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PROTOZOOLOGY - SHORT COMMUNICATION

# Molecular epidemiology and prevalence of babesial infections in dogs in two hyperendemic foci in Brazil

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## Abstract

Babesial parasites are some of the most ubiquitous blood pathogens and consequently have considerable worldwide veterinary impact. Dogs living in the tropics are highly exposed to babesial parasites, particularly to *Babesia vogeli*. Limited data on the seroprevalence and molecular prevalence of *Babesia* spp. in dogs are available in Latin America. We conducted a cross-sectional study combining serological and molecular tests to estimate the seroprevalence and molecular epidemiology of *Babesia* spp. infections in dogs in two hyperendemic foci in Brazil. A total of 630 privately owned dogs (417 from Goiana municipality, Pernambuco state, north-eastern Brazil, and 213 from São Joaquim de Bicas municipality, Minas Gerais state, south-eastern Brazil) were sampled and molecularly and serologically tested for *Babesia* spp. Overall, 519 dogs (82.4%) presented detectable IgG antibodies against *Babesia* spp., and seropositivity was significantly higher in dogs older than 1 year. Molecularly, 34 dogs (5.4%) were positive for a ~ 200 bp fragment of the *18S rRNA* gene of *Babesia* spp. and 88 (14.0%) for a longer fragment (~ 450 bp) of the same gene of *Babesia* spp. and other protozoa. The *18S rRNA* gene sequences generated herein corresponded to *B. vogeli* ($n = 52$) or *Hepatozoon canis* ($n = 20$). This study confirms a high level of exposure to *B. vogeli* in two areas of Brazil and highlights that most of the dogs living in these areas are infected during the course of their life, reflected by increased seroprevalence in older dogs. Increased awareness and prevention of tick-borne protozoa infections in dogs from Brazil and Latin America are urgently needed.

**Keywords** Age · *Babesia* · Dogs · Molecular epidemiology · Risk factor · Seroprevalence

## Introduction

Canine babesiosis is a clinically relevant disease of dogs, which affects disproportionately dogs living in the tropics (Panti-May and Rodríguez-Vivas 2020). Scientific evidence gathered during the past decades unravelled that dogs can be infected by an expanding number of recognized babesial species (Baneth et al. 2019, 2020; Penzhorn, 2020). Nonetheless, *Babesia vogeli* remains as the most widespread species globally, which may be partly explained by the vast geographical distribution of its tick vectors (Dantas-Torres et al. 2018).

Canine babesiosis is widely distributed and highly endemic in tropical regions (Maggi and Krämer 2019; Panti-May and Rodríguez-Vivas 2020). For instance, a review of molecular studies of babesial infections in dogs in Latin America and the Caribbean revealed that prevalence varies widely according

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to country and features of the canine population, ranging from < 1.0 to > 20.0% (Panti-May and Rodríguez-Vivas 2020). However, these figures should be interpreted with caution. Most of the studies reporting high prevalence values (> 20%) in this region included a small sample size and were based on hospital populations, which may not reflect the prevalence in the general population. Additionally, other studies with larger sample sizes (e.g., Araújo et al. 2015) were not included in the review by Panti-May and Rodríguez-Vivas (2020). In any case, large-scale, randomized studies are still limited and thus are required to build a better picture of babesial infections in dogs from Latin America and the Caribbean.

In Brazil, canine babesiosis is mainly caused by *B. vogeli*, but other species have sporadically been reported (Maggi and Krämer 2019; Panti-May and Rodríguez-Vivas 2020). The prevalence of babesial infections in dogs in Brazil has been studied in different regions, with variations in the prevalence of *B. vogeli* reported in various Brazilian states and in different studies conducted in the same state (Panti-May and Rodríguez-Vivas 2020). These differences may be related to several factors, including features of the canine population studied and also diagnostic techniques employed.

