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HELMINTHOLOGY - ORIGINAL PAPER

Molecular characterization of canine filarioids in a previously non-endemic area of Rio de Janeiro State, Brazil

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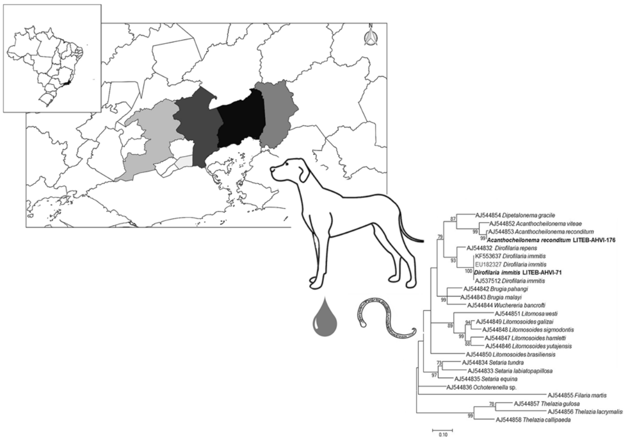
Abstract

Dirofilaria immitis is the causative agent of canine heartworm disease, a severe health problem in dogs, especially in coastal areas of tropical and subtropical regions of the world. We employed molecular methods to investigate the occurrence of canine infection by filarioids in five municipalities of Baixada Fluminense (Magé, Duque de Caxias, Guapimirim, Nova Iguaçu, and São João de Meriti), a non-endemic area of Rio de Janeiro State, Southeast Brazil. A total of 110 canine blood samples collected from 2017 to 2018 and positive for microfilariae at the modified Knott's test were screened by cPCR targeting DNA fragments of the 12S rDNA gene for filarial nematodes. Seventy-seven samples (70%) tested positive at the molecular analysis. Of these, 72 were identified as *D. immitis* and 5 (4.5%) as *Acanthocheilonema reconditum*. *Dirofilaria repens* was not detected in the studied municipalities of Baixada Fluminense. This is the first record of *D. immitis* and *A. reconditum* in the Baixada Fluminense region, Rio de Janeiro State, Brazil. The prevalence of *D. immitis* cases in the five municipalities suggests the establishment and maintenance of its enzootic cycle in the studied region, which indicate vulnerability to the occurrence of epidemic cycles and, possibly, human cases.

Graphical abstract



Keywords *Dirofilaria immitis* · *Acanthocheilonema reconditum* · Heartworm disease · Parasitic zoonosis · 12S rDNA · Molecular diagnosis

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Introduction

Dirofilariasis is a disease caused by filarioids of the genus *Dirofilaria* (Spirurida: Onchocercidae), which are capable of infecting more than 30 species of vertebrate hosts. The genus includes two species of greater medical and veterinary importance: *Dirofilaria immitis* (Leidy, 1856) and *Dirofilaria repens* Raillet and Henry, 1911 (Acha e Szyfres 2003; AHS 2020; Alberigi et al. 2020; Mendes-de-Almeida et al. 2021).

Among other filarioids of veterinary importance that infect domestic and wild canids (Ionică et al. 2017), *Acanthocheilonema reconditum* (Spirurida: Oncochercidae) occurs mainly in European countries (Pacifico et al. 2021). Transmission occurs by apteral vectors, that is, fleas (*Ctenocephalides felis*), where the life cycle in these intermediate hosts has been well established (Napoli 2014). The adult worm parasitizes the subcutaneous tissue, but with little clinical importance (Hoseini et al. 2020).

