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Original article

Canine piroplasmids: Molecular detection and laboratory characterization in dogs from Brasilia, Brazil, with the first molecular evidence of dog exposure to a novel opossum-associated *Babesia* sp.

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ABSTRACT

Canine piroplasmid infections can be caused by *Babesia* spp., *Theileria* spp. and *Rangelia vitalii*. In Brazil, canine babesiosis caused by *Babesia vogeli* is endemic and reported throughout the country. On the other hand, Rangeliosis caused by *R. vitalii* has only been described so far in the South and Southeast regions. Despite that, studies analyzing the laboratory and molecular characterization of these hemoprotozoa are still scarce. To investigate the occurrence, the laboratory features, the molecular characterization, and the diversity of piroplasmids from Midwestern Brazil, a survey was performed using blood samples obtained from 276 domestic dogs from Brasília, Federal District, Midwestern Brazil. A broad-range quantitative PCR (qPCR) targeting the mitochondrial large subunit ribosomal DNA (LSU4) was used to detect piroplasmid DNA. The overall molecular occurrence of piroplasmids was 11.2% (31/276), with 9.7% (27/276) of the sequences identified as *Babesia vogeli* (98–100% identity to *B. vogeli* isolate from the USA). Based on a partial 18S rRNA sequence pairwise alignment (-250 bp), 1.4% (4/276) of the sequences showed only 76.8% identity with *B. vogeli* but 100% identity with opossum-associated *Babesia* sp. (MW290046–53). These findings suggest the exposure of dogs from Brazil to a recently described *Babesia* sp. isolated from white-eared opossum. None of the analyzed dogs was positive for *Theileria* spp. or *R. vitalii*. Subsequently, all positive sequences were submitted to three additional PCR assays based on the 18S rRNA, *cox-1,* and *cytb* genes, aiming at performing a haplotype network analysis. Haplotype network using *cox-1* sequences showed the presence of six different haplotypes of *B. vogeli*; one of them was shared with isolates from Brazil, the USA, and India. When including animals co-infected with other vector-borne diseases, piroplasmid-positive dogs had 2.3 times higher chance of having thrombocytopenia than the negative ones. The molecular results demonstrated that the compared *Babesia vogeli* sequences showed a low variability as well as evidence of exposure to a putative novel opossum-associated *Babesia* sp. in dogs from Midwestern Brazil.

1. Introduction

Piroplasmids are among the most important tick-borne agents of domestic and wild animals worldwide. These hemoprotozoa (Apicomplexa: Piroplasmida), comprise the genera *Babesia* spp., *Theileria* spp., *Cytauxzoon* spp. and *Rangelia vitalii* (Uilenberg, 2006; Lack et al., 2012; Schnittger et al., 2012; Alvarado-Rybak et al., 2016). Although *Rangelia vitalii* has been given the rank of an additional genera, molecular phylogeny based on the 18S RNA and the *hsp70* genes demonstrated that this piroplasmid species belongs to *Babesia* sensu stricto (Clade VI, as defined by Schnittger et al., 2012) and reclassification is therefore pending (Soares et al., 2011; Schnittger et al., 2022). However, unlike

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other *Babesia* sensu stricto (s.s.) organisms it does appear to have an extra-erythrocytic life stage in the mammalian host.

Based on morphology, molecular methods, and phylogenetic analyses (Jalovecka et al., 2019; Mans, 2022; Schnittger et al., 2022), there are currently at least nine different species of *Babesia*, namely *Babesia canis, Babesia rossi,* and *Babesia vogeli* (Zahler et al., 1998; Carret et al., 1999), *Babesia gibsoni, Babesia* sp. Coco (Schnittger et al., 2022), *Babesia conradae* (Kjemtrup and Conrad, 2006; Solano-Gallego and Baneth, 2011), *Babesia vulpes* (Baneth et al., 2015, 2019), *Babesia negevi* (Baneth et al., 2020; Far et al., 2021), and *Rangelia vitalii,* belonging to *Babesia* s. s. group (Schnittger et al., 2022). In Brazil, only *Babesia gibsoni* (Trapp et al., 2006a; 2006b), *Babesia vogeli* (Passos et al., 2005), and a genotype closely related to *Babesia caballi* (de Sousa et al., 2018) have been reported in dogs so far, although molecular analysis of these piroplasmids is incipient.