A pilot study conducted in different Brazilian regions revealed very high levels of exposure to babesial parasites using an indirect immunofluorescence assay (Figueredo et al. 2017). In particular, seroprevalence percentages from 72 to 83% were detected in dogs from São Joaquim de Bicas (south-eastern Brazil) and Goiana (north-eastern Brazil). However, this study included a small number of dogs and did not report molecular data on the identity of babesial organisms present in these areas. A more recent study reported the prevalence and incidence of different vector-borne pathogens in a randomly sampled population of 307 *Leishmania* spp.-negative dogs (Dantas-Torres et al. 2020a). These analyses indicated high prevalence and annual incidence of *Babesia* spp. infections, corroborating the above-mentioned results of Figueredo et al. (2017). Again, molecular data on the identity of the babesial organisms present in these areas was not reported.

Herein, we conducted a cross-section analysis of the study reported by Dantas-Torres et al. (2020a), specifically with regard to *Babesia* spp. In particular, we combined serological and molecular tests to estimate the prevalence of *Babesia* spp. infections and to identify the babesial organisms in a larger number of dogs living in these two apparently hyperendemic foci in Brazil.

## Material and methods

### Study areas and dog populations

The present cross-sectional study was carried out in Goiana municipality (7°33′39″S, 35°0′10″W; altitude: 13 m), Pernambuco state, north-eastern Brazil, and São Joaquim de Bicas municipality (20°02′56″S, 44°16′26″W, altitude: 755 m), Minas Gerais state, south-eastern Brazil. Goiana has a tropical savanna climate with dry-summer, whereas São Joaquim de Bicas has a humid subtropical climate with dry-winter. Further information about these municipalities is available elsewhere (Figueredo et al. 2017; Dantas-Torres et al. 2020a).

A total of 630 dogs (417 were from Goiana and 213 from São Joaquim de Bicas) sampled during September–November 2015 were included herein. These dogs are a subset of the 823 dogs mentioned in Dantas-Torres et al. (2020a). In particular, we included all the 307 *Leishmania* spp.-negative dogs reported in Dantas-Torres et al. (2020a), plus 323 dogs. These additional dogs were also mostly *Leishmania* spp.-negative (i.e., 318 negative and five positive). Dogs were defined as negative when negative to anti-*Leishmania* antibodies and *Leishmania* DNA (see Dantas-Torres et al. 2020a). Dogs were included regardless the clinical status, but most of them presented one or more clinical signs (e.g., enlarged lymph nodes, pale mucous membranes, and skin lesions) suggestive of vector-borne diseases.

### Detection of IgG antibodies against Babesia spp

Serum samples were tested by an indirect immunofluorescent antibody assay (IFA) that detected IgG antibodies against *Babesia* spp. (*Babesia canis* IFA IgG Antibody Kit, Fuller Laboratories), as reported elsewhere (Dantas-Torres et al. 2020a). IFA slides were examined using a fluorescence microscope (Leica, Wetzlar, Germany), and samples with a clear fluorescence at a cut-off dilution of 1:50 were considered as positive. Samples were not titrated.

### DNA extraction and PCR testing

DNA samples extracted from EDTA blood aliquots using PureLink® Mini Kit (Invitrogen, Carlsbad, CA, USA) (Dantas-Torres et al. 2020a) were initially screened by a PCR assay targeting a ~ 200-bp fragment of the *18S r*RNA gene of *Babesia* spp., using the primers Bcanis-F (5'-GCA TTT AGC GAT GGA CCA TTC AAG-3') and Bcommon-R (5'-CCT GTA TTG TTA TTT CTT GTC ACT ACC TC-3') (Kordick et al. 1999). PCR conditions were as described previously (Dantas-Torres et al. 2020a).