Human pulmonary dirofilariasis (HPD), caused by *D. immitis*, is a zoonosis that has been reported in different continents, with more than 50 cases recorded in Brazil (WHO 1979; Rodrigues-Silva et al. 1995, 2004; Campos et al. 1997; Cavallazi, et al. 2002; Araújo et al. 2019; Doutrário et al. 2019). Another species of medical importance, *D. repens*, is the main agent of human subcutaneous/ocular dirofilariasis, with diagnosed cases in the old world (Benzaquen et al. 2015; Maggi and Krämer 2019), although the species has already been detected in dogs and coatis (*Nasua nasua*) in Brazil (Lentz and Freitas 1937; Moraes et al. 2017).

Dirofilaria immitis and *D. repens* are mosquito-borne filarioids. The maintenance of their transmission is associated with geographical and climatic conditions, anthropic action, density of susceptible domestic dogs, and vector density (Simón et al. 2017). Their main transmitters are species of the culicid genera *Culex*, *Aedes*, *Ochlerotatus*, and *Anopheles* (Genchi et al. 2009; Silva e Langoni 2009; Klinge, et al. 2011; Simón et al. 2017; Maggi and Krämer 2019). The mosquitoes *Ochlerotatus taeniorhynchus* (Widemann, 1841) and *Ochlerotatus scapularis* (Rondani, 1848), both exophilic and predominantly wild, have already been identified as primary vectors of *D. immitis* in the coastal region of Rio de Janeiro State (RJ), Southeast Brazil (Labarthe et al. 1998; Labarthe and Guerrero 2005; Bendas et al. 2019).

Cases of canine dirofilariasis have recently been observed by veterinarians who perform clinical care in Baixada Fluminense, an area of RJ regarded as non-endemic for *D. immitis* (Vieira 2019). The region comprises a large area of permanent environmental conservation that includes areas where water collects, such as waterfalls and rivers, making it a favorable environment for the breeding of wild vector culicids. In addition, the areas outside these reserves are densely populated, many with socio-environmental vulnerability (IBGE 2010), which concentrates a canine population without preventive care, thus favoring the transmission and maintenance of parasite and, consequently, cases of HPD (Rodrigues-Silva et al. 2004; Garcez et al. 2006). Here, a new molecular data set was generated for filarial nematodes detected in canine blood samples from the municipalities of Baixada Fluminense (RJ) and used to present phylogenetic inferences from the comparison of the mitochondrial small-subunit ribosomal RNA gene (12S rDNA).

Material and methods

Sample screening

A total of 10,031 dogs from the municipalities Nova Iguaçu (22° 45′33″S; 43° 27′04″W), Magé (22° 39′10″S; 43° 02′26″W), Guapimirim (22° 32′14″S; 42° 58′55″W), Duque de Caxias (22° 47′08″S; 43° 18′42″W), and São João de Meriti (22° 48′14″S; 43° 22′22″W) of Baixada Fluminense (RJ) and who received veterinary medical care between 2017 and 2018 were, regardless of clinical signs or suspicion of heartworm, carefully inspected in a clinical analysis laboratory. All canine blood samples were subjected to filarioid survey by rapid immunochromatography, immunoenzymatic assay (ELISA), modified Knott's concentration test (Newton and Wight 1956), and routine blood counts. Screening tests found 286 blood samples to have filarial nematodes detected by at least one of the above tests. Of these, a total of 110 microfilaremic blood samples were stored at − 20 °C in a tube containing EDTA for subsequent molecular analysis.

Molecular survey

Blood samples (200 µl) were used for DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's protocol. Freshly purified DNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Multiplex polymerase chain reaction (PCR) for filarid research was performed using two primer sets in the same reaction, one pair for detecting the 12S rDNA gene of filarial nematodes (12SF and 12SR) (Casiraghi et al. 2004), one specific for *D. immitis* (12SF2B) (Gioia 2010) and one specific for *D. repens* (12SR2) (Gioia 2010), according to a previously established protocol (Simsek and Ciftci 2016). The analyses included 300 ng of *D. immitis* DNAg reactions as positive controls and DNA-free samples as negative controls. Amplified DNA products were electrophoresed through a 2% agarose gel, stained with ethidium bromide, and examined by UV transillumination. Amplicons of the expected sizes were purified using Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced in an automated ABI 3730xl DNA analyzer (Applied BioSystems) applying BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

