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Research paper

Tick-borne infections in dogs and horses in the state of Espírito Santo, Southeast Brazil

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ABSTRACT

This work aims to identify and quantify the percentage of *Rickettsia* spp., *Ehrlichia* spp., *Babesia* spp. and *Hepatozoon* spp. positive pet dogs, and to identify ticks collected on these animals in the state of Espírito Santo, in the Southeast region of Brazil. The study included 378 dogs, 226 females and 152 males, of various breeds and ages (mean age of 4.1 years). All animals were examined for ticks, and whole blood was collected and processed by conventional PCR protocols for *Babesia* spp., Anaplasmataceae, *Hepatozoon* spp. and by real-time PCR for *Ehrlichia canis*. Of the 378 dogs examined, 157 (41.53%) had ticks at the time of the study, which were identified as *R. sanguineus* s.l. in 154 animals (98.10%), *Amblyomma ovale* in one animal (0.63%), and *Amblyomma sculptum* in 2 animals (1.27%). In the PCR for *Babesia* spp., 5 animals (1.32%) were positive, producing DNA sequences 100% identical to *Babesia vogeli*. For Anaplasmataceae, 34 animals (9%) were positive, 10 of which generated DNA sequences 100% similar to *Ehrlichia canis*. The other 24 samples generated fragments 100% identical to *Anaplasma platys*. In the PCR for *Hepatozoon* spp, 39 animals (10.31%) were positive, producing sequences 100% identical to *Hepatozoon canis*. Finally, in the real-time PCR specific for *E. canis*, 28 animals (7.40%) were positive. Coinfection with 2 or 3 agents was observed in 20 animals (5.29%). Of the 378 dogs sampled, 312 were analyzed by indirect immunofluorescence assay (IFA) for *E. canis* and five species of *Rickettsia* (*R. rickettsii, R. parkeri, R. amblyommatis, R. rhipicephali* and *R. bellii*). Among them, 71 dogs (22.75%) had a positive reaction for *E. canis* and 16 dogs (5.13%) had antibody titers higher than 64 to at least one *Rickettsia* species, 5 of them (1.60%) to *R. rickettsii*. Samples of blood were collected from 10 equines in the regions where dogs were found with positive serology for any one of the *Rickettsia* sp. tested. In the municipality of Vila Velha, two equines were positive for *R. bellii* with inverse titers of 64 and 128. These results indicate that tick-transmitted agents, such as *E. canis, A. platys, B. vogeli, H. canis*, and several spotted fever group *Rickettsia* are circulating in the canine population of the Brazilian state of Espirito Santo.

1. Introduction

Currently, an increased risk of exposure to pathogens transmitted by ticks is observed among animals worldwide, and due to the close relationship of these pathogens to humans, diseases may significantly impact public health (Beugnet and Marié, 2009). In this context, dogs are considered sentinels for infections by agents transmitted by ticks (Diniz et al., 2007), and also act as potential carriers of these infectious agents into the domestic environment. Dogs are susceptible to infections by *Rickettsia rickettsii* and can increase the risk of infection in humans living in the same environment due to close contact with infected ticks carried by dogs from the external environment into the home (Piranda et al., 2008). Horses also play an important role in the epidemiology of the disease as main reservoirs of the ticks involved in

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the transmission of the disease (Labruna et al., 2002). Based on serological surveys for *R. rickettsii* in sentinels, new areas with Brazilian Spotted Fever (BSF) can be determined (Paddock et al., 2002). Although equines are important in the epidemiology of the disease, under natural conditions they are not significant amplifier hosts for *R. rickettsii,* since infected equines transmit the bacteria to a minimal number of ticks, even though horses develop a good humoral response following infection (Ueno, 2014).

In Brazil, the principal pathogens transmitted to dogs by ticks are *Babesia vogeli*, *Ehrlichia canis*, *Anaplasma platys*, *Hepatozoon canis,* and *Mycoplasma haemocanis* (Spolidorio et al., 2011).

Rickettsia rickettsii, a gram-negative bacteria belonging to the order *Rickettsiales* (Gurgel et al., 2009), is the principal etiological agent of BSF (Dias and Martins, 1939). Ehrlichiosis and anaplasmosis are diseases caused by obligate intracellular gram-negative bacteria belonging to the order Rickettisiales, family Anaplasmataceae, genera *Ehrlichia* and *Anaplasma* that affect both humans and animals (Dagnone et al., 2009). Babesiosis is a disease caused by an apicomplexanhemoprotozoan of the order Piroplasmida, family Babesiidae, genus *Babesia* (Irwin, 2009). It is a common disease in veterinary medicine, but rare in humans (Alecrim et al., 1983; Taboada and Lobetti, 2006). Hepatozoonosis is a disease described in several countries caused by protozoans of suborder Adeleorina, family Hepatozoidae, genus *Hepatozoon* that can affect a great variety of vertebrate hosts worldwide (Smith, 1986). In Brazil, the transmission of *H. canis* to dogs in urban areas has been associated with *Rhipicephalus sanguineus* (Spolidorio et al., 2009; Miranda et al., 2014), while in rural areas, it has been associated with *Amblyomma ovale* (Forlano et al., 2005) and *Rhipicephalus microplus* (Miranda et al., 2011).

