

Babesia spp. in Dogs from Córdoba, Colombia

Carmen GALVÁN ^{1,a} Jorge MIRANDA ^{1,b} Salim MATTAR ^{1,c} Juan BALLUT ^{1,d}

¹ Instituto de Investigaciones Biológicas del Trópico, Facultad de Medicina Veterinaria y Zootecnia, Universidad de Córdoba, Montería, COLOMBIA

^a ORCID: 0000-0002-3782-1473; ^b ORCID: 0000-0002-7110-7791; ^c ORCID: 0000-0003-0526-4630; ^d ORCID: 0000-0002-1008-5496

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Abstract

Canine babesiosis is a tick-borne disease with worldwide distribution, caused by intra-erythrocytic protozoa of the genus *Babesia*. The present study describes the clinical features and molecular detection of *B. canis vogeli* in dogs in Córdoba, Colombia. Blood samples were taken from dogs with clinical signs compatible with tick-borne diseases. Peripheral blood samples were examined microscopically for the presence of *Babesia* spp. merozoites and trophozoites. Blood sample obtained by jugular or cephalic venipuncture were used for DNA extraction. PCR assay was carried out using primers for the 18S rRNA gene of *Babesia* spp. of the 42 dogs suspected of babesiosis, 23 were females and 19 males. Peripheral blood examination demonstrated intraerythrocytic piroplasms compatible with *Babesia* spp. in 23 of 42 (54.7%) dogs. The morphometric study showed that 73% (17/23) were large babesias (2.4x5 µm) and 26% (6/23) were small *Babesia* (1x3.2 µm). Eleven of 42 (26%) blood samples were positive for *B. canis vogeli*. Clinical signs found in positive dogs were: anorexia 63% (7/11), lethargy and apathy 63% (7/11), fever 54% (6/11), pale mucous membranes 54% (6/11), lymphadenomegaly 18% (2/11), vomiting 9% (1/11) and diarrhea 9% (1/11). Blood counts showed that 70% (8/11) dogs had anemia. Leukocyte disorders were variable; 27% (3/11) had leukocytosis and 46% (5/11) had leukopenia. Sequenced samples of the 18S rRNA gene showed 99% identity with *B. canis vogeli* sequences from several countries.

Keywords: Animal diseases, *B. canis vogeli*, Colombia, 18S rRNA gene

Kolombiya, Cordoba'daki Köpeklerde *Babesia* spp.

Öz

Köpek babesiosisi (Canine babesiosis), *Babesia* cinsinin intraeritrositik protozoası tarafından oluşturulan ve dünya çapında yaygınlık gösteren kene kaynaklı bir hastalıktır. Bu çalışmada, Córdoba, Kolombiya'daki köpeklerde *B. canis vogeli*'nin moleküler tespiti ve hastalığın klinik özellikleri tanımlanmıştır. Kene kaynaklı hastalıklarla uyumlu klinik bulguları olan köpeklerden kan örnekleri alındı. Periferik kan örnekleri *Babesia* spp. merozoit ve trofozoitlerinin mevcudiyetini belirlemek amacıyla mikroskopik olarak incelendi. DNA ekstraksiyonu için juguler veya sefalik venipunktur ile elde edilen kan örnekleri kullanıldı. PCR analizi, *Babesia* spp. 18S rRNA genine yönelik primerler kullanılarak gerçekleştirildi. Babesiosis şüphesi olan 42 köpeğin 23'ü dişi ve 19'u erkek idi. Köpeklerin 42'sinin 23'ünde (%54.7), periferik kan muayenesinde *Babesia* spp. ile uyumlu intraeritrositik piroplazmalar saptandı. Morfometrik çalışma, %73'ünün (17/23) büyük *Babesia* (2.4x5 µm) ve %26'sının (6/23) küçük *Babesia* (1x3.2 µm) olduğunu göstermiştir. Kan örneklerinin 42'sinden 11'i *B. canis vogeli* için pozitif idi. Pozitif köpeklerde gözlenen klinik bulgular: anoreksiya %63 (7/11), uyuşukluk ve apati %63 (7/11), ateş %54 (6/11), soluk mukozal zarlar %54 (6/11), lenfadenomegali %18 (2/11), kusma %9 (1/11) ve ishal %9 (1/11). Kan sayımı, köpeklerin %70 (8/11)'inde anemi olduğunu gösterdi. Lökosit bozuklukları değişkendi; %27'sinde (3/11) lökositoz ve %46'sında (5/11) lökopeni vardı. 18S rRNA geninin sekanslanmış örnekleri çeşitli ülkelerden elde edilen *B. canis vogeli* sekansları ile %99 benzerlik göstermiştir.