DNA sequences were edited with SeqMan II Expert Sequence Analysis Software 5.00 (DNASTAR package, Lasergene) and identity values were obtained by BLAST analysis of sequences published in GenBank. The obtained 12S rDNA sequences were aligned using Clustal W (Thompsonet al. 1994) and the maximum-likelihood tree was inferred using MEGA 7.0 software (Kumar et al. 2016) with TN93 + G + I correction model, selected by Bayesian Information Criterion. The reliability of the tree topology was evaluated using bootstrap support (1000 repeats).

Data analysis

General microfilariae prevalence for the five municipalities was calculated from the analysis of 10,031 reports of dogs submitted to at least one of the filarid survey tests. Molecular identification of filarial nematode species through multiplex PCR and sequencing of canine blood samples was expressed in simple frequency using R software (version 4.0.2) considering a 95% confidence interval.

Results

The overall prevalence of microfilaremic dogs in the five studied municipalities was 2.85% (286/10,031) (Table 1).

Most samples selected for molecular biology tests were from the municipality of Magé (51%; 56/110), followed by Duque de Caxias (40%; 44/110), Guapimirim (5. 4%; 6/110), São João de Meriti (1. 8%; 2/110), and Nova Iguaçu (1.8%; 2/110). After PCR assays, the 12S rDNA fragment was successfully amplified in 70% (77/110) of the analyzed samples, and nucleotide sequences of all PCR-positive samples were obtained (Table 2).

Table 2 Frequency of *D. immitis* and *A. reconditum* in dogs from Baixada Fluminense, Rio de Janeiro State, Brazil according to PCR and sequencing result

Municipalities	*n*	PCR-positive *n* (%)	Sequencing	
			D. immitis *n* (%)	*A. reconditum* *n* (%)
Guapimirim	6	4 (5.2)	3 (4.2)	1 (20.0)
São João de Meriti	2	1 (1.3)	1 (1.4)	-
Nova Iguaçu	2	2 (2.6)	1 (1.4)	1 (20.0)
Magé	56	37 (48.0)	35 (48.6)	2 (40.0)
Duque de Caxias	44	33 (42.9)	32 (44.4)	1 (20.0)
Total	110	77 (100.0)	72 (100.0)	5 (100.0)

n, canine population; %, relative frequency

Pairwise homology analysis revealed that 65.4% (72/77) of the obtained sequences were identical to each other and showed maximum homology (475/475, 100%) (Table 2) with *D. immitis* DNA sequences available at GenBank, which were from Australia (AJ537512), China (EU182327, KF707476,78–82), and French Guiana (MT252014–24). Also, 4.5% (5/77) (Table 2) of the sequences obtained were identical to each other and had 99%-similarity (464/467) with *A. reconditum* originally isolated from French Guiana (MT252011). The species *D. repens* was not detected in the dog samples from the studied municipalities of Baixada Fluminense.

Nucleotide sequence data reported in this paper are available in the GenBank™ databases under the accession numbers: MZ678855–MZ678931.

As the nucleotide sequences of *D. immitis* obtained here were identical, the sequence of one sample (code: LITEB-AHVI-71) was used in the phylogenetic reconstruction as a representative of the others ($n = 72$). Likewise, one sample (code: LITEB-AHVI-176) of the sequences of *A. reconditum* was used as a representative of the others ($n = 5$) in the phylogenetic reconstruction (Figs. 1 and 2).

