






## Molecular detection of *Ehrlichia canis* and *Anaplasma platys* in dogs from municipality of Belém, State of Pará, Brazil

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**ABSTRACT:** The occurrence of diseases transmitted by ticks in dogs is very frequent in Brazil, among these diseases we can highlight the ehrlichiosis and anaplasmosis, which are caused by *Ehrlichia canis* and *Anaplasma platys*, respectively. The objective of this study was to survey the occurrence of these pathogens in blood samples from domiciled and stray dogs from the city of Belém, Pará. Two hundred and seventy six dogs were sampled for convenience, and the DNA extracted from the blood of these animals was submitted to nested-PCR for research of *E. canis* and *A. platys*. *E. canis* DNA was detected in 39.4% (109/276) and *A. platys* DNA in 23.1% (64/276) of the samples, there was a statistically significant difference between the frequency of these agents ( $P < 0.0001$ ), and there was coinfection in 13.4% (37/276) of animals. The frequency of detection of these parasites was higher in stray dogs than in those domiciled for both *E. canis* (OR=2.84) and *A. platys* (OR=10.5). Considering the results, it was possible to conclude that *E. canis* and *A. platys* are present in the studied population, with stray dogs being more affected by these parasites.

**Key words:** Canine monocytic ehrlichiosis, Canine cyclic thrombocytopenia, hemoparasites, Anaplasma, Pará.

### Detecção molecular de *Ehrlichia canis* e *Anaplasma platys* em cães do município de Belém, Estado do Pará, Brasil

**RESUMO:** A ocorrência de doenças transmitidas por carrapatos em cães é muito frequente no Brasil, dentre estas enfermidades podemos destacar a erliquiose e a anaplasmoze, que são causadas por *Ehrlichia canis* e *Anaplasma platys*, respectivamente. O objetivo deste trabalho foi fazer um levantamento da ocorrência destes patógenos em amostras de sangue de cães domiciliados e errantes do município de Belém, Pará. Foram amostrados 276 cães por conveniência, sendo que o DNA extraído do sangue desses animais foi submetido à nested-PCR para a pesquisa de *E. canis* e *A. platys*. O DNA de *E. canis* foi detectado em 39,4% (109/276), e o DNA de *A. platys* em 23,1% (64/276) dos cães amostrados. Houve diferença estatisticamente significativa entre a frequência desses agentes ( $p < 0,0001$ ), pois foi encontrada coinfeção entre os agentes em 13,4% (37/276) dos animais. A frequência de detecção desses parasitos foi maior em cães errantes do que nos domiciliados tanto para *E. canis* (OR=2,84) quanto para *A. platys* (OR=10,5). Diante dos resultados, foi possível concluir que *E. canis* e *A. platys* estão presentes na população canina estudada, sendo os cães errantes mais acometidos por esses parasitos.

**Palavras-chave:** Erliquiose monocítica canina, Trombocitopenia cíclica canina, hemoparasites, Anaplasma, Pará.

## INTRODUCTION

Canine diseases exhibiting high morbidity and mortality that are caused by agents transmitted by ticks form a significant proportion of the cases seen in veterinary clinics. These diseases included canine monocytic ehrlichiosis (CME) and canine cyclic thrombocytopenia (CCT), that are caused by etiological agents *Ehrlichia canis* and *Anaplasma platys*, respectively (PAULINO et al., 2018; MACCHI et al., 2019).

In Brazil, the tick *Rhipicephalus sanguineus* is the main carrier of these pathogens and is abundant in large parts of the national territory. In addition to these pathogens, the tick can also transmit other pathogenic microorganisms such as *Babesia* spp. and *Hepatozoon canis* that cause common mixed infections (CICUTTIN et al., 2015; HAPPI et al., 2018).

Both CME and CCT are characterized by fever, weight loss, anorexia, and apathy, which are usually more evident in cases of co-infections (BOUZOURAA, 2016; LIMA et al., 2019). Since

the clinical signs of these infections are not specific, laboratory support is required in order to establish a confirmatory diagnosis. Studies have shown that molecular diagnostic techniques have high sensitivity and specificity for these infections (LAUZI et al., 2016; AZHAHIANAMBI et al., 2018; VLAHAKIS et al., 2018).

The importance of these diseases has been highlighted by the small number of epidemiological studies that have already been carried out in the northern region of Brazil using molecular biology tools (RIBEIRO et al., 2017; SOARES et al., 2017; VIEIRA et al., 2018; PAULINO et al., 2018). Thus, the objective of our study was to determine the rates of incidence of these pathogens in domesticated and stray dogs in the municipality of Belém, Pará, Brazil.