Babesia vogeli has already been widely reported in dogs from Brazil (Passos et al., 2005; Costa-Júnior et al., 2009; O'Dwyer et al., 2009; Oliveira et al., 2009; Ramos et al., 2010; Spolidorio et al., 2011; Costa-Júnior et al., 2012; Lemos et al., 2012; Silva et al., 2012; Sousa et al., 2013; Moraes et al., 2014; Rotondano et al., 2015, 2015; Ribeiro et al., 2017; de Sousa et al., 2018; Vieira et al., 2018; Paulino et al., 2018; Castro et al., 2020; Barbosa et al., 2020; Camilo et al., 2021; de Macedo et al., 2022; Fonsêca et al., 2022). Regarding Rangeliosis, cases of the disease remain restricted to the South and the Southeast regions of the country (Fighera et al., 2010; Soares et al., 2011; Lemos et al., 2012; Malheiros et al., 2016; Gottlieb et al., 2016; Mongruel et al., 2017; Lemos et al., 2017; Silva et al., 2019; Fournier et al., 2020). Nevertheless, there are no epidemiological studies investigating its occurrence in Midwest Brazil, despite the presence of its reservoir (*Cerdocyon thous*, crab-eating fox) in this region (Nascimento et al., 2013) and the recent report in Paraguay, a neighboring country to Brazil (Inácio et al., 2019).

Despite an increase of *Theileria* spp. reports in dogs from Europe (Criado-Fornelio et al., 2003; Criado et al., 2006; Beck et al., 2009; Fritz, 2010), Asia (Qablan et al., 2012; Bigdeli et al., 2012; Aktas et al., 2015; Xu et al., 2015; Díaz-Regañón et al., 2020; Habibi et al., 2020), Africa (Matjila et al., 2008a, 2008b; Kamani et al., 2013; Adamu et al., 2014; Rosa et al., 2014; Rjeibi et al., 2016; Sili et al., 2021), there is only one recent report described in one asymptomatic domestic dog in South America, Paraguay (Inácio et al., 2019). The sequence was closely positioned to a *T. equi*-like sequence from horses and a *Theileria* sp. from a domestic cat, both from Brazil (Inácio et al., 2019).

In order to investigate the occurrence and diversity of piroplasmids in dogs from Brasilia, Federal District, Midwestern Brazil, a place where there are no previous published data about piroplasmids in domestic dogs, we have evaluated blood samples for the presence of these hemoprotozoa through hematological, biochemical and molecular analyses.

2. Material and methods

2.1. Animals and sampling sites

This study was approved by the Ethics Committee of the University of Brasilia, under protocol number 40/2017 - UnB DOC. Between January/2017 and December/2019, 276 domestic dogs (*Canis familiaris*) were selected in Brasilia (15° 47′ 38″ S 47° 52′ 58″ O), regardless of age, sex, breed, and the presence or the absence of any clinical signs of vector-borne diseases. These animals were attended at private clinics, shelters, and kennels of the Federal District (FD) as well as at the Veterinary Hospital of the University.

The sample size of 246 animals was initially calculated according to Thrusfield (2005), based on a 95% confidence level, 5% absolute precision, and 20% expected prevalence (more than twice the mean number of dogs infected with piroplasmids according to previous surveys in Brazil). Our study started with 300 samples. However, due to pre-analytical and analytical issues (clots, small volume of samples, or negative results on the GADPH - the housekeeping gene), 276 samples were analyzed. Hematological and biochemical analyses were performed using all sampled dogs ($n = 276$) as well as excluding co-infected dogs ($n = 269$) with other vector-borne diseases.

2.2. Hematological and biochemical analysis

Blood samples were collected from dogs from cephalic or jugular veins into ethylenediamine tetra-acetic acid (EDTA)–coated tubes for complete blood count (CBC) and DNA extraction, and into tubes containing a clotting activator for biochemical analysis. All the hematological and biochemical analyses were performed at the Veterinary Clinical Pathology Laboratory, College of Agronomy and Veterinary Medicine, University of Brasilia, Brasilia, FD. The CBC and the concentration of hemoglobin were obtained using an automatic cell counter (ABC Vet Horiba® ABX diagnostics, Brazil). The Packed Cell Volume (PCV) was determined by microhematocrit centrifugation. Differential leukocyte counts were obtained by direct observation of 100 leukocytes in Diff-quick stained blood smears using a light microscope (CX40RF200, Olympus, Japan). All blood smears were checked for the presence of platelet aggregates and protozoan inclusions. Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were also calculated. Plasma protein concentration was determined by refractometry (models SZJ-D and RTP-20 ATC). Serum samples were analyzed for the activity of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and concentration of total serum protein, albumin, urea, and creatinine, in an automatic biochemistry analyzer (Cobas c111 Roche®). The reference intervals for CBC and biochemistry parameters were employed according to Weiss and Wardrop (2011) and Kaneko et al. (2008), respectively. Hematological abnormalities were considered as anemia (PCV < 37%, or Red blood cells $< 5{,}5 \times 10^6/\mu L$ or Hemoglobin < 12,5 g/dL), leukopenia (White Blood Cells $< 6.000 \times 10^3/\mu L$) or thrombocytopenia (Platelets $< 200.000 \times 10^3/\mu L$).