Table 1 Prevalence of canine filarioids in 5 municipalities in the Baixada Fluminense, in the years 2017 and 2018

Municipalities	2017			2018			Total		
	n	Events	Prevalence (%)	n	Events	Prevalence (%)	n	Events	Prevalence (%)
Guapimirim	877	14	1.6	495	6	1.21	1372	20	1.46
São João de Meriti	438	1	0.23	229	-	-	667	1	0.15
Nova Iguaçu	31	-	-	195	2	1.03	226	2	0.88
Magé	1050	78	7.43	846	78	9.22	1896	156	8.23
Duque de Caxias	2957	48	1.62	2913	59	2.03	5870	107	1.82
Total	5353	141	2.63	4678	145	3.1	10,031	286	2.85

n, canine population

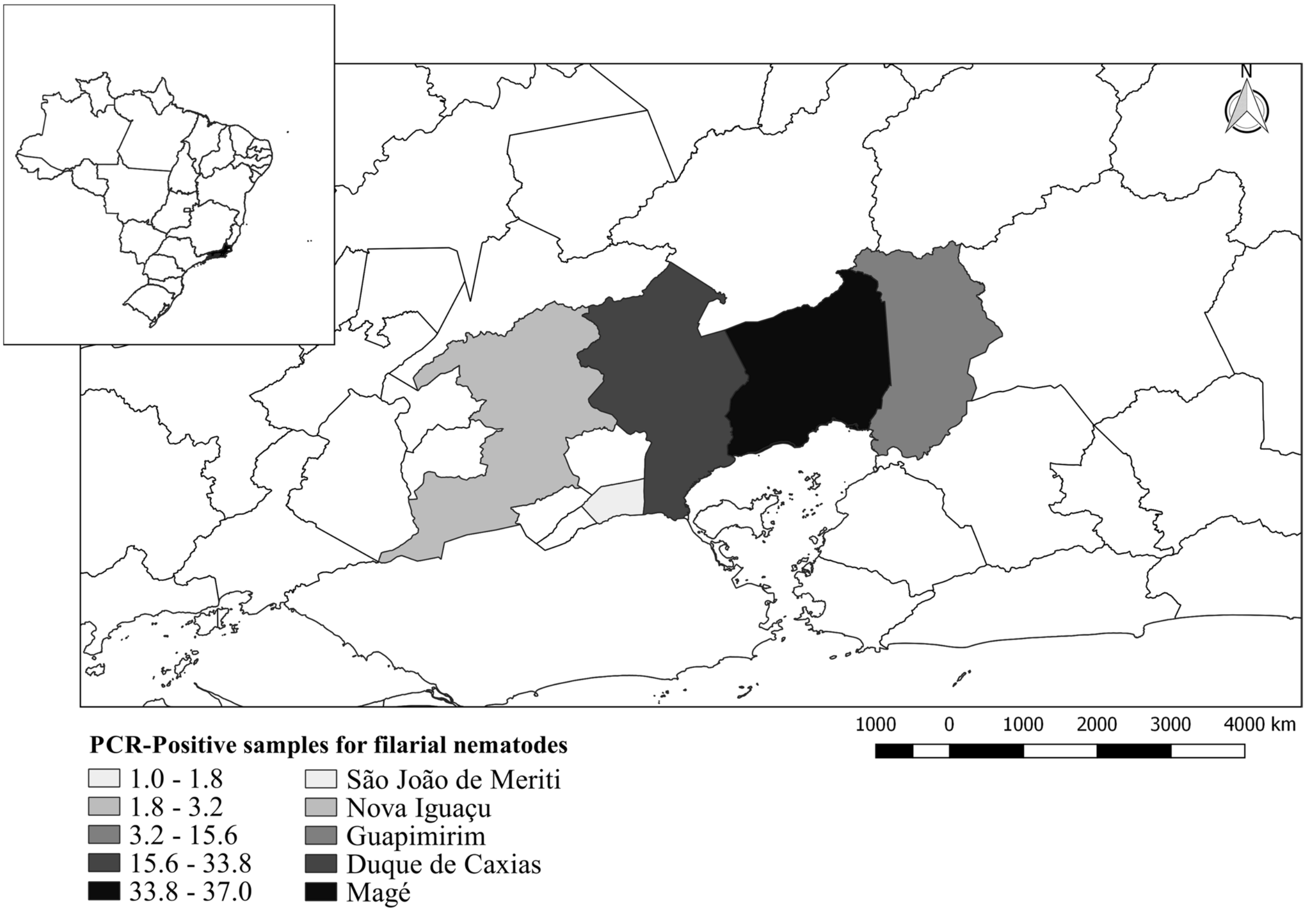

Fig. 1 Frequency of dogs infected with filarioids in Baixada Fluminense, Rio de Janeiro State, Southeast Brazil. Subtitle: The PCR-positive samples for the 12S rDNA gene from five municipalities of the Baixada Fluminense are presented in grayscale, according to the concentration

Phylogenetic analysis of the partial sequences of the 12S rDNA (Fig. 2) grouped sequences of samples from all the five municipalities investigated with the *D. immitis* cluster (100% bootstrap) and other sequences from Duque de Caxias, Guapimirim, Magé and Nova Iguaçu municipalities with *A. reconditum* (99% bootstrap).

Discussion

To our knowledge, the present study provides the first report of *D. immitis* isolated from dogs in a non-endemic area of Rio de Janeiro State, Southeast Brazil, which has favorable climatic conditions for the proliferation of the parasite (IBGE 2010).