Anahtar sözcükler: Hayvan hastalıkları, *B. canis vogeli*, Kolombiya, 18S rRNA geni

INTRODUCTION

Canine babesiosis is a tick-borne protozoa disease caused by different *Babesia* species. The disease produces hemolytic alterations of variable intensity, which include fever, lethargy, anorexia, anemia, hemoglobinuria, and may even

compromise other organ systems, thereby causing a wide variety of clinical signs ^[1]. The pathogenicity of the agent involved, the immune competence of the infected dog and the possibility of co-infections with other agents are the main factors that cause this variability in clinical presentation ^[2].



İletişim (Correspondence)



+57 034 7569027



mattarsalim@hotmail.com; smattar@correo.unicordoba.edu.co

At a global level, 12 piroplasm species capable of infecting dogs have been identified [2]; the identification is based on host specificity and the morphology of the intraerythrocytic forms [1]. However, this method fails to identify individually and accurately the species or subspecies that causes the infection, whereas the use of molecular techniques has allowed an accurate identification of the species and subspecies of *Babesia* and an understanding of their dynamics and distribution [3].

The species *Babesia canis* and *Babesia gibsoni* traditionally have been considered the only piroplasms that parasitize dogs. *Babesia canis* is classified as a large piroplasm and *B. gibsoni* as a small piroplasm, both have being reported in all five continents [2].

Currently, three subspecies of *B. canis* are recognized. *Babesia canis canis* is found in Europe and transmitted by *Dermacentor reticulatus*, *Babesia canis vogeli* is found in Europe, North Africa, America, Asia and Australia and is transmitted by *Rhipicephalus sanguineus*. *Babesia canis rossii* found in South Africa and North America is transmitted by *Haemaphysalis leachi* [4]. A new large *Babesia* sp. has also been reported in United States [5]. Within the small piroplasms, there are further reports of *B. gibsoni* on five continents. Moreover, recent molecular characterization has identified other small piroplasms, called *Theileria annae* endemic to Spain [6] and *Babesia conradae* reported in Southern California [7]. These species are morphologically identical, but have different vectors and variations in pathogenicity and clinical manifestations [8].

The aim of this study was to characterize the clinical presentation and molecular species of *Babesia* spp. collected from infected dogs in Cordoba, Colombia.

MATERIAL and METHODS

Dog and Blood Examination

Between November 2013 and December 2014, 42 dog blood samples were collected at the veterinary clinic of University of Cordoba and three private clinics. The dogs had clinical signs of ticks-borne disease, including fever, pale mucous membranes, lethargy, anemia, apathy, muscle tremors and hematuria and reported a diagnosis positive to *Babesia* spp. by microscopic examination with

Wright-stained blood smears.

Anticoagulated blood samples were collected from all dogs for complete blood counts. The blood was centrifuged, separating plasma from erythrocytes; the white cell layer was removed by pipetting, obtaining a concentrate of erythrocytes which was divided into two aliquots and stored at -90°C for further processing for nucleic acid extraction.

Samples of peripheral blood from the ear tip were taken from each animal to prepare thin smears. The slide smears were Wright-stained and examined under light microscopy (magnification 1000X) for detection of intraerythrocytic piroplasms. A scale to establish the degree of parasitemia was used: + (1-5 erythrocytes parasitized per blood smear), ++ (6-20 erythrocytes parasitized per blood smear), +++ (21-50 erythrocytes parasitized per blood smear) and ++++ (>50 erythrocytes parasitized per blood smear) [9]. The parasitic morphometry was determined using the Leica Application Suite version 3.1.1 software (LAS EZ, Leica Microsystems, Switzerland).

DNA Extraction

DNA from 200 µL of concentrated erythrocytes was extracted using the QIAmp blood kit (Qiagen, Chatsworth, CA) kit, according to the manufacturer's instructions. The extraction product was stored at -20°C.

