with infective sporozoites (BANETH et al., 2001).

Canine hepatozoonosis can be caused by two species, *Hepatozoon canis* and *Hepatozoon americanum*. In Brazil, although species genetically related to *H. americanum* have been described parasitizing wild canids (CRIADO-FORNELIO et al., 2006; ANDRÉ et al., 2010), only *H. canis* has been detected in domestic dogs (PALUDO et al., 2005; MUNDIM et al., 2008; RAMOS et al., 2010; GONÇALVES et al., 2014).

The transmission of *H. canis* to dogs in urban areas has been attributed mainly to the tick species *Rhipicephalus sanguineus* (LATROFA et al., 2014; MIRANDA et al., 2014), while in the rural environment, *Amblyomma ovale* and *Rhipicephalus* (*Boophilus*) *microplus* have been incriminated (FORLANO et al., 2005; MIRANDA et al., 2011). However, differences in the vector competence of these ticks can influence the prevalence of the parasite in different dog populations (SILVA et al., 2014).

Few studies have been conducted in Campo Grande to determine the occurrence of *H. canis* infection in domestic dogs (SALGADO, 2006). In fact, the only study was based on the microscopic examination of blood smears, making it impossible to accurately define the species involved, and possibly underestimating the prevalence owing to the low sensitivity of the technique (SALGADO, 2006).

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Therefore, the aim of this study was to use molecular methods to assess the occurrence of infection by *Hepatozoon* sp. in dogs from Campo Grande, Mato Grosso do Sul (MS), Brazil.

Materials and Methods

From 2007 to 2009, blood samples of 165 dogs, exhibiting clinical signs compatible with hemoparasitosis and treated at private veterinary clinics located in the municipality of Campo Grande, were collected. Samples were collected in tubes containing EDTA (Ethylenediaminetetraacetic acid) as anticoagulant and stored at -20°C for subsequent DNA extraction, according to the methodology described by Araújo et al. (2009). The concentration and integrity of the DNA extracted from the samples were assessed by means of spectrophotometry and electrophoresis in 1% agarose gel stained with *SYBR Gold* (Invitrogen), respectively.

The genus *Hepatozoon* was identified using a PCR reaction method previously described by Inokuma et al. (2002), and the primer set HepF 5'-ATACATGAGCAAAATCTCAAC-3' and HepR 5'-CTTATTATTCCATGCTGCAG-3'. The reaction amplified a fragment of 666 bp in a conserved *Hepatozoon* region of the 18S *rRNA* gene. The PCR amplification products were viewed under an ultraviolet light after electrophoresis on 1% agarose gel stained with *SYBR Gold* (Invitrogen).

In order to test for PCR inhibitors, aliquots of DNA (10 µL, 500 ηg) of all samples were spiked with a DNA sample positive to *H. canis* (1 µL, 50 ηg) and tested in the PCR reaction described above (2 µL / reaction). The *H. canis* positive sample was obtained in a previous study, and the DNA sequence was deposited in GenBank under accession number FJ943578 (RAMOS et al., 2010).

To prevent cross-contamination and sample carryover, pre- and post-PCR sample processing was performed in separate rooms. All the fluids were transferred using plugged pipette tips to eliminate aerosols.

For the sequence analysis, amplicons were purified from agarose gel, using a QIAEX II Gel Extraction Kit (Qiagen), and sequenced in both directions using a BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyzer (Applied Biosystems). Triplicate sequencing of each amplicon was performed and one consensus sequence was built for each amplicon, using the Sequencher v. 4.1.4 program (Gene Codes). The consensus sequences were subjected to a BLASTn (ALTSCHUL et al., 1990) search (http://www.ncbi.nlm.nih.gov) to determine the sequence identity by comparison with sequences available in the GenBank database.

Multiple alignment was performed and a phylogenetic tree was constructed based on the neighbor joining method, using the MEGA 4 program (TAMURA et al., 2007). Bootstrap resampling (1000 replicates) was performed for the statistical support of the reliabilities of the nodes on the trees (FELSENSTEIN, 1985). *Theileria equi* and *Babesia microti*were used as outgroups.

Results and Discussion

Out of the samples analyzed, 3.63% (6/165) showed amplicons (666 bp) compatible with *Hepatozoon* sp., according to Inokuma et al. (2002). Through partial sequencing of the 18*S rRNA*gene it was possible to identify the species *H. canis*. A similarity of 98 to 99% was observed between the sequences obtained in this study and those available in GenBank.