Several clinical and molecular studies have shown that the public health significance of filarial worms has increased worldwide (Theis 2005; Pampiglione et al. 2009; Dantas-Torres and Otranto 2013; Benzaquen et al. 2015; Cabrera et al. 2018; Bendas et al. 2019), mainly due to the large number of people affected and undergoing treatment for infection (Dantas-Torres and Otranto 2020). The nematode *D. immitis* is a well-known zoonotic parasite and the most widespread filarial worm infecting dogs throughout the USA (Dantas-Torres and Otranto 2013, 2020). Indeed, this species has been frequently reported as involved in canine dirofilariosis in endemic areas of Brazil (Bendas et al. 2017).

The phylogenetic topology showed that most of the worms from dogs of Baixada Fluminense gathered together in one clade, comprising *D. immitis* isolates from Australia, China, and Brazil in the related branches, with high bootstrap support. These findings demonstrate the low genetic variability that exists among *D. immitis* isolates, corroborating other molecular studies (Otranto et al. 2011; Silva et al. 2019).

There has been no previous description of the occurrence of dirofilariasis in the municipalities of the Baixada Fluminense, which is considered a non-endemic region for canine heartworm disease. However, the molecular techniques used here confirmed the presence of *D. immitis* in this region, and by presenting socioeconomic, demographic, and environmental vulnerability factors (IBGE 2010; Rocha

