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Molecular Diagnosis of Hemoparasitis in dogs attended at the Veterinary Hospital of the Universidade Santo Amaro, São Paulo, SP, Brazil

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ABSTRACT

OBJECTIVE

Detection of *Ehrlichia canis, Rickettsia rickettsii, Anaplasma platys, Rangelia vitalii,* and *Babesia canis vogeli* using real-time PCR in dogs treated at the veterinary hospital of the Universidade Santo Amaro, located in the south zone, in the city of São Paulo, SP, Brazil.

METHODS

DNA was extracted from 63 whole blood samples using the "PureLink Genomic DNA" extraction kit (Invitrogen®) according to the manufacturer's instructions and real-time PCR was performed to detect *Ehrlichia canis, Rickettsia rickettsii, Anaplasma platys, Rangelia vitalii,* and *Babesia canis vogeli*.

RESULTS

In total, 23.8% (15/63) of the samples were positive by real-time PCR for at least one pathogen. Of these, 9.52% (6/63) were positive for *Babesia canis vogeli* and 14.2% (9/63) for *Ehrlichia canis*. No samples were positive for *Rickettsia rickettsii, Rangelia vitalii*, and *Anaplasma platys*.

CONCLUSIONS

This study demonstrated, in an unprecedented way, the presence of *B. canis vogeli* and *E. canis* in dogs from fragmentation areas of the Atlantic Forest around the Guarapiranga Reservoir in the city of São Paulo, expanding knowledge on the dispersion of this agent in Brazil.

DESCRIPTORS

Ehrlichia canis, Rangelia vitalii, Babesia canis vogeli, Rickettsia rickettsii, Anaplasma platys.

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INTRODUCTION

Arthropods and their associated hemoparasitoses[1] have expanded to different regions of the world, due to climate change and the ensuing access of these parasites to new environmental habitats[2]. Thus, the molecular diagnosis of these pathogens in the studied geographical area provides important epidemiological data for the prophylaxis, control, and treatment of diseases[2].

Among the clinical manifestations that affected dogs may present are hyperthermia, weight loss, lethargy/apathy, mucous pallor, lymphadenopathies, diffuse hemorrhages, hematuria, jaundice, and petechiae, possibly progressing to death[3],[4]. The most common laboratory abnormalities are thrombocytopenia, hemolytic anemia, leukocytosis or leukopenia, hyperglobulinemia, hypoalbuminemia, hemoglobinuria, and proteinuria[3],[4].

Among the agents that cause canine hemoparasitoses are *Anaplasma platys, Babesia canis and Babesia gibsoni, Ehrlichia canis, Rangelia vitalii*, and *Rickettsia spp.*

The species *Anaplasma* platys belongs to the Order *Rickettsiales*, Family *Anaplasmataceae* and Genus *Anaplasma*[5]. These are obligate intracellular gram-negative bacteria of platelets, and are responsible for the development of a clinical condition called canine cyclic thrombocytopenia[6]. The *A. platys* vector remains uncertain, but there are several occurrences of co-infections with *Ehrlichia canis* and *Babesia canis*, suggesting the involvement of the tick *Rhipicephalus sanguineus* s.l.[7],[8],[9].

Brazilian canine Babesiosis is a disease whose vectors are ticks of the species *Rhipicephalus sanguineus* s.l., being caused by hematozoa that preferentially parasitize young erythrocytes[10]. The species *Babesia canis* and *Babesia gibsoni* are the etiologic agents of the disease[11], [12]. Three subspecies of *B. canis* are known: *B. canis* transmitted by *Dermacentor reticulatus* in Europe; *B. canis vogeli*, transmitted by *Rhipicephalus sanguineus* s.l. in tropical and subtropical regions; and *B. canis rossi*, transmitted by *Haemophysalis leachi* in South Africa[13].

Regarding the epidemiology of Babesia canis subspecies in Brazil, B. canis vogeli is the most commonly diagnosed in dogs, when compared to other subspecies, regardless of the age or breed of the animal[14]. However, there are recent molecular studies carried out in the country reporting cases of the disease caused by B. gibsoni[15].

Bacteria of the genus *Ehrlichia* are gram-negative obligate intracellular parasites that parasite hematopoietic cells, such as monocytes, macrophages, and neutrophils. In different parts of the world, including the American continent, the *R. sanguineus* tick is the main, if not the only, vector of the bacterium *Ehrlichia canis*, the etiological agent of Canine Monocytic Ehrlichiosis (CME)[7]. In Brazil, there has been an increasing number of cases of CME in hospitals and veterinary clinics, being considered, by many, as one of the most important communicable diseases in small animal clinics[16],[17].