3. Molecular analysis

3.1. DNA extraction

DNA was extracted from EDTA-whole blood samples using a commercial kit (Blood Genomic Prep Mini Spin Kit, Promega Corporation®, WI, USA, according to the manufacturer's recommendations. To assess the DNA sample concentration and quality (260/280 ratio) we analyzed all samples by optical spectrophotometry (Nanodrop, Thermo Scientific®). We also performed a housekeeping PCR targeting the mammal glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene to confirm the presence of genomic DNA of the vertebrate host and the absence of PCR inhibitors (Birkenheuer et al., 2003). DNA was stored at −20 °C until PCR analysis.

3.2. PCR assays

The molecular analyses involved PCR and quantitative PCR (qPCR) assays, which were performed at the Veterinary Molecular Biology Laboratory from the College of Agronomy and Veterinary Medicine, University of Brasilia, Brasilia, FD, Brazil, and the Vector-Borne Disease Diagnostic Laboratory (VBDDL) (College of Veterinary Medicine, North Carolina State University, Raleigh, NC, U.S.A.), respectively.

3.2.1. Molecular screening for piroplasmids using qPCR assay based on the mitochondrial large subunit (mtLSU4)

Starting from the qPCR assay, all 276 samples were screened for the presence of piroplasmids DNA using a broad-range qPCR assay targeting a 108–173 bp region from the mitochondrial large subunit (mtLSU4) DNA as previously described (Qurollo et al., 2017). The qPCR was carried out using the primers BAB-LSU4 F

(ACCTGTCAARTTCCTTCACTAAMTT), BMIC-LSU4 F (TTGCGATAGTAATAGATTTACTGC), and BAB-LSU R (TCTTAACCCAACTCACGTACCA). Briefly, the amplification reaction was performed using the Thermocycler Biorad CFX96 Real-Time System C1000 Touch. The qPCR assays contained 12,5 μL of SSO Advanced SYBR Universal Supermix 2X (BioRad, Hercules, USA), 5 μL DNA template, 0,3 μL of BAB primers (0,6 μM), 0,2 μL of BMIC primer (0,4 μM) and molecular grade water to a final volume of 25 μL. The amplification protocol used was as follows: 3 min at 98 °C, followed by 40 cycles of 15 s at 98 °C, 15 s at 60 °C and 15 s at 72 °C. The melting curve was acquired using 0.5 °C steps, withholds of 2s, from 65 to 95 °C. The results were assessed through observation of amplification and melting curves. In all qPCR assays, plasmids encoding mt LSU fragments of *B. microti*-like (GenBank access number: KC20782) and *B. rossi* (KC207823.1) were used as positive controls, and a negative dog DNA and no template control (NTC), using Ultra-pure water (Sigma-Aldrich Inc, Germany), were used as negative controls. All amplicons were submitted to an electrophoresis gel on 2% agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, Inc, US), regardless of the presence or absence of amplification curves in qPCR. In addition, only those samples whose mtLSU4 sequencing results showed poor identity (< 90% Identity) to piroplasmids by Blast analysis were submitted to a qPCR assay targeting the Apicomplexa-specific 18S rRNA gene (Tyrrell et al., 2019). This additional 18S rRNA qPCR was done as an attempt to obtain sequences from a molecular marker that had more GenBank available sequences to perform a new Blast analysis. That could, in turn, provide a better characterization of those sequences.

3.2.2. Conventional pcr targeting babesia spp. and possible co-infections

Subsequently, the positive samples on LSU4-based qPCR were subjected to further molecular characterization using PCR assays targeting three molecular markers (Supplementary material 1), namely 18S rRNA, *cox-1,* and *cytb*. Moreover, these positive samples were also screened for the presence of *Leishmania* spp. SSU rDNA (-603 bp) (Schönian et al., 2003) and *Ehrlichia canis* 16S rRNA gene (396 bp) (Murphy et al., 1998). The PCR reactions described herein were adapted from the original sources for reaction setup and cycling. Amplifications of all rounds were performed in a total volume of 25 μL containing 1 μL of template DNA, except for the amplification of *cytb* fragments that were carried out using 2 μL of DNA for the first round, and the amplification of the *Leishmania* spp. SSU fragments were carried out using 2,5 μL of DNA. Amplified DNA was electrophoresed and visualized using UV illumination on 1,5% agarose gel stained with ethidium bromide. For all PCR assays, DNA-positive controls were obtained from naturally *Babesia*-infected dogs (MZ648124), and, for negative controls, MilliQ® Ultra-pure water (Sigma-Aldrich Inc, Germany) was used. All samples were run in duplicate.

