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Original Article

Molecular epidemiology of *Babesia vogeli* in dogs from the southeastern region of Rio de Janeiro, Brazil

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ABSTRACT

Hemoparasitic diseases are prominent in domestic animals, particularly in Brazil, a tropical country with a wide range of vectors. This study investigated the epidemiology of *Babesia vogeli* in the whole blood of dogs from the southeastern region of Rio de Janeiro, Brazil. Whole blood samples from 390 dogs were screened for the presence of *B. vogeli* DNA by qPCR using the *heat shock protein 70 kDa* (*hsp70*) gene of *B. vogeli*. Characteristics related to the host and its environment were collected using a questionnaire. Bivariate analysis was used to evaluate each factor individually. A phi correlation test was used to verify collinearity. The variables with $p < .1$ and a low or moderate correlation with the other variables were selected for the multivariate analysis. Multiple models were created, and the best logistic regression model was chosen using the Akaike Information Criterion (AIC). The final model was used to determine which variables were closely related to *B. vogeli* infections in dogs. Of the 390 dog blood samples, 15.66% were positive for *B. vogeli*. The variables cat contact, age, shelter, street or woods access, tick infestation and fur lengthwere included in the final model. Per the logistic regression analysis, three variables explained *B. vogeli* detection in dogs: age (odds ratio [OR] = 2.12; *p*-value < .05; confidence interval [CI]: 1.13–3.96), tick infestation (OR = 2.08; *p*-value < .05; CI: 1.10–3.93) and shelter (OR = 2.22; p-value < .05; CI: 1.16–4.26). These variables were determined to be associated with *B. vogeli* detection in domiciled dogs in the southeastern region of Rio de Janeiro, Brazil. These data indicate that the age of the animal, the presence of ticks and the lack of shelter directly affect the epidemiology of *B. vogeli*.

1. Introduction

Babesia vogeli is the primary etiological agent of canine babesiosis in Brazil. The *Rhipicephalus sanguineus* sensu lato tick is the biological vector of this protozoan in the country (Passos et al., 2005). Clinical signs of babesiosis are unspecific, but anorexia, pyrexia, petechiae, epistaxis and splenomegaly are often reported (Solano-Gallego et al., 2016). Laboratory findings include anemia, hemoglobinuria and thrombocytopenia (Martin et al., 2006). The routine diagnosis of canine babesiosis is based on clinical signs, hematological profiling and the presence of the parasite in blood smears (Vidotto and Trapp, 2004).

Therefore, due to the unspecific clinical signs, as well as the high frequency of false negatives on blood smears examined by light microscopy, other laboratory techniques, such as molecular methods and immunodiagnostic tests, are necessary to confirm the infection and proceed with treatment (Solano-Gallego et al., 2016). Thus, chronic or less virulent cases may not be detected by examination of the smear due to its low parasitemia (Solano-Gallego et al., 2016). In addition, examining blood smears can be tedious and requires the experience and time of a laboratory technician.

Confirming a babesiosis diagnosis is not common in routine veterinary practice. The infection caused by *B. vogeli* is often mistaken for the infection of other hemoparasites. Maciera et al. (2005) reported that many clinicians in Brazil, including those in Rio de Janeiro, use the presence of only thrombocytopenia to make a presumptive diagnosis of *Ehrlichia canis* infection in dogs. The use of doxycycline is often adopted to eliminate blood parasites, especially *E. canis*. Although antibiotics are not the therapy of choice for piroplasmids, Vercammen et al. (1996) reported that doxycycline may reduce the severity of clinical signs in canine babesiosis. However, this treatment does not eliminate *Babesia* spp., and the disease may resurface. Brandão et al. (2003) reported that the use of imidocarb dipropionate in two doses of 7 mg/kg administered

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over 14 days seemed to be effective in eliminating large *Babesia* spp. parasites. Therefore, babesiosis occurrence is often under diagnosed, and epidemiological studies are needed to determine the frequency of parasitized animals in a particular area, as well as the environmental variables and other host-related factors leading to *B. vogeli* infections in dogs (Maia et al., 2007).

In Brazil, epidemiological research on the frequency, as well as other factors related to canine babesiosis, has been conducted in both endemic and non-endemic areas. Many of these studies determined the presence of antibodies against *Babesia* spp. by indirect immunofluorescence antibody (IFA) assays (Mrljak et al., 2017). However, cross-sectional epidemiological studies based on serological techniques can lead to temporal bias, since the presence of circulating antibodies does not necessarily indicate a current infection. Moreover, there is a trend for the serological tests to overestimate the protozoan frequency as circulating antibodies may remain for long periods following treatment or resolution of clinical signs (Solano-Gallego et al., 2016).

A variety of molecular tools, including loop-mediated isothermal amplification assay (LAMP), real-time quantitative polymerase chain reaction (qPCR), and high resolution melting analysis (HRM), are used to directly detect hemoparasite DNA from clinical or environmental samples, providing relatively low-cost, rapid molecular tests of high yield (Lymbery and Thompson, 2012). Quantitative PCR allows visualization of the reaction process cycle by cycle, shortening the time to obtain results and avoiding contamination post-amplification. Therefore, quantitative PCR was chosen to estimate *B. vogeli* detection in this study.