## MATERIALS AND METHODS

### Study area

The study was conducted in the municipality of Belém (01°23.6''S and 048°29'.5''W) (capital of the state of Pará), which has approximately 1,485,732 inhabitants (IBGE, 2018). The climate in Belém is hot and humid throughout the year and the average temperature is 30 °C with a relative humidity of around 78% (INPE, 2019).

### Sampling

Blood samples were collected from 276 adult dogs that were then divided into two groups. Group A consisted of 146 stray dogs that were captured by the Center for Zoonoses Control (CCZ), while group B consisted of 130 pet dogs that belonged to four neighborhoods and were selected during an anti-rabies vaccination campaign.

Samples were collected by puncturing the cephalic vein using 5 mL syringes and sterile needles (25 × 7 mm). The samples were subsequently stored in vacuum tubes containing EDTA and frozen at -20 °C until DNA extraction.

### Polymerase chain reaction (PCR)

DNA extraction from whole blood samples was performed using the commercial kit Illustra® Blood Genomic Prep Mini Spin Kit (GE Healthcare), in accordance with the manufacturer's protocol. A total of 300 µL of blood was used in each extraction. The DNA obtained was analyzed using spectrophotometry by the Picodrop microliter® (Picodrop Limited).

A nested PCR was performed for the detection of *E. canis* DNA using the primers ECC

(5'-AGAACGAACGCTGGCGGCAAGCC-3') and ECB (5'-CGTATTACCGCGGCTGCTGGC-3'). These primers amplified the 478 base pairs (bp) fragment in the first reaction. In the second reaction, we used the primers HE (5'-TATAGGTACCGTCATTATCTTCCCTAT-3') and ECA (5'-CAATTATTTATAGCCTCTGGCTATAGGAA-3') in order to amplify the final product containing 389 bp.

The amplification solution that was used for the amplification of *E. canis* was composed of buffer (100 mM Tris-HCl, pH = 8.5, 500 mM KCl), 50 mM MgCl<sub>2</sub>, 2 U of Taq DNA polymerase (Ludwig Biotec®), 0.2 mM of each dNTP, and 11 pmol of each of the primers. In the first reaction, 5 µL of test DNA (~ 100 ng) was used and in the second reaction, we used 1 µL of the product of the first reaction, yielding a final volume of 25 µL. This reaction amplifies only part of the *E. canis* 16S rRNA gene.

The reactions were performed using a Veriti 96 Well Thermal Cycler (Applied Biosystems®). In order to detect the DNA of *E. canis*, the first reaction that we carried out was an amplification protocol with an initial denaturation step that was performed for three minutes at 94 °C. This was followed by 35 cycles for one minute at 94 °C, 30 seconds at 60 °C and 40 seconds at 72 °C. A final extension step was performed for three minutes at 72 °C. For the second reaction, the initial denaturation step was carried out for one minute at 94 °C. This was followed by 35 cycles for one minute at 94 °C, 30 seconds at 62 °C and 40 seconds for 72 °C. Subsequently, the final extension was performed for 3 minutes at 72 °C.

For the detection of the DNA of *A. platys* we used the primers 8F (5' - AGTTTGATCATGGCTCAG-3') and 1448R (5' - CCATGGCGTGACGGGCAGTGT-3') in the first reaction, while in the second reaction the primers used were APLATYS (5'-GATTTTTGTCGTAGCTTGCTATG-3') and EHR16SR (5'-TAGCACTCATCGTTTACAGC-3'). The primers in the second reaction amplified the final product of the 16S rRNA gene containing 678 bp.

The amplification solution used in the reaction was composed of the buffer (100 mM Tris-HCl, pH = 8.5, 500 mM KCl), 50 mM MgCl<sub>2</sub>, 2.5 units of Taq DNA polymerase (Ludwig Biotec®), 3 mM of each dNTP (dATP, dGTP, dCTP, and dTTP), and 11 pmol of each primer. In the first reaction, 5 µL of the test DNA (~ 100 ng) was used and in the second reaction, we used 1 µL of the product of the first reaction, to obtain a final volume of 25 µL. This reaction amplified only part of the 16S rRNA gene of *A. platys*.

The temperature protocol of the first reaction for detection of the DNA of *A. platys* was as follows: an initial denaturation step that was performed at 94 °C for 7 minutes, followed by 40 cycles at 94 °C for 1 minute, at 48 °C for 1 minute and at 72 °C for 40 seconds. The final extension step was performed at 72 °C for 10 minutes. For the second reaction the initial denaturation was performed at 94 °C for 5 minutes, followed by 40 cycles at 94 °C for 1 minute, at 56 °C for 30 seconds and at 72 °C for 30 seconds, and the final extension step was performed at 72 °C for 7 minutes.