3.3. Amplicon purification, sequencing, and blast analysis

Amplicons from all qPCR positive samples were submitted to bidirectional Sanger sequencing to confirm the results (GENEWIZ, Inc., Raleigh, NC). Regarding the PCR assays, the amplicons were previously purified using the Nucleo Spin® Gel and PCR Clean-up purification kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. Subsequently, the purified amplicons were sequenced using Sanger sequencing at the Center of Biological Resources and Genomic Biology (CREBIO), Jaboticabal, São Paulo, Brazil. Geneious Prime (v. 9.0.5.) was used to align and analyze DNA results with reference sequences from GenBank. The primers regions were manually trimmed. Identity, query coverage, and e-values were assessed by the BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST) - using default parameters and non-redundant (nr) database, available in the NCBI GenBank (Altschul et al., 1990). The Smith-Waterman pairwise algorithm was applied for better comprehension and molecular elucidation of the identities between the *Babesia vogeli* and *Babesia* sp. isolates of this study based on an alignment of ~250 bp. A multiple sequence alignment (MSA) was constructed with 82 piroplasm sequences using the Clustal Omega algorithm. The sequences included the ten phylogenetics clades proposed by Jalovecka et al. (2019). The length of the alignment was 231 bp.

3.4. Haplotype network

TCS Network was performed in PopART v.1.7 software using all 20 available sequences of *cox-1* from *B. vogeli* deposited in GenBank and the *cox-1* sequences obtained in the present study. DnaSP v. 6 was applied to calculate nucleotide diversity (π), number of haplotypes (h), diversity of haplotype (Dh), the average number of nucleotide differences (K), and number of segregating sites (S).

4. Statistical analysis

The effects of the results of the molecular test for piroplasmids (positive or negative), gender (male or female), age in years (0–1; > 1- 2 years; > 2–7; > 7), or origin (kennel, shelter, urban or rural area) on blood parameters were evaluated in a General Linear Model (PROC GLM) and means compared using Duncan's Multiple Range Test, with $P<0.05$ used as a significant difference. The effect of sex, age, and origin of the animal on the test outcome was evaluated using logistic regression (PROC LOGISTIC). A chi-square test of frequencies was used to check the effect of the test result on sex, age, origin, anemia, leukopenia, and thrombocytopenia (PROC FREQ). A logistic regression model with the odds ratio value was performed for the cases that showed an association with the positive results, to test the independent chance factors for the infection. All data were analyzed in SAS (Statistical Analysis System Institute, Cary, North Carolina).

5. Results

5.1. Epidemiological findings

The overall molecular occurrence for piroplasmids using the LSU-based qPCR was 11.2% (31/276): 9.7% (27/276) were identified as *Babesia vogeli* and 1.4% (4/276) of the samples were described as *Babesia* sp., since they had 100% Identity and coverage with *Babesia* sp. isolated from white-eared opossum (*Didelphis albiventris*) (MW290046–53) from a recent study performed in Brazil (Gonçalves et al., 2021). All samples were positive on agarose gel electrophoresis. None of the analyzed dogs were positive for *Theileria* spp. or *Rangelia vitalii* by qPCR analysis. Six *Babesia*-positive dogs had co-infections based on PCR results. Out of these co-infected dogs, two animals were positive for *Leishmania* sp., while *Ehrlichia canis* was identified in four dogs (Supplementary material 2).

5.2. Hematological and biochemistry profile

Out of all 27 dogs *Babesia vogeli* positive dogs, only three animals (3/276, 1.1%) had intracytoplasmatic inclusions detected on blood smears by light microscopy. Two samples had *Babesia*-like large merozoites, and one had *Hepatozoon* sp. gametocytes. No other inclusions suggestive of hemoparasites, such as *Ehrlichia* sp. morulae, were found in the other samples, even those with a positive result on PCR for the same agent.

None of the mean values for both hematological and biochemical parameters were out of the normal range, (Table 1). However, when including the co-infected dogs, thrombocytopenia was found in higher frequency in the positive group, compared to the negative one ($p = 0.0309$; Table 2). Furthermore, a platelet decrease was observed in 75% (3/4) of dogs also infected with *E. canis*, while 100% (2/2) of the dogs co-infected with *Leishmania* sp. were thrombocytopenic (Supplementary material 2). The odds ratio analysis showed that piroplasmid-positive animals were 2.3 times or 133% (95% CI: 1.0634 – 5.1353) at a higher chance of having thrombocytopenia than the negative dogs (chi-square = 4.6563; $n = 266$; $p = 0.0309$).

