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Short communication

Hepatozoon canis infecting dogs in the State of Espírito Santo, southeastern Brazil

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ABSTRACT

From May 2007 to March 2008, blood samples were collected from 92 healthy dogs living in 21 households (17 farms in rural area, and 4 homes in urban area) in 6 counties of the State of Espírito Santo, southeastern Brazil. In addition, ticks were collected from these dogs. A mean of 4.4 ± 3.0 dogs (range: 1–12) were sampled per household; 78 and 14 dogs were from rural and urban areas, respectively. Polymerase chain reaction (PCR) designed to amplify fragments of the 18S rDNA gene of *Babesia* spp or *Hepatozoon* spp revealed amplicons of the expected size in 20 (21.7%) dogs for *Babesia,* and 54 (58.7%) dogs for *Hepatozoon*. All *Babesia*-positive dogs were also *Hepatozoon*-positive. Among the 21 households, 15 (71.4%) from 3 counties had at least one PCR-positive dog, including 13 farms (rural area) and 2 homes (urban area). A total of 40 PCR products from the *Hepatozoon*-PCR, and 19 products from the *Babesia*-PCR were submitted to DNA sequencing. All generated sequences from *Hepatozoon*-PCR were identical to each other, and to corresponding 18S rDNA sequences of *H. canis* in GenBank. Surprisingly, all generated sequences from the *Babesia* PCR were also identical to corresponding 18S rDNA sequences of *H. canis* in GenBank. Dogs from 10 rural and 2 urban households were found infested by *Rhipicephalus sanguineus* ticks. Immature of *Amblyomma cajennense* ticks were found in dogs from only 4 rural households (also infested by *R. sanguineus*). All but one household with *R. sanguineus*-infested dogs had at least one *Hepatozoon*-infected dog. Statistical analysis showed that the presence of ticks (i.e. *R. sanguineus*) infesting dogs in the households was significantly ($P < 0.05$) associated with at least one PCR-positive dog. There was no significant association ($P > 0.05$) between PCR-positive dogs and urban or rural households. Canine hepatozoonosis caused by *H. canis* is a high frequent infection in Espírito Santo, Brazil, where it is possibly vectored by *R. sanguineus*. Since all infected dogs were found apparently healthy, the pathogenicity of *H. canis* for dogs in Espírito Santo is yet to be elucidated.

1. Introduction

Canine hepatozoonosis is a tick-borne disease of dogs found almost worldwide, caused by two different species of apicomplexan protozoa, *Hepatozoon canis* and

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Hepatozoon americanum (Baneth et al., 2003; Vincent-Johnson, 2003). *H. canis* was first described in India in 1905, and was incriminated as the cause of a mild disease, mainly characterized by anemia and lethargy (Baneth and Weigler, 1997). Dogs infected with *H. canis* generally appear healthy, since the disease is usually unapparent and subclinical, and the diagnosis is occasionally confirmed during laboratory examinations (Mundim et al., 1994; Gondim et al., 1998; Paludo et al., 2005). Immunosuppression caused by concurrent disease or other factors appear to play an important role in manifestation of clinical signs. In contrast, dogs infected with *H. americanum* typically develop severe disease (Vincent-Johnson, 2003). The distribution of *H. americanum* has been restricted to the United States, whereas *H. canis* has been reported in Africa, Southeast Asia, Middle East, Europe, and South America (Baneth et al., 2003; Gavazza et al., 2003; Vincent-Johnson, 2003; Oyamada et al., 2005; Rubini et al., 2008).

In Brazil, canine hepatozoonosis was first described by Massard (1979) from dogs of a rural area in Rio de Janeiro. Recent molecular studies have revealed the species *H. canis* infecting dogs from the States of São Paulo and Rio de Janeiro, and the Federal District (Brasília) (Paludo et al., 2005; Forlano et al., 2007; Rubini et al., 2005, 2008). Clinical evaluation in infected dogs was performed in one of these studies, which revealed mild disease similar to canine hepatozoonosis previously reported in the Old World (Paludo et al., 2005).

The known vector of *H. canis* in the Old World is the tick *Rhipicephalus sanguineus* (Baneth et al., 2001), although *Haemaphysalis* spp have been suggested to be possible vectors in Japan (Murata et al., 1995). In the United States, *Amblyomma americanum* is the only recognized tick vector of *H. americanum* (Vincent-Johnson, 2003). In Brazil, *Amblyomma* spp have been suggested as vectors of canine hepatozoonosis, although *R. sanguineus* has also been found infesting *H. canis*-infected dogs (O'Dwyer et al., 2001; Forlano et al., 2005; Rubini et al., 2008).