The objective of this work was to investigate and obtain epidemiological data about tick-transmitted pathogens, *Rickettsia* spp., *Ehrlichia* spp., *Babesia* spp. and *Hepatozoon* spp., to domesticated dogs in urban areas, predominantly in the state of Espírito Santo, Brazil, to serve as data for the implementation of future control measures for these pathogens in dogs, and of zoonoses transmitted by ticks.

2. Material and Methods

2.1. Sample Collection

2.1.1. Dogs

The samples from dogs in this study came from dogs living in six municipalities in the state of Espírito Santo, Brazil (Alegre, Colatina, Santa Teresa, Serra, Vila Velha, and Vitória) (Fig. 1). In this study, whole blood and serum, but also ticks material was collected from 378 dogs (226 females and 152 males) of different breeds, ages 4 months–15 years (mean age = 4.1 years), and with ticks present or a history of recent parasitism by ticks within the past 30 days as provided by the owner. These animals were screened in veterinary consultations for annual wellness visits and for sick-animal visits at the Veterinary Hospital of the University Vila Velha (UVV), the Veterinary Hospital of the School of Veterinary Medicine of UNESC Colatina, and the Veterinary Hospital of the UFES Alegre campus, and during a castration campaign run by the school of veterinary medicine of the Escola Superior São Francisco de Assis (EFSA), in Santa Teresa.

Blood samples were collected in tube with EDTA and tube without anticoagulant from the jugular or cephalic vein of the dogs and sera were separated by centrifugation (12,000 *g*, 10 min) and stored at −20 oC until tested. Blood was used for molecular and serologic analyses aimed at identifying the bacterial and protozoan agents, as well as the presence of antibodies produced against

agents transmitted by ticks. Ticks were collected directly from the skin of the dogs, preserved in absolute alcohol, and identified according to the criteria published by Martins et al. (2016).

2.1.2. Horses

Serum samples from equines (n = 10) came only from the municipalities of Vila Velha (VV) and Vitória. These horses lived in or traveled through regions (neighborhoods) where *Rickettsia* spp. seropositive dogs were found for any of the *Rickettsia* tested in this study (Neighborhoods: Soteco–VV, Itapuã − VV, Ulisses Guimarães–VV, Fradinhos–Vitória). The samples were collected by jugular venipuncture into Vacutainer tubes containing sodium citrate and sera were separated by centrifugation (12,000 *g*, 10 min) and stored at −20 °C until tested. Data such as Age and breed were not collected. In addition, on the day of the group visit to these 10 animals, no ticks were found.

2.2. Ethical Aspects

Collection of the biological materials from the animals was previously approved by the Animal Ethics Committee of the Universidade Vila Velha (number 215/2012). Samples were collected only after the owner of each animal had signed an informed consent form.

2.3. DNA Extraction

Total DNA was extracted from the blood using the commercial kit from Qiagen (DNeasy Tissue and Blood Kit, Qiagen, Chatsworth, CA), according to the manufacturer's instructions. The samples were eluted in 100 ml of TE buffer. An extraction control (DNA-free distilled water) was included for every 20 samples extracted.

2.4. Polymerase Chain Reaction (PCR) and sequencing

For the detection of DNA of *Babesia*, *Hepatozoon,* and the Anaplasmataceae family (*Anaplasma* spp and *Ehrlichia* spp.), conventional PCR's were performed with the following primer sets (Table 1): BAB-143-167 forward and BAB-694-667 reverse primers which correspond to the preserved regions of the 18S rRNA gene of *Babesia* spp. (551 bp) (Almeida et al., 2012a); HEP-144-169 forward and HEP-743-718 reverse primers to amplify a fragment of the 18S rRNA gene of *Hepatozoon* spp. (574 bp) (Almeida et al., 2012a); and GE2 forward and HE3 reverse primers to amplify a fragment of the 16S rRNA gene of Anaplasmataceae (360 bp) (Breitschwerdt et al., 1998). In each PCR assay, negative controls (water) and an appropriate positive control sample (DNA of *Babesia vogeli*, *Hepatozoon canis* or *E. canis*-cultured DH82 cells) were included for all PCR assays.

The PCR products were run on agarose gel in electrophoresis, stained with ethidium bromide and visualized the bands at heights of the positive control, which characterized the positive samples.

The samples of canine blood that were positive by conventional PCR for Anaplasmataceae, *Babesia* spp., and *Hepatozoon* spp. were sequenced at the Center for Human Genome Studies of the USP Biology Institute (IB). Purification of the amplified product was performed with EXOSAP®, following the protocol described by the manufacturer. The samples were then sent to the IB for the sequencing reaction using the BigDye® Terminator v3.1 Cycle Sequencing Kit (code 4337456) and sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA).

Assays to detect specific *E. canis* DNA were performed using a TaqMan real- time PCR (qPCR) system targeting a portion of the *Ehrlichia* disulfide bond formation protein-encoding gene (dsb) with the primers dsb-321 (5′- TTGCAAAATGATGTCTGAAGATATGAAACA-3′) and dsb-671 (5′-GCTGCTCAACCAAGAAATGTATCCCCTA–3′) and the *E. canis* −specific probe (5′-FAM-AGCTAGTGCTGCTTGGGCAACTTT GAGTGAA-BHQ-1–3′) (Table 1) at a concentration of 25 µmol/L as previously described (Doyle et al., 2005). Positive (DNA from *E. canis*-cultured DH82 cells) and negative (DH82 cells only) controls were included for all PCR assays.