Table 1
Mean values of the hematological and biochemistry data on negative and positive piroplasmids dogs based on LSU4 mitochondrial region qPCR. Data not including co-infected dogs ($n = 269$).

Variable	Negative dogs	Positive dogs	R^2	CV
Hematologic features				
Red blood cells (x10^6/μL)	6.3	6.0	0.114562	24.94
Hemoglobin (g/dL)	14.2	11.9	0.177941	24.90
PCV (%)	42.7	40.9	0.147969	23.52
Total Plasmatic Protein (g/dL)	7.2	6.8	0.082174	17.49
MCV (fL)	68.1	71.6	0.034367	10.60
MCHC (%)	33.3	32.8	0.052451	9.83
White blood cells (x10^3/μL)	10.755	8.750	0.039490	57.32
Band neutrophils (%)	16.0	0.9	0.342760	277.47
Band neutrophils (/μL)	997	56	0.376476	204.04
Segmented neutrophils (%)	66.6	63.3	0.092038	20.68
Segmented neutrophils (/μL)	7.313	5.643	0.025240	68.14
Lymphocytes (%)	20.7	15.1	0.091975	58.39
Lymphocytes (/μL)	2.025	1.180	0.117087	72.11
Monocytes (%)	6.3	7.1	0.074877	86.77
Monocytes (/μL)	683	617	0.044510	122.98
Eosinophils (%)	6.4	6.0	0.109137	86.22
Eosinophils (/μL)	625	739	0.097826	116.28
Platelets (x10^3/μL)	323.198	258.750	0.069280	43.46
Biochemistry				
ALT (UI/L)	67.0	65.3	0.180614	0.18
Alkaline phosphatase (UI/L)	173.2	128.0	0.055211	187.89
Creatinine (mg/dL)	1.0	0.8	0.058626	95.31
Urea (mg/dL)	46.4	39.1	0.059100	91.41
Total protein (g/dL)	6.5	7.2	0.102050	17.27
Albumin (g/dL)	3.5	3.9	0.152457	20.79

$R^{2\,-}$ coefficient of determination; CV – coefficient of variation, PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Volume.

Table 2
Frequency of hematological abnormalities data on negative and positive piroplasmids dogs based on LSU4 mitochondrial region qPCR. Data including co-infected dogs ($n = 276$).

PCR result/ Variable n (%)	Anemia		Leukopenia		Thrombocytopenia *	
	No	Yes	No	Yes	No	Yes
Negative dogs	168 (71.2)	68 (28.8)	208 (88.2)	28 (11.9)	185 (78.7)	50 (21.3)
Positive dogs	17 (54.8)	14 (45.2)	28 (90.3)	3 (9.7)	19 (61.3)	12 (38.7)
Total	185 (69.3)	82 (30.7)	236 (88.4)	31 (11.6)	204 (76.7)	62 (23.3)

Statistical test: chi-square; Significance level: 95%; p-values: Anemia: 0.0636; Leukopenia: 0.7208; Thrombocytopenia.
* : 0.0309.

5.3. Sequencing analysis

The consensus sequences obtained with the LSU4 qPCR amplification for all 27/31 sequences (GenBank numbers OM687171 - OM687187) showed a 98–100% identity with a *B. vogeli* isolate from the USA (KC207825.1) with 100% coverage in BLASTn. We also obtained three partial 18S rRNA amplicons (MZ595100, MZ595101, MZ595102) from four samples that presented 100% coverage and nucleotide identity with *Babesia* sp. isolates from white-eared-opossums (MW290046–53). (Fig. 1). In BLASTn, these sequences (MZ595100) have high-quality, range between 215 and 257 bp in length, and shared 100% nucleotide identity.

Regarding the molecular markers used in this study, *Babesia vogeli* was the only agent amplified in all PCR assays. *B. vogeli* had seven partial 18S rRNA and *cox-1* sequences amplified, and also ten sequences were amplified by *cytb* PCR reactions (Supplementary material 3). The putative novel *Babesia* sp. isolated from white-eared opossum was not amplified in any of these PCR assays.

Our seven *B. vogeli* sequences (649–751 bp) (MZ594998–5004) showed 100% identity among them and with isolates from Italy (AY072925.1) and Brazil (AY371194.1) in pairwise alignment based on a modified Smith-Waterman algorithm. Comparing the three *Babesia* sp. sequences with the GenBank isolates, the highest identity found were 83.8, 82.4%, and 82.0%, related to *Babesia* sp. EEZA-CRETAV (MW287597.1, host: Argas tick, Spain), *Babesia percei* AP174–09/Rambo (MF288025.1, host: *Spheniscus demersus*, South Africa), and *Babesia ardeae* (KY436057.1, host *Ardea cinerea*, Singapore), respectively.