Many epidemiological studies perform only bivariate analysis to determine infection-related factors (Nalubamba et al., 2011; Bashir et al., 2009). Thus, only single epidemiological variables are evaluated, making the statistical analysis incomplete, as other factors are not considered. Multiple logistic regression analysis is used to learn which other factors influence the epidemiology of canine babesiosis in a region (Medronho, 2009). In this study, multiple variables are analyzed together, providing a more comprehensive result.

This study used qPCR based on the *hsp70* gene to determine the frequency of *B. vogeli* and developed an epidemiological model using multiple logistic regression analysis to determine the factors associated with the presence of *B. vogeli* DNA in dogs from the southeast region of Rio de Janeiro, Brazil.

2. Materials and methods

2.1. Description of the studied area

This study was conducted in rural and urban areas of the Itaguai Microregion located in southeast of the State of Rio de Janeiro (Fig. 1), which encompasses the municipalities of Mangaratiba (22° 57′ 35″S; 44° 02′ 26″W; altitude: 18 m), Seropedica (22° 44′ 38″ S; 43° 42′ 27” W; altitude: 26 m) and Itaguai (22° 51′ 08″ S; 43° 46′ 31″ W; altitude: 13 m).

The area has a tropical climate (Aw) characterized by a dry season, summer rains and a well-defined winter season according to Köppen-Geiger classification (Kottek et al., 2006). This climate presents an average temperature during the coldest month of the year > 18 °C and an average maximum temperature above 25 °C.

The population of Itaguai has 100,362 inhabitants followed by Seropedica with 76,045 and Mangaratiba with 34,966. The region has a total of 211,373 inhabitants (IBGE, 2010).

2.2. Sampling

The absence of frequency data on *B. vogeli* in dog whole blood samples through molecular techniques led to an expected prevalence of 50%, which represents the maximum number for the sample size, an error of 5% and a precision of 5% (Medronho, 2009). The sample number was set per the equation described by Sampaio (2002): $n = nx1.962P_{exp}(1\text{-}P_{exp})/d^2$, where n = sample size; P_{exp} = expected prevalence; and d^2 = absolute accuracy desired. Per this equation, 384 dog blood samples were needed in the study region. Dog blood samples were collected by cephalic venipuncture in sterile vacuum tubes containing EDTA (ethylenediaminetetraacetic acid). Samples were randomly collected in a volume of 2 to 5 mL from 390 domestic dogs from the rural and urban areas of southeast Rio de Janeiro, Brazil.

During the visits, the dogs were systematically examined by manual inspection for tick infestation for 20 min. The specimens found were collected and properly identified according to a taxonomic key described by Barros-Battesti et al. (2006). Ticks were collected and stored in polypropylene tubes containing isopropyl alcohol.

2.3. Standard control

The blood used as a positive control was obtained from a dog presenting clinical signs of babesiosis, with intraerythrocytic inclusions being observed in cytological examination of a blood smear, and by conventional PCR using 18S rDNA as molecular marker and (Carret et al., 1999) and the *hsp70* gene of *B. vogeli*, as described by Paulino et al. (2018).

The fragment was purified with CleanSweep (Applied Biosystems®) and sequenced. The 18S rDNA sequence showed 100% identity with *B. vogeli* and was deposited into GenBank under the accession number MF459002.

The negative standard control was obtained from a naïve dog, which has never been exposed to ticks. Next, the blood was extracted and tested using the molecular marker 18S rDNA as a target to detect *B. vogeli* by conventional PCR, as described by Carret et al. (1999), and using the *hsp70* gene of *B. vogeli* by conventional PCR, as described by Paulino et al. (2018). The results were negative in all of the molecular assays. Nuclease-free water (Ambion®, Thermo Scientific, Wilmington, DE, USA) was also used as a negative control.

To avoid contaminating the reactions, the DNA extraction step, preparation of the PCR reagents mixture, the addition of DNA samples and electrophoresis were carried out in different environments, respecting a unidirectional flow.

2.4. Extraction of total DNA

DNA extraction was performed using a commercial kit (Qiagen DNeasy-tissues-Blood, Valencia, CA, USA) as per the manufacturer's recommendations. After extraction, the total DNA was quantified in a spectrophotometer (Nanodrop ND-2000 ®, Thermo Scientific, Wilmington, DE, USA) and standardized at a concentration of 100 ng/μL. The samples were frozen in triplicate at −20 °C until the molecular analyses.

2.5. Detection by qPCR

All samples were tested by qPCR based on the *hsp70* gene using the primers *BvqF* (5'-GCTGGTGACACCCACCTT-3′) and *BvqR* (5'-CAACAA GCGTGCCCTCC-3′) and the hydrolysis probe Bvq (5'-*NED*-CCTCCTCG TTGAGCACT-*MGB*-3′) as described by Paulino et al. (2018).Reactions were performed in a final volume of 12 μL on StepOnePlus (Applied Biosystems®, ThermoFisher Scientific, Wilmington, DE, USA) equipment, containing 1× TaqMan® Universal PCR Master Mix (2×), 800 nM of each primer, 250 nM of probe and 300 ng of total DNA. The thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in duplicate. Samples with amplification < 40 quantification cycles (Cq) were considered positive.