The protozoan *R. vitalii* belongs to the Order *Piroplasmorida* and infects erythrocytes and endothelial cells of canids[18]. In a recent work carried out by Soares[19], it was reported that the species A. *aureolatum* demonstrated vector competence for *R. vitalii*, as it was able to acquire and transmit the agent between domestic dogs, differently from the species *A. ovale, A. tigrinum, A. cajennense*, and *R. sanguineus*.

The *Rickettsia* genus comprises obligate intracellular gram-negative bacteria, some with zoonotic potential in different parts of the world[31]. *R. rickettsii* is considered the most pathogenic species[33], being reported in different countries like the United States of America (USA), Mexico, Canada, Costa Rica, Panama, Colombia, Argentina, and Brazil[20]. Ticks of the genus *Amblyomma* are the vectors of Brazilian Spotted Fever (BSF), with *A. sculptum* (formerly *A. cajennense*), *A. aureolatum*, and *A. ovale* (vector of *R. parkeri*) considered the most important species in the transmission of the disease[21,22].

Knowledge of the most frequent pathogens in the studied region, as well as the association with the vectors most commonly found in this type of ecosystem, will aid in the direction of a definitive diagnosis and collaborate with the maintenance of the health and social good of the human community that resides in the area. In view of these factors, the current work aimed to perform molecular diagnosis regarding the presence of *Ehrlichia canis, Rickettsia rickettsii, Anaplasma platys, Rangelia vitalii*, and *Babesia canis vogeli* in dogs treated at the veterinary hospital of the Universidade Santo Amaro, located in the south zone of the city of São Paulo, SP.

METHODS

Whole blood was collected between January and December 2017 from 63 dogs treated at the Veterinary Hospital of the Universidade Santo Amaro (UNISA), resident of the south zone of the city of São Paulo, in fragmentation areas of the Atlantic forest around the Guarapiranga Reservoir (23°42'0"S; 46°42'0"W)[23]. The study was approved by the Animal Use Ethics Committee of Universidade Santo Amaro (CEUA 17/2018).

The whole blood samples were processed individually. DNA extraction was performed using the Purelink Genomic DNA Extraction Kit (Invitrogen®), according to the manufacturer's instructions. The eluates obtained from DNA were properly identified and stored at -20ºC for later molecular analysis.

Real-time PCR for *Anaplasma* platys was performed by amplifying the 18s rRNA gene, with primers An1-F and An2-R, associated with a specific internal probe (6FAM-CGGATTTTTGTCGTAGCTTGCTATGATQSY)[24].

Real-time PCR for *Babesia canis vogeli* was performed using hsp70-F and antisense hsp70-R primers associated with a specific fluorogenic internal probe (5'- Hex/AGCGCCAGGCCACCAAGGACGCT-3´-IABlkFQ), obtaining the amplification of a fragment of the hsp70 gene[25].

For E. canis, real-time PCR was performed using primers Dsb-321 and Dsb-671, in addition to the specific probe TaqMan (5`- AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA - 3´) 5' FAM/BHQ – 1 3', obtaining an amplified nucleotide sequence of the *dsb* gene[26].

Real-time PCR for *R. vitalii* used the primers called sense Rv751-770 and antisense Rv930-91, in addition to a TaqMan probe [5'- 6-FAM (CCT TAT CAA ATC ATT CTT C) MGB NFQ -3']. This pair of primers corresponds to the amplification of a fragment of the *hsp*70 gene[27].

For the detection of *Rickettsia rickettsii* through real-time PCR, the primer pair CS5 and CS6 were used, which amplify a fragment of the *Rickettsia* spp. citrate synthase gene, associated with an internal fluorogenic probe (5´ 6-FAM – BHQ-1 3´)[28].

The reactions were carried out in 96-well plates subjected to thermal variations corresponding to an initial cycle of 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute[28]. Data amplification, acquisition, and analysis were performed using the multicolor detection system for Real-Time PCR (7500 Real-Time PCR Systems - Applied BioSystems, Foster City, CA, USA).

RESULTS

Of the 63 samples from the dogs tested, fifteen (23.8%) were positive in real-time PCR for at least one agent studied. Of these: 6 (9.52%) were positive for *Babesia canis vogeli* and 9 (14.2%) for *Ehrlichia canis*. No samples were positive for *Rickettsia rickettsii, Rangelia vitalii*, and *Anaplasma platys*.

