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A molecular survey of three tick-borne pathogens in dogs from Algodoal village/Maiandeua island on the northeast coast of Pará, Brazil

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Abstract: *Ehrlichia* spp., *Anaplasma* spp. and *Babesia* spp. are obligate intracellular parasitic microorganisms found in the blood of domestic animals. Until then, there were no reports of these hemoparasites in dogs in the northeast of the State of Pará. The aim of this study was to record cases of natural infection by *Ehrlichia* sp., *Anaplasma* sp. and *Babesia* sp. in dogs from Ilha de Algodoal/ Maiandeua, State of Pará, Brazil, through the detection of DNA from these agents. Whole blood samples were collected from 52 animals, without considering breed, sex or age. Include results for different species of animals and parasites. Molecular analysis data showed 50% co-infection for *Ehrlichia* sp. and *Anaplasma* sp. This study allowed the detection of *Ehrlichia canis* and *Anaplasma platys* in domestic dogs on Algodoal Island, an important ecological tourism site in northern Brazil.

Keywords: hemoparasites; mammals; Brazilian amazon; zoonosis; PCR

1. Introduction

Vector-Borne Diseases (VBDs) are illnesses caused by viruses, bacteria, spirochetes, rickettsia, and parasites which are transmitted between humans, or from animals to humans through blood-feeding arthropod vectors. Since clinical manifestations can vary from no visible symptoms to severe and possibly deadly conditions, VBDs play an important role in veterinary medicine because they affect both pets and economically valuable livestock, and they are a growing public health concern due to their zoonotic potential (Savi et al., 2014; Chala et al., 2021).

Ticks are the second most common agents of VBDs, as they are hematophagous arthropods distributed worldwide, which is due to their ability to adapt to various hosts, environments, and climates (Dantas-Torres et al. 2012). In recent decades, ticks and tick-borne diseases have experienced geographic range shifts, resulting in changing rates of tick exposure and the spread of tick-borne zoonoses as Lyme disease, babesiosis, ehrlichiosis, anaplasmosis, and tularemia, which have a significant impact on public health (Araújo et al., 2015; Kilpatrick et al., 2017). Although there are hundreds of tick species found around the world, not all are known to be disease vectors, and the epidemiological importance of a given species is related to its geographic distribution. Ixodes (2 species), *Dermacentor* (2), *Amblyomma* (2), and *Rhipicephalus* (1) are the most common disease-transmitting ticks in the United States (Choi et al., 2016; Pace and O'Reilly, 2020). According to Zhao et al. (2021), in China, the most widely distributed tick genus is *Dermacentor* (574 counties), followed by Heamaphysalis (570), Ixodes (432), *Rhipicephalus* (431), *Hyalomma* (298), Argas (90), *Ornithodoros* (38), *Amblyomma* (37), and *Anomalohimalaya* (5). Brazilian ticks include 70 species, 47 in the family Ixodidae and 23 in the family Argasidae. The genera *Amblyomma* (32 species) and *Ornithodoros* (18) are the most representative (Dantas-Torres et al., 2019). Another vector species of great importance is *Rhipicephalus sanguineus* sensu lato, a cosmopolitan tick found throughout Brazil (Caetano 2016; Labruna and Pereira, 2001). It has a predilection for domestic dogs and is the vector of a series of pathogens that can infect dogs and humans, causing diseases such as ehrlichiosis, anaplasmosis, and babesiosis (Groves et al., 1975; Greene and Harvey 1990; Nava et al., 2017).

In the context of public health, VBDs represent a challenge, as they require the implementation of control and prevention strategies, both to protect health of animals and to prevent transmission of these diseases to humans. Regarding to animal health, identifying agents of VBDs is an essential instrument for veterinarians in deciding the appropriate measures for treatment and prevention (Pinto et al., 2018). Since there is no research data available, in this study we performed a molecular survey aiming to evaluate the prevalences of *Ehrlichia canis*, *Anaplasma platys*, and *Babesia vogeli* in the dog population of the Algodoal village/Maiandeua island on the northeast coast of Pará, Brazil.

2. Materials and Methods

2.1. Ethical aspects and Study area

From November 2019 to August 2021, 123 surgical castrations of dogs and cats of Algodoal village (-0.6066301, -47.5611076) in Algodoal-Maiandeua Environmental Protection Area (EPA), which is located in the municipality of Maracanã, Pará, Brazil, were performed within the scope of the Amazônia Veterinária Project - AVP. These procedures were authorized by the Ethics in the Use of Animals Committee (CEUA) nº23084.010805/2017 of the Federal Rural University of the Amazon, UFRA, approved in 2017, effective from November 13, 2017, to August 10, 2021.