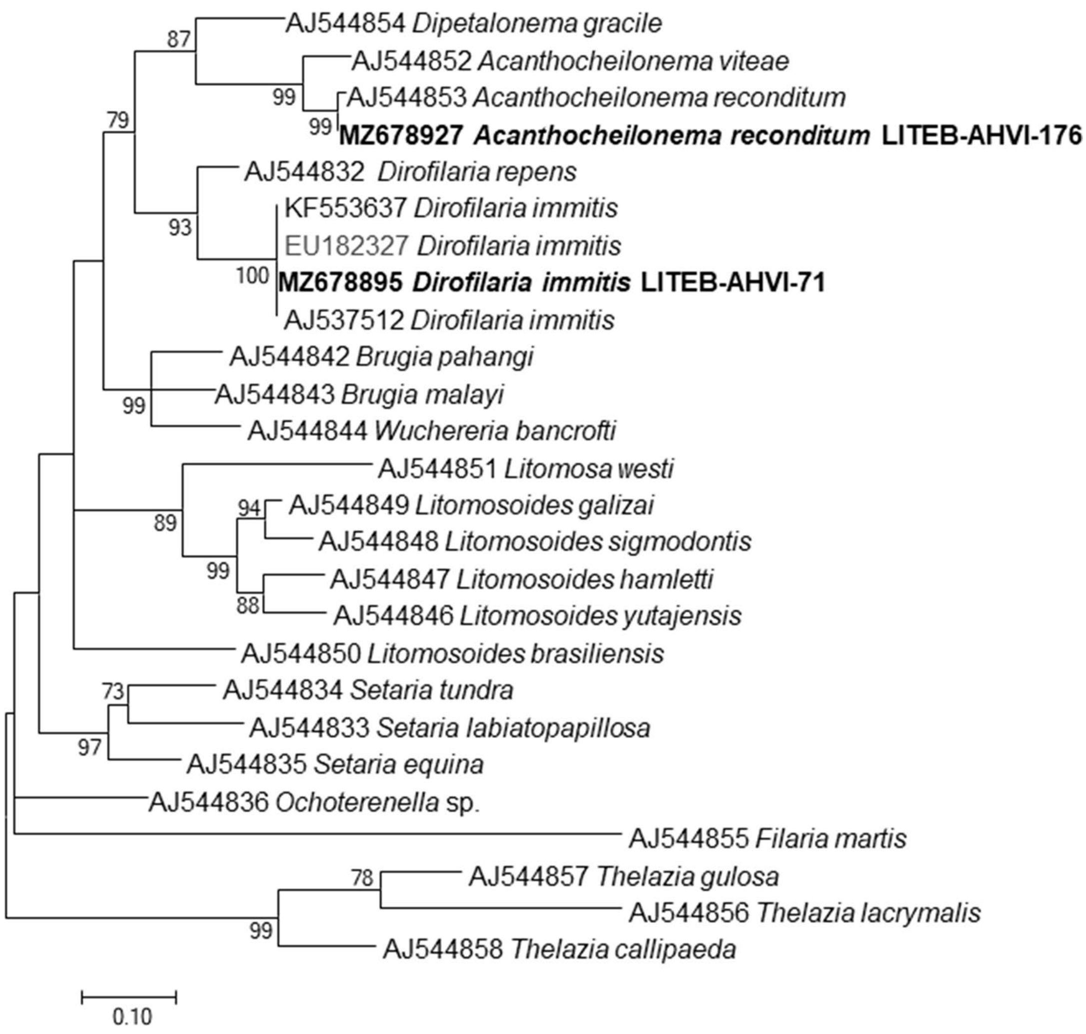

Fig. 2 Phylogenetic inference based on the 12S rDNA gene fragment of the filarioids and related nematodes. Subtitle: The phylogenetic construction was conducted using maximum likelihood from 1000 replicated trees. Evolutionary distances were estimated by the TN93 + G + I model. Bootstrap values > 70% are shown. GenBank accession numbers precede the sequence names and the sequences obtained from *Canis familiaris* blood samples from Baixada Fluminense, Rio de Janeiro, are highlighted in bold. Scale bar indicates nucleotide substitutions per site

and David 2015), these locations have the potential for the emergence of human dirofilariasis. Previous studies have shown a decreasing prevalence of canine heartworm disease, from 7.9% in 1988 to 2% in 2001 (Labarthe et al. 2003; Costa et al. 2004; Labarthe and Guerrero 2005), due to chemoprophylactic measures performed by veterinarians. However, the epizootiological panorama has changed in recent years with it being considered a re-emerging disease (Bendas et al. 2019) since the last survey carried out on the Brazilian coast showed an overall seroprevalence of 23.1% while the prevalence for coastal regions of RJ, which are considered endemic for heartworm, ranged between 16.3 and 62.2% (Labarthe et al. 2014).