Amplification and Sequencing

Amplification of the 18S ribosomal RNA (rRNA) gene of *Babesia* spp. was performed using primers as shown in Table 1. The PCR assay for each subspecies of *Babesia canis* PCR assay was evaluated using the following combinations of primer pairs: BAB1/BAB3 (*B. canis canis*), BAB1/BAB4 (*B. canis vogeli*) and BAB1/BAB5 (*B. canis rossii*) [10]. Amplification was carried out under the following conditions: an initial denaturation step at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 20 sec; a final extension step at 72°C for 5 min.

PCR for detection of *B. gibsoni* was performed using primers GIB599/GIB1270 [11] (Table 1) with the following conditions: initial denaturation step at 95°C for 2 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C

Table 1. Primers used for amplification for PCR *Babesia* spp.

Name	Description	Sequence (5'-3')	Product (pb)	Ref.
BAB 1	All <i>Babesia canis</i> spp.	GTGAACCTTATCACTTAAAGG (forward)		[10]
BAB 3	<i>B. canis canis</i>	CTACACAGAGCACACAGCC (reverse)	746	
BAB 4	<i>B. canis vogeli</i>	CAACTCCTCCACGCAATCG (reverse)	590	
BAB 5	<i>B. canis rossii</i>	AGGAGTTGCTTACGCACTCA (reverse)	342	
GIB599	<i>B. gibsoni</i>	CTCGGCTACTTGCTTGTC (forward)	665	[11]
GIB1270		GAAGCCGAAATAACGGC (reverse)		

for 30 sec and extension at 72°C for 90 sec; a final extension step at 72°C for 5 min.

Amplifications were performed using a programmable thermocycler. The PCR mixture contained 0,4N_M of each dNTP (Invitrogen, California), 2 mM MgCl₂, 50 mM KCl, 10 mM Tris - HCl pH 9.0 (GenTaq[®], Colombia), 0.4 mM each primer (Oligo Macrogen, Korea), 1.5 U polymerase GenTaq (GenTaq[®], Colombia) and 5 µL of DNA previously extracted, to a final volume of 25 µL.

The PCR products were visualized following electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.4 mg/mL) under UV transillumination. Results were captured and recorded using a digital imaging system. For sequencing, amplicons with high DNA concentration were selected. Both strands of amplified fragments were directly sequenced using ABI3730XL (Life Technologies, Applied Biosystems). The sequences were compared for similarity to sequences in GenBank, using the BLAST program hosted by NCBI, National Institutes of Health, USA <http://www.ncbi.nlm.nih.gov>. Alignments and phylogenetic analyses using the MEGA 6 software package were conducted [12]. A phylogenetic tree was constructed using the Neighbor-Joining model.

Statistical Analysis

The Chi-squared or Fisher's exact tests were used to compare proportions. Differences between independent groups were analyzed with the Mantel-Haenszel test [13]. Analyses were performed with SAS 9.1 software for Windows, with a probability (P) value <0.05 as statistically significant.

RESULTS

Epidemiological and Blood Smear Examination

Forty-two dogs suspected of having babesiosis were included; 23 were females and 19 males, with an age range of 12-84 months. There were 23 dogs from 11 defined breeds and 18 mixed. The most represented breed was Labrador (8/23).

Peripheral blood smear evaluation showed intraerythrocytic piroplasms morphologically consistent with *Babesia* spp. in 54.7% (23/42) of the clinically suspected dogs (Fig. 1). In the morphometric study, 73% (17/23) had dimensions corresponding to large babesias (2.4x5 µm) and 26% (6/23) were classified as small babesias (1x3.2 µm).

Babesia spp. PCR Analysis

For differentiation of the subspecies of *B. canis*, PCR was performed separately with each pair of primers. No amplification with primers for *B. canis canis* or *B. canis rossi* was detected. However, PCR with primer of *B. canis vogeli* yielded an amplicon for 26% (11/42) of the samples. No