The pairwise alignment obtained by the Smith-Waterman algorithm showed 76.8% identity between the *Babesia vogeli* and *Babesia* sp. isolates of this study. Additionally, the pairwise identity based on 18S rRNA partial sequence among the piroplasmids isolates from this study, compared with the GenBank isolates, that were provided by their Multiple Sequence Alignment (MSA), is shown in 18S rRNA-based Identity Matrix A (Supplementary Material 4). The MSA was constructed using the Clustal Omega algorithm with visual assessment for the identification of possible incoherencies and is available in the Supplementary Material 5.

A haplotype network based on the *cox-1* gene showed the presence of only one haplotype (H1) of *B. vogeli* in Brazil, which was also shared with isolates from the USA and India. Conversely, India showed to have 6 different haplotypes in total. Nucleotide diversity (π) was calculated as 0,00,310; number of haplotypes (h),6; diversity of haplotypes (Dh),0,516; segregating sites (S),9; average number of nucleotide differences (K),1247 (Fig. 2; Table 4). We were unable to perform a Haplotype Network using the 18 s rRNA and the *cytb* sequences obtained in this study, as the few available sequences found in the GenBank had 100% Identity among them.

6. Discussion

The molecular tests performed in the present study showed a moderate prevalence of *B. vogeli* (11.2%) when compared to studies previously reported in Brazil from the Midwest, Southeast, and North regions in the country, with rates of 14.2% (de Sousa et al., 2018), 15.7% (Paulino et al., 2018) and 15.7% (Moraes et al., 2015), respectively. These findings are not surprising, since *B. vogeli* is endemic in Brazil (De Valgas et al., 2004; Dantas-Torres and Figueredo, 2006; Trapp et al., 2006b; Dantas-Torres, 2008; Da Silva et al., 2016; Maggi and Krämer, 2019; Panti-may and Rodríguez-vivas, 2020), and the use of molecular techniques as an additional tool for diagnosis has been improving the ability to detect these hemoparasites (Maggi et al., 2013; Qurollo et al., 2017; Kidd, 2019; Garcia-Quesada et al., 2021).

Consistent with previous studies in Brazil, this survey found no statistical difference with respect to sex, age, and origin of infected and non-infected dogs (Trapp et al., 2006b; O'Dwyer et al., 2009; Barbosa et al., 2020). However, the sampled dogs from this study were selected from veterinary care and a bias cannot be excluded.

We obtained just one sample where intraerythrocytic inclusions were detected by microscopic examination of a stained blood smear. Since almost half of the dogs in the positive group were asymptomatic and most of them were adults, they were probably chronic carriers of *B. vogeli* with low parasitemia in a state of premunition (Solano-Gallego et al., 2016; Bilić et al., 2018). In cases of low and often intermittent parasitemia, it is often difficult to observe *Babesia* species by microscopic evaluation (Baneth, 2018). These subclinical dogs could present later with clinical signs (Solano-Gallego et al., 2016) or could contribute to the spread of infection via ticks (Checa et al., 2019). Since this study only evaluated dogs at a single time point it is unclear whether these