Although *D. repens* was not detected in the present study, it is important to continue the investigation in the Baixada Fluminense region since only a few morphological studies have reported the occurrence of this species throughout the Brazilian territory (Lentz and Freitas 1937; Moraes et al. 2017). New molecular studies will allow the differentiation of species of nematodes (Latrofa et al. 2012; Argôlo et al. 2018), the analysis of parasite circulation, and investigation into whether cases can be autochthonous.

The 12S rDNA fragment of *A. reconditum* was sequenced in 4.5% (5/77) of PCR-positive samples, representing four of the five investigated municipalities (Table 2). Although this filarioid is generally regarded as non-pathogenic to animals (Knight 1987), it has been reported parasitizing the subcutaneous tissue of domestic and wild dogs and hyenas in Africa, India, and North and South America, with flea and lice species acting as vectors (Torres and Figueredo

2007; Napoli et al. 2014). However, there was also a report of *A. reconditum* parasitizing a human eye (Huynht, et al. 2001; John et al. 2012), with dubious claims about possible zoonotic potential (Napoli et al. 2014). In Brazil, this species has been detected by molecular assays only in Pará State in the North Region of the country (Argôlo et al. 2018). For now, it is important to study the prevalence of wingless vectors of this filarioid to better determine its abundance and establish prophylactic guidance to dog owners for the control of these ectoparasites, aiming at the welfare and health of their animals.

Conclusion

Dirofilaria immitis was the most frequently identified species in the present study, although *A. reconditum* was also documented. This is the first record of *D. immitis* in the Baixada Fluminense region, Rio de Janeiro State, Brazil. The prevalence of cases of *D. immitis* in the five studied municipalities is the establishment and maintenance of its enzootic cycle in the studied region, which indicates vulnerability for the occurrence of epidemic cycles and, possibly, human cases.

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Author contribution Designed the study: Viviane Marques de Andrade Vieira, Nicole Oliveira Moura Martiniano, Gilberto Salles Gazêta, Antonio Henrique Almeida de Moraes Neto; collected and analyzed clinical data from the animals: Viviane Marques de Andrade Vieira, Priscila Pinho da Silva, Norma Labarthe, Erica Tex Paulino, Gilberto Salles Gazêta, Antonio Henrique Almeida de Moraes Neto; performed the screening blood analysis: Priscila do Amaral Fernandes, Viviane Marques de Andrade Vieira; performed the molecular and phylogenetic analyses: Viviane Marques de Andrade Vieira, Nicole Oliveira Moura Martiniano; interpreted the results and wrote the article: Viviane Marques de Andrade Vieira, Nicole Oliveira Moura Martiniano, Gilberto Salles Gazêta, Antonio Henrique Almeida de Moraes Neto, Erica Tex Paulino; reviewed and edited the final version of the manuscript: Viviane Marques de Andrade Vieira, Nicole Oliveira Moura Martiniano, Gilberto Salles Gazêta, Antonio Henrique Almeida de Moraes Neto; supervise: Gilberto Salles Gazêta, Antonio Henrique Almeida de Moraes Neto.

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Data Availability The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request. The nucleotide sequences of the dataset generated here are available on the Genbank website (https://www.ncbi.nlm.nih.gov/nucleotide/) with the accession numbers: MZ678855-MZ678931.

Code availability Not applicable.

Declarations

Ethics approval The study was approved by the Animal Use Ethics Committee of the Oswaldo Cruz Institute/Oswaldo Cruz Foundation (CEUA-IOC-L009/2020) and by the Oswaldo Cruz Institute/Oswaldo Cruz Foundation Human Research Ethics Committee (CEP CAAE: 30759620.1.0000.5248).

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest Norma Labarthe is a consultant for Boehringer Ingelheim, Idexx, and Zoetis in Brazil. Viviane Marques de Andrade Vieira, Nicole Oliveira Moura Martiniano, Priscila do Amaral Fernandes, Priscila Pinho da Silva, Érica Tex Paulino, Gilberto Salles Gazêta and Antonio Henrique Almeida de Moraes Neto declare that they have no competing interests.

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