The negative results could not be attributed to the presence of inhibitor, since all aliquots contaminated with *H. canis* DNA showed amplification.

The multiple alignment performed with the consensus sequences obtained in this study (635 bp) revealed an identity of 100% among *18S rRNA* sequences of local isolates of *H. canis,* suggesting the presence of a single isolate in the region under study. Therefore, only one sequence was deposited in GenBank under accession number JX118828.

The phylogenetic analysis confirmed that the dogs of this study were infected with the species *H. canis*. The phylogenetic tree based on *18S rRNA* sequences revealed that the *H. canis* isolate (Campo Grande) formed a cluster with other *H. canis* isolates in a segregated branch of *H. americanum*. Both clusters (*H. canis* and *H. americanum*) were confirmed by high bootstrap values (95 and 100%, respectively) (Figure 1).

Hepatozoon canis has been detected in dogs in several regions in Brazil, with frequencies ranging from 0.48% (1/205) (RAMOS et al., 2010) to 75.9% (161/212) (MIRANDA et al., 2014) in dogs from urban areas of Recife and Uberlândia, respectively. In a single survey conducted in Campo Grande, a *Hepatozoon* spp. infection rate of 2.4% (4/165) was observed by microscopic examination of blood smears of stray dogs caught by the Center for Zoonosis Control of the city (SALGADO, 2006). Although the methodology used in the present study (PCR) is more sensitive than optical microscopy, and the current sampling has been performed in sick dogs, in which a higher frequency of infected animals would be expected, no relevant difference was observed with respect to the previous study (SALGADO, 2006). On the other hand, a comparative analysis of data from different regions of Brazil revealed significant differences in *H. canis* infection rates in urban areas (RAMOS et al., 2010; PEREIRA et al., 2011; MIRANDA et al., 2014).

In Brazil, the brown dog tick *Rhipicephalus sanguineus* has been incriminated as the main vector of *H. canis* in urban areas (SPOLIDORIO et al., 2009; MIRANDA et al., 2014) because it is the most common tick in these areas (LABRUNA & PEREIRA, 2001; DANTAS-TORRES et al., 2004). However, an association has not always been observed between high levels of tick infestation and high frequency of *H. canis* infection in dogs (SPOLIDORIO et al., 2009; MIRANDA et al., 2014). In some cases, low infection rates have been reported despite high tick infestation (SALGADO, 2006).

A previous study by Ramos et al. (2010) in the metropolitan region of Recife, Pernambuco, Brazil, detected a *H. canis* infection rate of only 0.48% in dogs treated at the UFRPE Veterinary Teaching Hospital. Although the tick infestation of the animals in that study was not evaluated, the high frequency of infection by other tick-borne pathogens such as *Ehrlichia canis* (38.04%) and *Anaplasma platys* (48.78%) suggests that tick infestation in dogs occurs frequently in the region. Similar findings were reported in Campo Grande, where high frequencies of infection by other tick-borne pathogens have been observed (DAGNONE et al., 2009; SOUSA et al., 2013). Therefore, considering the wide distribution