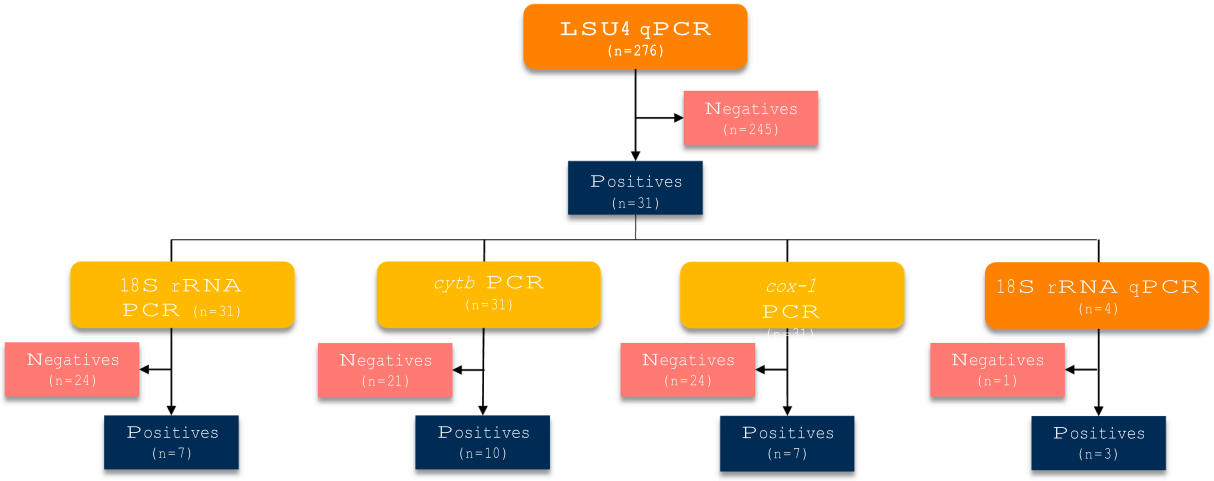


Fig. 1. Molecular results used in this study of the molecular markers targeting Piroplasmids.
Notes: 18S rRNA qPCR was performed only for those samples whose sequences obtained did not match *Babesia vogeli* on BLASTn analysis.

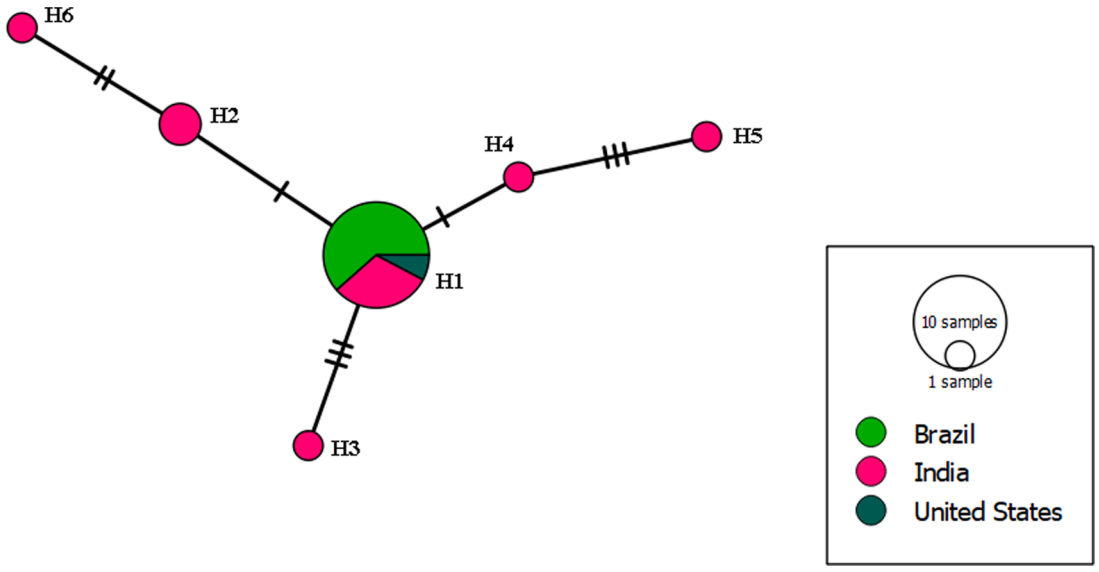


Fig. 2. TCS Network of twenty *cox-1* partial sequences (402 nt) from *Babesia vogeli* performed on PopART v.1.7 software.
The size of circles corresponds to the haplotype frequency; hatch marks represent SNPs separating haplotypes.

dogs would have eventually developed a clinical illness.

It seems that co-infections (at least those detected by PCR alone) were not extremely common in our study. We only found a few samples co-infected with *Ehrlichia canis, Hepatozoon* sp., or *Leishmania* sp. However, it is noteworthy that we performed neither serological assays nor PCR on spleen and bone marrow samples (Kidd, 2019) and are likely to have underestimated the prevalence of co-infections. Nonetheless, co-infection with these vector-borne agents seems have worsened laboratory abnormalities in co-infected dogs (Sasanelli et al., 2009; Harrus and Waner, 2011; Rawangchue and Sungpradit, 2020; Zaki et al., 2021), since thrombocytopenia was found to be statistically associated with vector-borne infection only when co-infected animals were included in the analysis. This reinforces the likely chronic carrier state of the dogs studied and agrees with a previous study that found hematological abnormalities only in chronically infected or splenectomized dogs (Wang et al., 2018).