A second PCR assay using the primers PIRO A1-F (5'-AGG GAG CCT GAG AGA CGG CTA CC-3') and PIRO B-R (5'-TTA AAT ACG AAT GCC CCC AAC-3') (Carret et al. 1999; Jefferies et al. 2003) was also run. This assay was chosen because it targets a larger fragment (~ 450 bp) of the *18S rRNA* gene of *Babesia* spp. and other tick-borne protozoa, including *Hepatozoon* spp., which is also endemic in Brazil. PCR assays were performed in a final volume

of 25 µl containing 7.5 µl of DNA-free water, 12.5 µl of GoTaq® Colorless Master Mix (Promega, Madison, USA), 1.5 µl of each primer at a concentration of 12.5 pmol/µl, and 2 µl of template DNA. The cycling conditions were as follows: initial denaturation at 95 °C for 15 min, 62 °C/1 min, 72 °C/2 min; then, 94 °C/30 s, 62 °C/20 s and 72 °C/30 s for 35 cycles; and a final extension at 72 °C for 7 min. A master mix without DNA (no template control) and a DNA sample extracted from the blood of a *B. vogeli*-positive dog (positive control) were included in each assay. PCR assays were run on a Veriti® 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA), and PCR products stained with ethidium bromide (10 mg/ml) were loaded on 1.5% agarose gel and visualized using a UV light. Amplification was considered successful when a single band of the expected size was visualized.

### DNA sequencing and phylogenetic analysis

PCR products were purified using PureLink™ PCR micro kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions. The purified PCR products were prepared using a BigDye™ terminator v3.1 matrix standard kit (Applied Biosystems, Foster City, CA), and bi-directional sequencing reactions were carried out using the same PCR primers in a 3500xL genetic analyzer (Applied Biosystems, Foster City).

The sequences generated were analyzed using the Staden package (http://staden.sourceforge.net/), and contig assembly was performed based on the *Phred* quality score ≥ 30. The sequences were aligned using the ClustalW algorithm implemented in MEGA7 software. Sequence similarity searching was performed against the GenBank database using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Representative consensus *18S rRNA* gene sequences generated herein have been deposited in GenBank (accession numbers: MW290105–MW290108).

For sequences identified as *Hepatozoon canis*, we conducted a phylogenetic reconstruction including sequences of different strains of *Hepatozoon* spp. available in GenBank (see Fig. 1 for accession numbers). Phylogenetic reconstruction was inferred based on neighbor-joining (NJ) method using MEGA7 software with bootstrap test (1000 replicates). The parameters used for the reconstruction of the tree were the same as in Díaz-Sánchez et al. (2021). *Toxoplasma gondii* was used as outgroup.

### Data analysis

The seroprevalence of *Babesia* spp. was calculated based on the IFA results. Exact 95% confidence intervals (95% CI) were calculated for both IFA and PCR positivity. The chi-square test ($\chi 2$) was used to compare the positivity in relation to sex, age, place of origin and ectoparasite treatment. Significant differences were considered when $P < 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA) and BioEstat 5.3 (Instituto de Desenvolvimento Sustentável Mamirauá, Bélem, Brazil).

## Results and discussion

All 630 dogs were mongrels, with males ($n = 366$; 58.1%) predominating over females ($n = 264$; 41.9%). Also, > 1-year-old dogs (77.0%) were more abundant than ≤ 1 year old (33.0%), with age ranging from 2 months to 14 years. According to their level of dependence on human care and the level of restriction, dogs from São Joaquim de Bicas are mostly restricted (fully dependent; fully restricted), whereas those from Goiana are mostly family dogs (fully dependent; semi-restricted), which may increase their exposure to vector-borne pathogens (Figueredo et al. 2017; Dantas-Torres et al. 2020a). Semi-restricted dogs have free access to the streets (sometimes with no human supervision), which may increase their contact with other dogs and infested environments (Otranto et al. 2017). Besides, the vast majority of the dogs from Goiana is not treated against ectoparasites, which further increases their exposure to vector-borne pathogens (Figueredo et al. 2017).