The blood from a dog with parasitemia that was detected during blood smear examination using optic microscopy and was “positive” in the nested PCR was used as a positive control for *E. canis*. The positive control for *A. platys* was a blood sample obtained from a dog. The sample was “positive” in nested PCR with sequencing, thus confirming the presence of *A. platys*. The sample was provided by Professor Dr. Evonnildo Costa Gonçalves and DNA was extracted from the whole blood of a parasitologically negative and asymptomatic dog. This sample, which had no alterations in blood count and was “negative” in the nested PCR, was used as a negative control. In order to avoid false positives, a contamination control was used in all the reactions. The contamination control was an amplification solution and double-distilled water without DNA.

The amplified products were analyzed by horizontal electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL). The length of the amplified products was estimated using a 100 base pair standard (100 bp ladder-Ludwig Biotec) in each gel. Visualization of the amplified products was performed using a photo documentation system and a UV transilluminator (Quantum ST41000/26M).

#### Statistical analysis

Evaluation of the number of positive samples in the nested PCR and the comparative

analysis between the numbers of samples positive for both agents based on the sample group, were performed using the Chi-square test (significance level of 5%) and the BioEstat® v.5.3 program.

The Odds Ratio (OR) was applied in order to evaluate the effect of the characteristics of each sample group in relation to the presence of *E. canis* and *A. platys* DNA (MEDRONHO, 2006).

## RESULTS

DNA of at least one of the agents studied was detected in 49.2% (136/276) of the dogs sampled. The frequency of detection of the *E. canis* and *A. platys* DNA was 39.4% (109/276) and 23.1% (64/276), respectively ( $P= 0.0001$ ). Simple infection by *E. canis* and *A. platys* was observed in 26.0% (72/276) and 9.7% (27/276) of dogs, respectively, while mixed infection was detected in 13.4% (37/276) of animals. There was no statistically significant difference in the occurrence of *E. canis* and *A. platys* ( $P<0.0001$ ) between the sample groups. Distribution of positive samples based on the etiological agents and sample groups is presented in table 1.

The analysis of the OR showed that the likelihood of detection of *E. canis* and *A. platys* DNA in animals belonging to group A (stray dogs) was 2.84, which was 10.5 times greater than the likelihood of their detection in group B (pet dogs).

## DISCUSSION

Epidemiological studies on the etiologic agents of CME and CCT have already been carried out in several regions of Brazil (LEAL et al., 2015; MAKINO et al., 2016; SOARES et al., 2017; VIEIRA et al., 2018; LOPES et al., 2019). However, there are few studies on the molecular epidemiology of these agents in the northern region of the country.

Table 1 - Distribution of positive samples for *Ehrlichia canis* and *Anaplasma platys* from stray dogs (A) and pet dogs (B) from the municipality of Belém.

Agent	-----Group-----		Total
	A	B	
<i>E. canis</i>	32.1% (47/146) <sup>a</sup>	19.2% (25/130) <sup>b</sup>	26.0% (72/276)
<i>A. platys</i>	15.7% (23/146) <sup>c</sup>	3.1% (4/130) <sup>d</sup>	9.7% (27/276)
<i>E. canis</i> e <i>A. platys</i>	24.6% (36/146) <sup>c</sup>	0.7% (1/130) <sup>f</sup>	13.4% (37/276)
Total	72.6% (106/146) <sup>e</sup>	23.1% (30/130) <sup>h</sup>	49.2% (136/276)

<sup>a</sup>Different letters on the same line indicate statistical difference ( $P<0,05$ ).

RUFINO et al. (2013) conducted the first molecular diagnosis of infection by *E. canis* and *A. platys* in the State of Pará using multiplex PCR. However, they subjected only 30 animals to this test.

In the present study, nested PCR was performed with the aim of obtaining high accuracy. Nested PCR can also be considered a confirmatory test for these infections, because it has higher sensitivity than other routine techniques, such as the blood smear (DONI et al., 2016; HAN et al., 2017) and conventional PCR (YANG et al., 2017).

Nested PCR revealed that 49.2% of the dogs examined were infected by at least one of the etiological agents being studied, indicating a high frequency of infected dogs in the region. It is worth mentioning that the municipality of Belém is located within the area of prevalence of the tropical lineage of *R. sanguineus*, which has already been demonstrated the ability to transmit *E. canis* to dogs, this explains the movement of this agent between dogs in the region (MORAES-FILHO et al., 2015).