The present study employed molecular analysis to evaluate infection by *Hepatozoon* spp and *Babesia* spp in dogs from the State of Espírito Santo, southeastern Brazil. In addition, we evaluated the tick species that were infesting these dogs.

2. Materials and methods

During May 2007 to March 2008, three scientific expeditions were undertaken to the State of Espirito Santo, southeastern Brazil. In each expedition, farms in rural and homes in urban localities were visited in the six counties: Nova Venécia (18°43′S, 40°24′W), São Mateus (18°43′S, 39°51′W), Santa Leopoldina (20°06′S, 40°32′W), Ecoporanga (18°22′S, 40°50′W), Colatina (19°32′S, 40°37′W), and Vila Valério (18°59′S, 40°22′W). A total of 92 apparently healthy dogs were sampled from 21 households (17 farms and 4 homes). These households had been previously selected for another study with tick-borne zoonoses (N.H.Y., unpublished data), so they were not randomly selected.

Canine blood samples were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant, and kept frozen at −20 °C until DNA extraction. DNA was extracted from each blood sample using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA samples were eluted in 100 μL of TE buffer. Five-microliter of extracted DNA was used for polymerase chain reaction (PCR) amplification. DNAse-free water was used as a negative control for the extractions and PCR assays.

Primers HEP-1mod (5′-CGC GAA ATT ACC CAA TTC TA -3′) and HEP-4 (5′-TAA GGT GCT GAA GGA GTC GTT TAT-3′), were used to amplify a ≈670-bp portion of the 18S rRNA gene of *Hepatozoon* spp (Criado-Fornelio et al., 2006). Primer HEP-1mod was modified from the HEP-1 primer reported by Criado-Fornelio et al. (2006) through the insertion of the last three nucleotides (CTA), which are conserved among *Hepatozoon* sequences available in GenBank. This insertion was done with the intention to increase primer annealing temperature. In addition, primers BAB-33–57 (5′-GCC AGT AGT CAT ATG CTT GTC TTAA-3′) and BAB-432–409 (5′-TTC CTT AGA TGT GGT AGC CGT TTC-3′), corresponding to conserved regions of the 18S rRNA gene of *Babesia* spp were designed to amplify a ≈370-bp portion of the 18S rRNA gene. Polymerase chain reactions (PCR) were carried out in a total of 50 μL solution containing 1X PCR buffer minus Mg, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Platinum TaqDNA Polymerase (Invitrogen, Carlsbad, CA), and 0.2 μM of each primer. PCR cycle conditions for *Hepatozoon* spp consisted of a initial denaturation for 3 min at 95 °C, and 40 repetitive cycles of 15 s at 95 °C, 40 s at 53 °C, and 40 s at 72 °C, followed by a 5 min final extension at 72 °C. PCR cycle conditions for *Babesia* spp primers consisted of a initial denaturation for 3 min at 95 °C, and 35 repetitive cycles of 15 s at 95 °C, 30 s at 63 °C, and 30 s at 72 °C, followed by a 7 min final extension at 72 °C. Both cycles were previously submitted to a gradient of temperature to find the best annealing temperature to our laboratory conditions. PCR products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and examined by UV transillumination. Amplicons of the expected size were purified with ExoSap (USB) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA) according to the manufacturer's protocol. Partial sequences obtained were submitted to BLAST analysis (Altschul et al., 1990) to determine the closest similarities to corresponding sequences.

During blood collections, dogs were examined for the presence of ticks, which were collected and brought to the laboratory for taxonomic identification. For statistical analysis, the presence of at least one PCR-positive dog in each farm or home was analyzed qualitatively at a dichotomous level (0: farm or home with no PCR-positive dog; 1: farm or home with at least one PCR-positive dog) with the following two independent variables by univariate analysis (Fisher's Exact test): (i) presence of ticks infesting dogs in the farm or home (0: absent; 1: present); and (ii) living place (0: urban area; 1: rural area). Statistical association between variables was considered significant if $P < 0.05$. All analyses were performed using the program EpiInfo (version 3.3.2).