The high exposure to *Babesia* parasites was confirmed in the study areas. Overall, out of 630 dogs tested, 519 dogs (82.4%; 95% CI: 79.2–85.3%) presented detectable IgG antibodies against *Babesia* spp. Dogs > 1 year were more seropositive than ≤ 1-year-old dogs ($\chi 2 = 34.1$, $df = 1$, $P < 0.0001$). No significant differences are found in relation to sex, place of origin, or ectoparasite treatment (Table 1). Although not significant, there was a tendency to lower *Babesia* prevalence in dogs without ectoparasite treatment. This finding may be attributed to the unreliability of treatment information, which only considered treatments applied during the 4 weeks preceding the blood collection. Likewise, this could also be due to fact that dogs in the study areas are mostly treated when their owners detect an active ectoparasite infestation. So, treated dogs may actually have had a higher exposure to ectoparasites prior treatment and thus were at a higher infection risk.

Our results agree with previous studies conducted in Brazil pointing that older dogs are more exposed than younger ones (e.g., Araújo et al. 2015; Minervino et al. 2015; Paulino et al. 2018). However, a recent study reported that < 1-year-old dogs were more frequently parasitemic (Barbosa et al. 2020). Most of the studies conducted in Brazil have shown no breed and sex predilection (e.g., Costa-Júnior et al. 2009; Minervino et al. 2015; Paulino et al. 2018), but some studies have reported a higher proportion of infection among mixed


Hepatozoon canis (Rio Grande do Sul / Brazil) (EF622096.1 )
Hepatozoon canis (Italy) (KP644235.1)
Hepatozoon canis (Spain) (AY150067.2)
Hepatozoon canis (Brasília / Brazil) (AY864677.1)
Hepatozoon canis (Santa Catarina / Brazil) (KX816958.1)
542 Hepatozoon canis (Minas Gerais / Brazil)
576 Hepatozoon canis (Minas Gerais / Brazil)
551 Hepatozoon canis (Minas Gerais / Brazil)
628 Hepatozoon canis (Minas Gerais / Brazil)
427 Hepatozoon canis (Minas Gerais / Brazil) (MW290107)*
517 Hepatozoon canis (Minas Gerais / Brazil)
456 Hepatozoon canis (Minas Gerais / Brazil)
Hepatozoon canis (Pantanal / Brazil) (KJ831221.2)
Hepatozoon canis (Brasília / Brazil) (AY864679.1)
76
Hepatozoon canis (Pará / Brazil) (KU729738.1)
519 Hepatozoon canis (Minas Gerais / Brazil)
545 Hepatozoon canis (Minas Gerais / Brazil)
500 Hepatozoon canis (Minas Gerais / Brazil)
Hepatozoon canis (Pantanal / Brazil) (KP167594.1)
Hepatozoon canis (Rio de Janeiro / Brazil) (GQ176285.1)
Hepatozoon canis (Pernambuco / Brazil) (FJ943578.1)
432 Hepatozoon canis (Minas Gerais / Brazil)
596 Hepatozoon canis (Minas Gerais / Brazil)
369 Hepatozoon canis (Pernambuco / Brazil)
Hepatozoon canis (Cuba) (MN393911.1)
Hepatozoon canis (Pernambuco / Brazil) (MG241229.1)
Hepatozoon canis (Pará / Brazil) (KU729737.1)
Hepatozoon canis (Rio Grande do Sul / Brazil) (AY471615.2)
53
Hepatozoon canis (Austria) (KX712123.1)
Hepatozoon canis (Japan) (LC169075.2)
Hepatozoon canis (Czech Republic) (KU893127.1)
80
Hepatozoon canis (Czech Republic) (KU893123.1)
Hepatozoon canis (Romenia) (KX712126.1)
Hepatozoon canis (Venezuela) (DQ439540.1)
157 Hepatozoon canis (Pernambuco / Brazil)
153 Hepatozoon canis (Pernambuco / Brazil)
218 Hepatozoon canis (Pernambuco / Brazil) (MW290106)*
61 Hepatozoon canis (Pernambuco / Brazil)
60 Hepatozoon canis (Pernambuco / Brazil)
216 Hepatozoon canis (Pernambuco / Brazil)
105 Hepatozoon canis (Pernambuco / Brazil)
Hepatozoon canis (Cuba) (MN393913.1 )
67
Hepatozoon felis (HQ829445.1)
Hepatozoon felis (KX017290.1)
94
Hepatozoon americanum (AF176836.1 )
96
Hepatozoon americanum (KU729739.1 )
Toxoplasma gondii (L37415.1 )
0.020