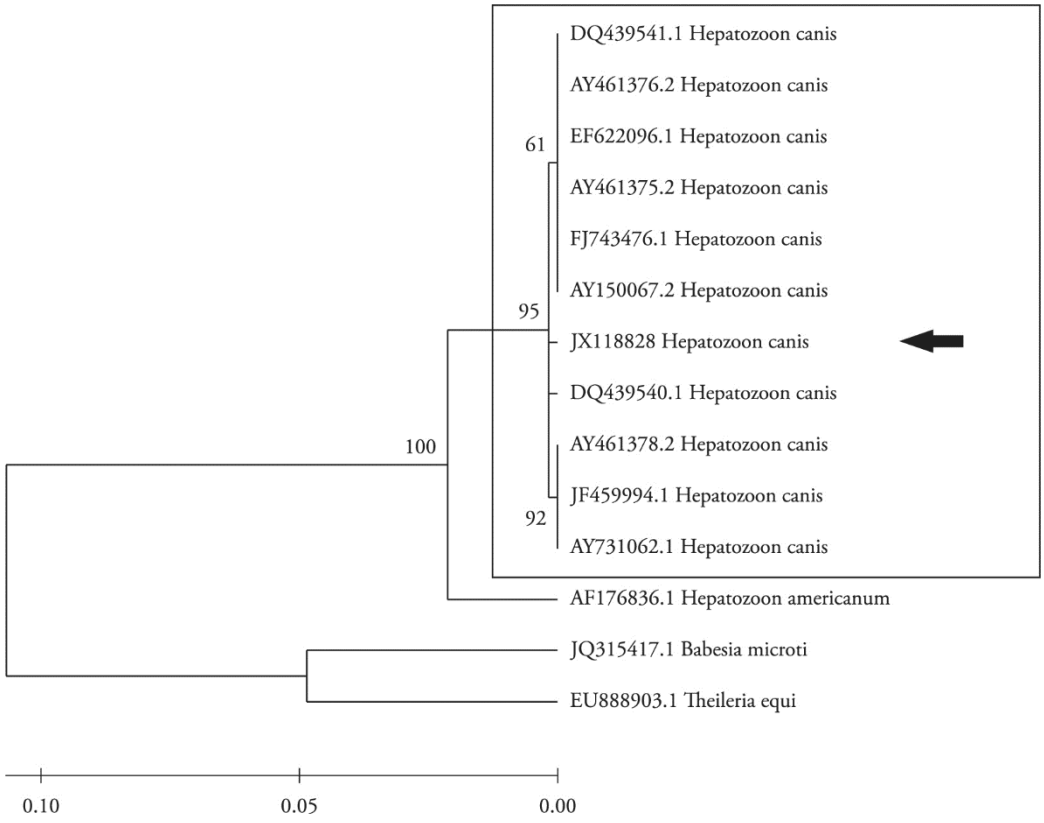

Figure 1. Phylogenetic tree constructed by the neighbor joining method, expressing the relationship between isolate of *Hepatozoon canis* from Campo Grande, MS (arrow) and other isolates available in the GenBank database. The cluster containing *H. canis* is highlighted. Each isolate is presented with the GenBank accession number, followed by the name of the species. The phylogenetic tree was built based on *18S rRNA* gene sequences.

of *R. sanguineus* in different urban areas, one question remains: How can the wide variations in *H. canis* infection rates in dogs from Brazil be explained?

One explanation could be the climate conditions of each region, which influence tick populations. However, even in regions whose climate conditions are favorable for tick reproduction, discrepancies have been observed among frequencies of infection by *H. canis.* For example, based on PCR frequencies of 3,63% (present study) and 75.9% have been reported in Campo Grande, MS and Uberlândia, MG, respectively (MIRANDA et al., 2014). These areas are distant approximately 770 km, and have similar climatic conditions.

Another important factor may be related with the methods of detection, which have different levels of sensitivity. However, again, many results do not corroborate this observation. For example, although Ramos et al. (2010) in Recife, Pereira et al. (2011) in Piraí and Miranda et al. (2014) in Uberlândia employed the same PCR reaction (Inokuma et al., 2002), all these researchers reported very different frequencies of infection (0.48%, 2.2% and 75.9%, respectively). It is still interesting to note that the lower rate of infection (0.48%) observed by Ramos et al. (2010), was detected in sick animals, while the other studies have been conducted in domiciled animals.

The low frequency of infection despite high tick infestation rates is most likely the result of the low vector competence of this tick (*R. sanguineus*) in acquiring and transmitting specific isolates of the protozoan. The vector competence of some tick species, including *R. sanguineus*, has been assessed recently (DEMONER et al., 2013). The authors reported an unsuccessful attempt to describe the development of *H. canis* in *R. sanguineus* and *A. cajennense* ticks (DEMONER et al., 2013). Similarly, previous studies performed by Forlano et al. (2005) and Gomes et al. (2010) also failed to detect any developmental stages of *H. canis* in ticks (*R. sanguineus*, *A. aureolatum* and *A. cajennense*) collected from naturally infected dogs in Rio de Janeiro and Uberlândia, respectively. On the other hand, the development of *H. canis* in populations of *R. sanguineus* in Europe has been reported frequently (BANETH et al., 2001; GIANNELLI et al., 2013).

Interestingly, over the last few years, genetic and morphological differences have been detected in *R. sanguineus* populations in different regions (SZABÓ et al., 2005; MORAES-FILHO et al., 2011). This variability can probably be ascribed to the tick's ability to transmit pathogens.

In conclusion, a systematic analysis of the tick species that parasitize dogs in urban areas, including Campo Grande, as well as their genetic structure, is needed to gain a better understanding of the transmission of *H. canis* in different regions.

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