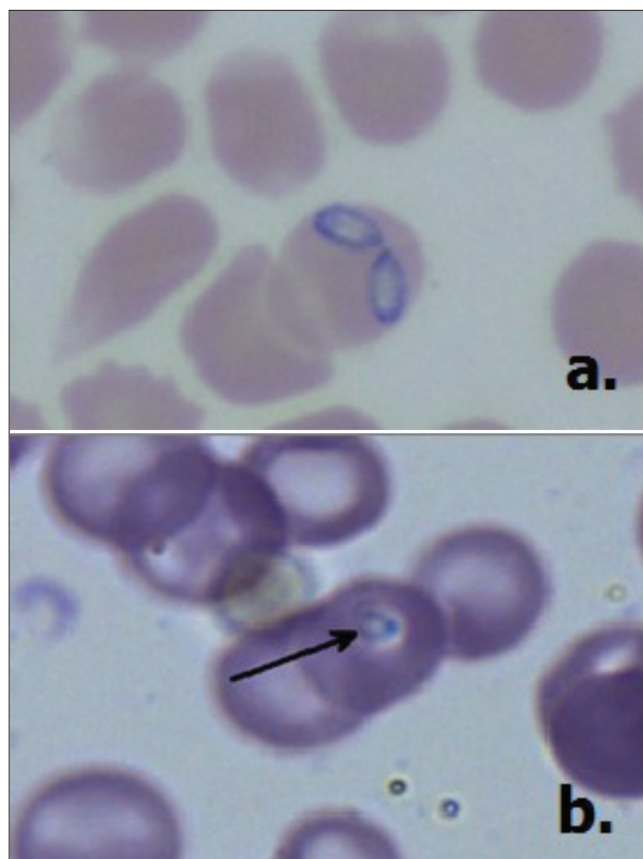


Fig 1. Intraerythrocytic forms of *Babesia* spp. observed in the extended blood of patients (1000x). a. Large *Babesia* sp. (4.4 µm x 2.2 µm); b. Small *Babesia* sp. (1.6 µm x 1 µm)

amplification with primers for the species *B. gibsoni* was detected.

Clinical and Pathological Findings in Dogs Infected with *B. Canis Vogeli*

Seven dogs were female and four were male. Ages ranged from 3 to 84 months, with three puppies and eight adults. The patients belonged to various breeds: mixed (4/11), Labrador (1/11), Schnauzer (1/11), Pitbull (1/11), Bullterrier (1/11), Beagle (1/11), Pug (1/11), Fila Brasileiro (1/11) (Table 2). Statistically significant differences for these variables (Fisher's exact test; P=0.72; P=0.45; P=0.08 respectively) were not found.

The clinical signs on physical examination of 11 dogs with *B. canis vogeli* were: anorexia 63% (7/11), lethargy and apathy 63% (7/11), fever 54% (6/11), pale mucous membranes 54% (6/11), lymphadenomegaly 18% (2/11), vomiting 9% (1/11) and diarrhea 9% (1/11). Statistically significant differences for the clinic signs were not found (P=0.02).

Complete blood counts showed that 70% (8/11) of the dogs had anemia, with a hematocrit value below the reference interval (37-45%). The leukocyte disorders were variable; 27% (3/11) of the patients had leukocytosis and 46% (5/11) had leukopenia (Table 2).

Table 2. Clinical and pathological findings in 11 dogs infected with *B. canis vogeli*

Dog ID/sex/age (m)	Breed	Clinical Signs	Hematological Abnormalities	Parasitemia Degree
25/F/36	Mixed	Anorexia, lethargy and apathy, pale mucous membranes, vomiting	Anemia	+
26/M/24	Mixed	Fever, anorexia, lethargy and apathy, pale mucous membranes	Anemia, thrombocytopenia	++
27/F/4	Fila Brasileiro	Anorexia, lethargy and apathy, pale mucous membranes, demodicosis	Anemia, leukocytosis	+++
30/F/3,5	Bulterrier	Lymphadenomegaly and demodicosis	Thrombocytopenia	++
31/F/24	Mixed	Fever, anorexia, lethargy and apathy	Leukopenia, thrombocytopenia	++
32/F/3	Schnauzer	Fever, anorexia, lethargy and apathy, pale mucous membranes,	Anemia, leukopenia	++++
34/F/48	Mixed	Pale mucous membranes	Anemia, leukocytosis	+
37/M/30	Pug	Fever, lethargy and apathy, pale mucous membranes	Anemia, leukopenia	++
38/F/48	Beagle	Anorexia, lethargy and apathy, diarrhea	Leukopenia, thrombocytopenia	+++
41/M/84	Piltbull	Health check for infertility	Anemia, leukopenia, thrombocytopenia	+
43/M/48	Labrador	Fever, anorexia, lymphadenomegaly	Anemia, leukocytosis, thrombocytopenia	+