◂**Fig. 1** Phylogenetic tree based on an alignment of 531 bp fragment of *18S rRNA* of *Hepatozoon* spp., using NJ method and Tamura 3-parameter model. Numbers at nodes correspond to bootstrap values above 50. *Hepatozoon canis* samples originally sequenced in the present study are highlighted in green (group 1) and red (group 2). The asterisk (*) indicates the representative consensus sequences deposited in GenBank. Sequences highlighted in bold represent strains from Brazil. Accession numbers are indicated in parentheses

breed dogs in comparison with purebred dogs (Araújo et al. 2015; Barbosa et al. 2020). In a study conducted in the semi-arid region of Pernambuco, dogs with access to the street and living in contact with forested environments were at a higher risk of *B. vogeli* infection (Araújo et al. 2015).

High seroprevalence rates were found both in Goiana (81.2%, 95% CI: 75.3–86.2%) and São Joaquim de Bicas (83.0%, 95% CI: 79.0–86.5%). The seroprevalence rates calculated herein nicely match with data generated during a pilot cross-sectional study (Figueredo et al. 2017) and a longitudinal study conducted with part of the dogs included herein (i.e., 204 from Goiana and 103 from São Joaquim de Bicas) (Dantas-Torres et al. 2020a). The seroprevalence of *Babesia* spp. in dogs varies widely in Latin American countries, reaching more than 70% in highly endemic foci in Brazil (Santos et al. 2009; Ramos et al. 2010; Paulan et al. 2013; Sousa et al. 2013; Spolidorio et al. 2013; Vieira et al. 2013; Krawczak et al. 2015). For instance, a high seropositivity (i.e., 81.6%) has been reported among a subset of 60 *Leishmania*-positive dogs from Campo Grande (Mato Grosso do Sul state, central-western Brazil) (Sousa et al. 2013). The high seroprevalence of anti-*Babesia* spp. antibodies in Latin America may be partly explained by the widespread distribution and frequently high abundance of *Rhipicephalus sanguineus* sensu lato ticks.

Molecularly, 34 dogs (5.4%; 95% CI: 3.7–7.5%) were positive for the first PCR assay (~200 bp fragment) and 88 (14.0%; 95% CI: 11.4–16.9%) for the second (~450 bp). Of these, 27 dogs were simultaneously positive to both assays, seven only to the first, and 61 only to the second. Our sequencing data (19 sequences from the first PCR and 53 from the second; Supplementary material) revealed that *B. vogeli* was the only species of *Babesia* infecting dogs in the study areas (34 sequences from Goiana and 18 from São Joaquim de Bicas) (100% identity: MN823219 and MT386936). Additionally, *H. canis* was detected in eight dogs from Goiana and 12 from São Joaquim de Bicas (>99% identity: MN393911, KT246304, and MH615006). Interestingly, while all sequences of *B. vogeli* were clones (i.e., no pairwise nucleotide difference), polymorphic sites were observed in *H. canis* sequences. These variations resulted in two "groups" of sequences: one comprising seven sequences from Goiana (group 1) and another one with the remaining sequences (group 2; one sequence from Goiana and 12 from São Joaquim de Bicas). Neighbor-joining tree analysis (Fig. 1) suggests that the samples of group 1 may be close to *H. canis* strains from Cuba (MN393913), Venezuela (DQ439540), and Romania (KX712126), whereas the samples of group 2 may be close to other *H. canis* strains reported in Brazil previously (Paludo et al. 2005; Criado-Fornelio et al. 2006; Perles et al. 2019; Díaz-Sánchez et al. 2021), including strains from Pernambuco (FJ943578 and MG241229). Further studies are required to better elucidate the genetic diversity of *H. canis* and its relationship with the epidemiology of canine hepatozoonosis in Brazil. For instance, it would also be interesting to assess the presence of *H. canis* in ticks in the study areas, where *R. sanguineus* s.l. is the dominant tick species on dogs (Dantas-Torres F., personal observation). Importantly, the limited use of ectoparasiticides on dogs is also a major issue in Brazil and other Latin American countries (Figueredo et al. 2017; Maggi and Krämer 2019). Therefore, increased awareness and promotion of guidelines for the prevention of canine babesiosis and other vector-borne diseases in dogs (Dantas-Torres et al. 2020b) in this region are urgently needed.