DISCUSSION

Tick-borne diseases represent an important part of the human and veterinary medical clinic worldwide, with a cycle involving vertebrates and invertebrates interacting in constantly changing biomes[29]. The city of São Paulo is characterized by variations in the tropical climate, with the presence of fragments of the Atlantic Forest[23]. Despite this biome having favorable climatic conditions for the development of ticks of the genus *Amblyomma spp*.[30], none of the samples were positive for *R. vitalii* and *R. rickettsii*.

Of the 63 samples analyzed, 15 (23.8%) were positive for at least one pathogen. In the past decade, molecular research in different locations in Brazil has reported different data. A study in the city of Recife, in an environmental area similar to the present study, detected 45.35% positivity in the samples of dogs analyzed for *E. canis, B. canis vogeli*, and *A. platys*[31], while in Maranhao, in a transition area between Equatorial Forest and the Cerrado, the DNA for *B. canis vogeli* and *E. canis* was detected in only 3.7% of 322 samples[32]. It is important to note that due to the breeding behavior of *R. sanguineus* s.l., environmental differences have little impact on the environmental infestation of this vector[29], suggesting that health management and responsible care are important behaviors in the control of these hemoparasitoses. Another factor that may have contributed to the divergence between the data was the sampling methods used in the studies.

In the present study, through real-time PCR, 9.52% (6/63) of the samples were detected as positive for *B. canis vogeli* and 14.2% (9/63) for *E. canis*. All of these agents are transmitted by *R. sanguineus* s.l. which presents rearing behavior and urban households as a habitat[29], is found in the large urban area of the metropolitan region of São Paulo and in a large number of domestic dogs, ideal conditions for their maintenance and the transmission of pathogens.

Although *Anaplasma platys* was not detected, its presence has already been described in Rio Grande do Sul[33] with a frequency of 14.05% in dog samples collected between 2007 and 2009. In Brazil, there are two different populations of *Rhipicephalus sanguineus* s.l., one that is found in tropical regions and the other in subtropical regions, and further studies are needed to verify whether the vector competence for this pathogen occurs in the same way between the two populations, which could explain the difference between the two studies; as observed for *E. canis*[34].

Of the samples analyzed, 9.52% (6/63) were positive for *Babesia canis vogeli* in a single infection. Castelli and collaborators[35] in a study carried out in a residential condominium in the city of Embu Guaçú, SP, a fragmentation area of the Atlantic Forest, found 16.25% (13/80) of positivity in samples of dogs for *Babesia canis vogeli*. Azevedo and collaborators[36], in a scientific study carried out in an animal shelter in the municipality of São Bernardo do Campo, SP, in an environmental area similar to our study, found 20.9% (17/81) of dogs positive for *Babesia canis vogeli*. One hypothesis that could explain these differences is the characterization of the population of the animals sampled. The high canine population density found in the shelter and in the condominium could favor both the increase in the number of ticks of the species *R. sanguineus* and their permanence in the environment, as well as the circulation of the pathogen in the canine population[35,36].

The real-time PCR for E. canis was positive, as a single infection, in 9/63 (14.2%) of the analyzed samples. Cases of human infection by *Ehrlichia canis* associated with the *R. sanguineus* tick have been reported in Venezuela, causing a disease called Human Monocytic Ehrlichiosis (HME)[37] and, there are reports in the scientific literature of parasitism in humans by *R. sanguineus* in Pernambuco[38], suggesting the need for further scientific studies to elucidate the agent's zoonotic character in Brazil.

No samples were positive for *R. vitalii*, even though it has already been described in the northern part of the city, in an area of fragmentation of the Atlantic Forest[39], which suggests the need for monitoring this pathogen in the southern region, as this infection can cause a severe disease in domestic dogs[40].

Although the animals tested live close to regions with fragmentation areas of the Atlantic Forest, an environment conducive to the development of vectors of the genus *Amblyomma*[21,30], none of the samples were positive for *R. rickettsii*. To date, there is only one report of *R. rickettsii* in dogs in Brazil[41]. As the dogs amplify the bacteria in systemic circulation for a short period and later become immune throughout life, we cannot conclude that the animals in the present study had not already had contact with the pathogen[42]. It is important to note that *R. sanguineus* s.l also proved to be a competent vector of *R. rickettsii* for dogs[42] and is recognized as the main vector of the bacterium for humans in some areas of northern Mexico[43].

The detection of DNA from *B. canis vogeli* and *E. canis* in dogs residing in fragmentation areas of the Atlantic Forest, around the Guarapiranga Reservoir in the southern area of the city of São Paulo in an unprecedented way expands the knowledge of the dispersion of this agent in the country. In addition, the role of *R. sanguineus* s.l. as a vector of pathogens in Brazil cannot be neglected.

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