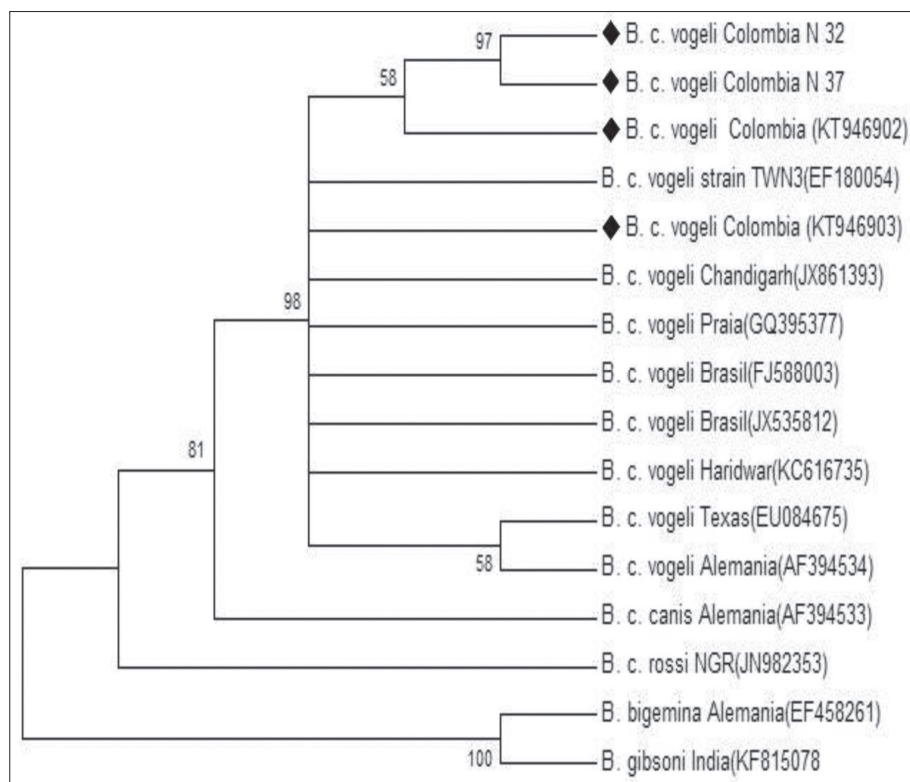


Fig 2. Phylogenetic analysis showing the position of the group of *B. canis vogeli* found in this study, obtained by comparison of partial sequence of the 18S rRNA gene with the sequences of *Babesia* spp. available in GenBank (accession numbers in parentheses). The neighbor-joining method was used, based in the kimura 2-parameter model. The numbers on the tree indicate bootstrap values for branch points (500 replications). The evolutionary analysis was made with the MEGA 6 program^[12]

Phylogenetic Analysis

Five of the 11 *B. canis vogeli* samples were sequenced and compared with the sequences available in the GenBank database. The samples showed 99% identity with the sequences of *B. canis vogeli* Chandigarh (India, GenBank JX861393), Praia (Cape Verde, GenBank GQ395377), Recife (Brazil, GenBank FJ588003), Taiwan (China, GenBank EF180054), Brazil (GenBank JX535812), Haridwar (India, GenBank KC616735) and Texas (GenBank EU084675).

Sequence analysis of *B. canis vogeli* found in this study grouped with *B. canis vogeli* sequences available in GenBank with high bootstrap levels of 98% (Fig. 2). Two *canis vogeli* strain Colombia 18S rRNA partial sequence were deposited in GenBank database under access numbers KT946902 and KT946903.

DISCUSSION

This study demonstrated for the first time in Córdoba, that

canine babesiosis is mostly caused by large piroplasms (73%). Molecular characterization confirmed that *B. canis vogeli* is the most common cause of babesiosis, and also showed high genetic similarity with *B. canis vogeli* in other countries. However, in 6 of 42 patients the morphological study showed characteristics of small *Babesia* sp. *B. gibsoni* is a small *Babesia* transmitted by *R. sanguineus* and reported to affect dogs in South America; it has been reported in countries like Brazil [14] and recently in Nicaragua [15]. In this study, there was no evidence of its presence and there are no reports in Colombia. However, its existence cannot be excluded due to the presence of the vector.