In similar studies performed in different Brazilian states, rates of infection by *E. canis* in dogs ranged between 3% and 76%. VIEIRA et al. (2018) detected an infection rate of 7.4% in pet dogs that were seen at a veterinary hospital and a castration campaign at the School of Veterinary Medicine in the state of Espírito Santo. PAULINO et al. (2018) reported an infection rate of 24.8% in dogs from rural and urban areas in the state of Rio de Janeiro. ROTONDANO et al. (2017) detected *E. canis* in 8.9% of pet dogs in the urban area of the state of Paraíba. MAKINO et al. (2016) reported an infection rate of 76.3% in pet dogs in the state of Mato Grosso. In all the studies mentioned above, as well as in our present study, molecular diagnostics were performed in animals without clinical suspicion of CME, indicating that a highly sensitive technique can reveal cryptic cases of infection by this agent.

DAGNONE et al. (2003) reported the first molecular diagnosis of infection by *E. canis* in Brazil, they related an infection rate of 21%. Several other authors also reported infection by *E. canis*. For example, WITTER et al. (2013) reported an infection rate of 23.3% in Cuiabá-MT, AGUIAR et al. (2013) reported 65% infection rate in several regions of Brazil and SOARES et al. (2017) reported an infection rate of 59.66% in Campo Grande-MS. The dogs in these studies were presented in veterinary hospitals with clinical or serologic signs of infection. This was in different from our present study in which the selection of dogs was entirely random. However, consistent with the observations in other regions, we

also reported that the number of infected animals was above 20%, thus implying the movement of the parasite in dogs in the region.

The number of samples in which *A. platys* DNA was detected was lower than that reported by RAMOS et al. (2009) in Pernambuco and by RAMOS et al. (2010) in Recife. The rates of infection reported were 55% and 48.78%, respectively, in dogs with some clinical signs that were suggestive of the disease. These dogs were also being treated in Veterinary Hospitals. This may have happened because the sample group in our study was not composed of animals that presented in clinics and hospitals; rather, the group was composed of animals that did not show any clinical signs of the disease.

We reported a higher rate of infection compared to the rates of infection reported in other studies that were carried out in animals that had no clinical signs suggestive of infection by *A. platys*. RIBEIRO et al. (2017) reported an infection rate of 32.9% in stray dogs in the state of Paraná. LASTA et al. (2013) observed infection in 14.07% of stray dogs in the state of Rio Grande do Sul. VIEIRA et al. (2018) detected an infection rate of 6.34% in pet dogs in the state of Espírito Santo.

One common factor that has been observed in different studies is coinfection even in animals without clinical evidence of infection (LAUZI et al. 2016). In the present study, such animals accounted for 13.4% (37/276) of the sample. RAMOS et al. (2010) and SOARES et al. (2017) reported a similar result using nested PCR and detected coinfection in 16% and 9.94% of dogs, respectively. Conversely, SILVA et al. (2012) and VIEIRA et al. (2018) detected a coinfection rate of 5.47% and 3.70%, respectively, using conventional PCR. Given the difficulty of detecting multiple infections, the use of more sensitive techniques such as nested PCR, can facilitate the detection of co-infected animals.

Other than the similarity between the geographical areas of occurrence, the fact that a significant number of animals were co-infected implies that *R. sanguineus* is probably capable of transmitting both agents. Studies that aimed to detect the DNA of *E. canis* and *A. platys* in both dogs and in ticks that infest these animals, reinforce the involvement of this tick in the transmission cycle of these agents (CICUTTIN et al., 2015; SILVA et al., 2016).

The comparative analysis between the rates of infection by *E. canis* and *A. platys* between the sample groups indicated that the errant behaviors or the management of the animals in the CCZ are risk factors for this infection. This may be due to greater

exposure of stray animals to tick infestation by the vector *R. sanguineus*, compared to pet animals. Pet animals also received more intense care, such as periodic examinations and correct treatment for possible diseases, in order to prevent infection by ectoparasites (LABRUNA et al., 2001; LIU et al., 2016; FIGUEIREDO et al., 2017).

## CONCLUSION

We concluded that the agents assessed are present in high frequency in the region studied. Stray dogs are at a greater risk of infection compared to pet dogs.

## ACKNOWLEDGEMENTS

This study was funded by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES - Finance code 001) and Federal University of Pará.

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was submitted for analysis by the Research Ethics Committee of experimental animals of the Federal University of Pará - CEPAE / UFPA - Protocol 083-12.

## DECLARATION OF CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest. The founding sponsors had no role in the design of the study, nor in the data collection, analyses, or interpretation of data, the writing of the manuscript, nor the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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