Based on both qPCR assays targeting mitochondrial LSU4 and 18S rRNA gene, we amplified four sequences that did not match *B. vogeli* sequences previously deposited in GenBank. DNA of these hemoprotozoa had very low identity with our *B. vogeli* isolates (65.8%) and no more than 83.76% identity to *Babesia* sp. EEZA-CRETAV (MW287597.1, host: *Argas* sp, Spain). Those sequences were found to have 100% Identity and coverage with sequences from *Babesia* sp. detected in opossums from midwestern Brazil. Furthermore, we did not find piroplasmid-like structures on blood smears from these four dogs and also only detected the DNA of this agent using qPCR assays (Qurollo et al., 2017) suggesting low parasitemia. It is worth noting that the *Babesia* sp. isolated from the white-eared opossum described by Gonçalves et al. was generated in a different laboratory from the present study, which makes DNA cross-contamination unlikely. Previously, the only reports of "non-*Babesia vogeli*" infected dogs in Brasil are from the states of Paraná and Mato Grosso do Sul, which described the molecular detection of *B. gibsoni* (Trapp et al., 2006a) and a sequence variant related to *Babesia caballi* (de Sousa et al., 2018). Ultimately, we suggest that these piroplasmids might represent a native *Babesia* species for which domestic dogs represent an accidental hosts (Criado et al., 2006; de Sousa et al., 2018). Further studies are needed to elucidate the source, molecular characterization, vectors involved, and relevance of this

Table 4
Haplotype characterization for *cox-1* sequences of *Babesia vogeli*.

Haplotype number	Gene Bank Access numbers	Origin	Host
H1	MZ577088, MZ577089, MZ577090, MZ577091, MZ577092, MZ577093, MZ577094	Brasilia, Brazil	Domestic dog
H1	KX426022	Brazil	Domestic dog
H1	KC207825	USA	Domestic dog
H1	MN176012; MN176014; MN176017; MN176018	India	Domestic dog
H2	MN176013; MN176016	India	Domestic dog
H3	MN176011	India	Domestic dog
H4	MN176015	India	Domestic dog
H5	MN176019	India	Domestic dog
H6	MN176020	India	Domestic dog

opossum-*Babesia* sp. identified in dogs.

To our knowledge, this is the first assessment of *B. vogeli* diversity based on the *cox-1* haplotype network. Despite the low number of obtained sequences, only one *cox-1 B vogeli* haplotype was obtained among seven sequences, whereas six were found in India. This same haplotype was shared with other *B. vogeli* isolates from the USA and India, which could reflect that this mitochondrial gene has some genetic conservation. Future studies using mitochondrial genes from *B. vogeli* isolates from different regions of the country should be carried out in Brazil. The low number of *B. vogeli* haplotypes found herein ($n = 1$) can be due, unfortunately, to the limited number of available sequences in the Genbank database. Alternative approaches such as cloning and sequencing multiple clones or next generation sequencing of amplicons may have facilitated the identification of different *B. vogeli* haplotypes in individual dogs. However, we believe that our first network of *cox-1* sequence variants can give visibility to this marker so that more sequences will be deposited soon. A previous study showed the presence of nine haplotypes of *B. canis* in dogs from Poland, where the authors worked with 93 *cox-1* sequences (Hrazdilová et al., 2019). Our results corroborate recent studies, which encourage the use mitochondrial markers as potential tools for studying the molecular evolution, phylogeny, and diversity of Piroplasm species (Schreeg et al., 2016; Hrazdilová et al., 2019; Wang et al., 2019; Panait et al., 2021; Willi et al., 2022).

7. Conclusion

We characterized, for the first time, piroplasmid infections in domestic dogs from Brasília, Federal District, Midwestern Brazil, by assessing both the laboratory and molecular features. When co-infected dogs were included in the statistical analysis, piroplasmid-infected dogs were more likely to be thrombocytopenic compared with dogs where infections were not detected. A *cox1*-based haplotype network analysis showed that *B. vogeli* isolates showed a low intraspecific variability, although further studies using more sequences are needed to confirm this finding. Besides the occurrence of the widespread *B. vogeli*, we showed molecular evidence of a putative novel opossum-associated *Babesia* species that could be infecting dogs.

CRediT authorship contribution statement

Camila Manoel de Oliveira: Investigation, Writing – original draft, Writing – review & editing, Project administration, Software, Formal analysis. **Tzushan Sharon Yang:** Writing – original draft, Software, Investigation. **Matheus Almeida Duarte:** Methodology, Software, Data curation, Writing – original draft. **Henry Marr:** Supervision. **Concepta Margaret McManus:** Methodology, Data curation. **Marcos Rogério André:** Conceptualization, Data curation, Writing – review & editing, Resources. **Adam Joseph Birkenheuer:** Methodology, Formal analysis, Resources, Data curation, Project administration, Supervision, Validation, Funding acquisition, Writing – review & editing. **Giane Regina Paludo:** Conceptualization, Formal analysis, Funding acquisition, Data curation, Project administration, Writing – review & editing, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

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