Clinical manifestations of *B. canis* in patients vary depending on the subspecies involved [1]. In the present study, only *B. canis vogeli* was found and patients presented with variable clinical signs; fever ($T > 39.5^{\circ}\text{C}$) (6/11), anorexia (7/11), lethargy and apathy (7/11), pale mucous membranes (6/11). The clinical variability presentation of the diseases in the present study is consistent with other studies, in which the same subspecies of *Babesia* was detected [8,16,17].

In the present study *B. canis vogeli* was frequently found in adults; however, higher levels of parasitemia were observed in the three puppy patients (≤ 1 year), confirming the increased susceptibility of these to the *Babesia* spp. [1,18]. This result is similar to reported by Solano Gallego in Italian puppies (1-2 months); in four of 11 dogs with *B. canis vogeli*, the puppies developed severe hemolytic anemia with fatal outcome for two puppies [8].

The clinicopathological data of dogs infected with *B. canis vogeli* of this study were similar to those reported by other authors, without observing a homogenous clinicopathological pattern as reported by Solano et al. [8] and Cardoso et al. [4] in their studies in dogs in Italy and Portugal, respectively. In the present study, most patients had alterations erythrocyte (73%: 8/11), similar results were found by Carli et al. [19] (67%). However, our results were lower than those reported by Solano Gallego et al. [8] (93%) in dogs of Italy.

Alterations in the white cell profiles, as leukocytosis and leukopenia, have been described irregularly in babesiosis, with leukopenia being more common. In this study, 46% of patients had leukopenia, similar to that reported by [1,8,9,19]. Leukocytosis in babesiosis is less frequent [1]; in our study it was observed in 27% (3/11) of patients. However, it has also been reported in other studies worldwide [8,9,20]. Leukopenia is a component of the systemic inflammatory response syndrome (SIRS) described in babesiosis and it is included in the criteria for the diagnosis of sepsis in dogs [9].

Thrombocytopenia is a typical finding in canine babesiosis; immune-mediated platelet destruction, co-infections and sequestration of platelets in the spleen are possible mechanisms [1,2]. In this study, 46% of the positive patients showed thrombocytopenia; similar findings have been

reported in other studies worldwide, such as Ruiz de Gopegui et al. [20] in Italy and Inokuma et al. [11] in Japan, with 100% and 60% thrombocytopenia respectively. The results reported for these authors are higher than those in this study. However, Solano Gallego et al. [2] reported a 36% thrombocytopenia in dogs in Italy, lower than the value found in our study.

B. canis vogeli is the most globally distributed subspecies of *Babesia*; it is found in Africa [21,22], Europe [4,16], Asia [11,23] and Australia [24,25]. In South America, *B. canis vogeli* has been reported in Colombia [26], Venezuela [17], Brazil [27,28] and Argentina [29]. This discovery is supported by the geographical distribution of tick *R. sanguineus* as vector, being the Ixodidae species most widely distributed in domestic dogs in different regions of the country [30].

The presence of *B. canis vogeli* and absence of *B. canis rossi* and *B. canis canis* in naturally infected dogs in Colombia contrasts with the results reported by Düzlü et al. [31], where *B. canis canis* (12.0%) and *B. gibsoni* (9.0%) were the most prevalent *Babesia* species in blood samples of dogs in Turkey, *B. canis vogeli* (2.3%) was found in lower proportion.

B. canis vogeli sequences found in this study, are closely related to isolates from Asia (India), Africa (Praia), North America (United States) and South America (Brazil). Despite the proximity with Venezuela, *B. canis vogeli* sequences found in the present study could not be compared with isolates reported in Venezuela [17]. The same is true for isolates reported by Vargas et al. [26] in the central area of Colombia, because the amplified gene fragment of 18S ribosomal does not correspond to the amplified fragment of this study.

In conclusions, the situation of the absence of others species or subspecies of *Babesia* may change in the future due to different factors. These include climate change, especially global warming and anthropogenic factors such as permanent travel companion animals, the resistance of vectors and pathogens to products and medications for control as well as failures in treatment and prevention. These factors are responsible for the spread of tick borne diseases to non-endemic areas.

This work demonstrated for the first time in the Caribbean the presence of *B. canis vogeli* in dogs and the clinical and epidemiological characteristics associated with infection by this hemoparasite. The molecular identification was extremely valuable as a tool for epidemiological analysis, allowing the inference that there are other species in the Caribbean that require more extensive studies in the region both in dogs and other domestic species.

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STATEMENT OF ANIMAL RIGHTS

This article does not contain any studies with animals performed by any of the authors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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