Further regarding seropositivity and PCR positivity, 16 dogs were seronegative but PCR positive, which suggest that the PCR assays picked up dogs with recent infections (i.e., prior to antibody production), dogs with antibodies below detectable levels, or even individuals infected by other organisms (e.g., *H. canis*). Indeed, six of these dogs were infected by *B. vogeli* and two by *H. canis*; for

**Table 1** Positivity to IgG antibodies against *Babesia* spp. in dogs in relation to municipality of origin, sex, age, and ectoparasitic treatment

| Variables | Neg | Pos | % (95% CI) | Statistical analysis |
|---|---|---|---|---|
| Origin | | | | |
| Goiana | 71 | 346 | 83.0 (79.0–86.5) | $\chi2=0.3$, $df=1$, $P=0.59$ |
| São Joaquim de Bicas | 40 | 173 | 81.2 (75.3–86.2) | |
| Sex | | | | |
| Female | 46 | 218 | 82.6 (77.5–87.0) | $\chi2=0.01$, $df=1$, $P=0.91$ |
| Male | 65 | 301 | 82.2 (77.9–86.0) | |
| Age | | | | |
| ≤1 year | 67 | 161 | 70.6 (64.2–76.4) | $\chi2=34.1$, $df=1$, $P<0.0001$ |
| >1 year | 44 | 358 | 89.1 (85.6–91.9) | |
| Ectoparasiticide treatment | | | | |
| Yes [a] | 20 | 137 | 87.3 (81.0–92.0) | $\chi2=3.4$, $df=1$, $P=0.06$ |
| No | 91 | 382 | 80.8 (76.9–84.2) | |

[a]"Yes" means that the dog or its environment had been treated with any ectoparasiticide during the 4 weeks preceding blood sampling. This variable is not controlled for frequency or type of product, and is based on owner verbal information

the remaining eight dogs, no DNA sequence could be generated.

Except for a single report of a genotype closely related to *B. caballi* in a dog from central-western Brazil and few reports of *B. gibsoni* from southern Brazil, all reports of *Babesia* infections in dogs in this country refer to *B. vogeli* (Araújo et al. 2015; Maggi and Krämer 2019; Barbosa et al. 2020; Panti-May and Rodríguez-Vivas 2020). The comparison of PCR assays used herein is beyond the scope of this study, but the PIRO A1/PIRO B PCR has a broader diagnostic spectrum than the Bcanis/Bcommon PCR (Carret et al. 1999; Kordick et al. 1999; Jefferies et al. 2003).

In conclusion, this study confirms a high level of infection by *B. vogeli* in two areas of Brazil and highlights that most of the dogs living in these areas are exposed to this parasite during the course of their life, with older dogs being consequently more positive than young ones.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00436-021-07195-8.

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## Declarations

**Ethical approval** The Committee on Ethics in the Use of Animals (CEUA, project number: 66/2014) of the Aggeu Magalhães Institute (Fiocruz) approved the procedures used in this study. Dog owners signed a consent form before the inclusion of their animals in this study.

**Conflict of interest** GA, KD, and MP are employees of Elanco Animal Health. FDT and DO are members of the CVBD World Forum, whose activities are sponsored by Elanco Animal Health. JA, DEOM, LAF, KGSS, LCSP, LGS, GRV, and VMR declare they have no competing interests.

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