The Algodoal-Maiandeua EPA (00º34'02" to 00º38'55" south latitude and 47º31'22" to 47º35'56" west longitude) has an area of about 3,100.34 hectares (7,661.1 acres), and is a conservation unit for the sustainable use of nature which protects two coastal islands, Algodoal and Maiandeua (Lisboa 2017; Ideflor-Bio 2019; Castro 2021), with beaches, dunes, mangroves, and wetlands that are home to fishing people and are popular with Brazilian and foreign tourists. The Amazonian equatorial climate is predominant, and the temperature averages between 26°C and 27°C, with a maximum reaching 34°C and a minimum of around 19°C, with a small thermal amplitude caused by the location conditions of the Municipality of Maracanã. It is in the Salgado region and benefits from strong sea winds. The highest rainfall index occurs in the first months of the year, from February to April, and the lowest is between September and October. These precipitations are around 2.000 mm/year (Governo do Estado do Pará, 2007).

Access to the island region from the state capital of Belém is via the BR-316 highway to the city of Castanhal and 120 km via the PA-136 and PA-318 highways, arriving at the port of Marudá District, municipality of Marapanim. Afterward, access to the island is done by boat, a crossing of about 40 minutes to the port of Vila de Algodoal (Ideflor, 2019; Castro 2021). There are still some areas covered by the original dryland forest in the municipality. With the intensity of deforestation, there is a predominance of secondary forests in the regeneration stage, and the floodplain vegetation is found on the banks of the Caripi and Maracanã rivers. In the semi-coastal and coastal areas, there is a predominance of mangroves (Governo do Estado do Pará, 2007).

Based on the registration carried out before the sterilization program, Algodoal village/Maiandeua island had 303 domiciled dogs in 2019. In addition to this, approximately 50 other non-domiciled dogs were sighted during the activities of the AVP team. The samples obtained for the present study were obtained in two work missions, the first in November 2019 and the second one in December 2020. During the first mission, 39 animals (32 dogs and 7 cats) were sterilized, and in the second one, 84 (63 dogs and 21 cats).

2.2. Sampling and collection of biological material

The study analyzed a total of 52 (32 and 20, from first and second missions, respectively) blood samples of dogs (*Canis lupus familiaris*) mixed breeds, which were randomly selected regardless of their clinical status. An epidemiological questionnaire was used by the animal guardians to obtain information about the dogs' breeding routines. The information included gender, presence of ectoparasites as ticks and fleas, if they have been living with other animals, the socioeconomic level (high, medium-high, medium, medium-low, and low), and the age group: young (up to 1 year old) and adults (1 to 5 years old).

Under chemical restraint, and by cephalic or jugular venipuncture, approximately 5 ml of blood of each animal were collected using tubes with the anticoagulant ethylenediaminetetraacetic acid (EDTA). These biological materials were properly transported in polystyrene boxes with recyclable ice. Then, in the laboratory, the samples were refrigerated at -20°C until processing.

2.3. Laboratory procedures

Genomic DNA of each sample was extracted from 300 µL of whole blood using a standard phenol-chloroform procedure, as described by Sambrook et al. (1989). DNA quality was checked by electrophoresis on an agarose gel, and the DNA was then quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, USA). Molecular detection of *Ehrlichia canis* and *Anaplasma platys* was performed following Rufino et al. (2013), while DNA research of *Babesia vogeli* was performed according to Moraes et al. (2014). Genomic DNA of blood samples of dogs previously identified as infected with E. *canis*, A. *platys*, and B. *vogeli* were used as positive controls, while sterile bi-distilled water was used as the negative control.

Since Rufino et al. (2013) and Moraes et al. (2014) are based on a nested and semi-nested PCR, respectively, to properly identify the pathogens species, amplicons of the second round PCR were cleaned using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fischer Scientific, Waltham, USA). Then, according to the manufacturer’s specifications, purified PCR products were sequenced in both directions using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer

Scientific, Waltham, USA) in conjunction with an ABI 3500 xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA). BioEdit software (Hall, 2011) was used to align forward and reverse sequences.

2.4. Statistical analyses

Data were tabulated and treated statistically by simple descriptive percentages, implemented by Microsoft Excel 2016 software.

3. Resultados

During the missions in the Algodoal village/Maiandeua island many dogs were seen roaming the streets and beach without adequate sanitary care (Figures 1A and B). The presence of ectoparasites (mites, fleas, and ticks) was observed in 61.5% (32/52) dogs, with flea infestation being greater than that of ticks (Table 1). Coinfestation by tick and flea was the most common observed. As recorded at the time of sample collection, none of the animals received any medicine from the tutor or veterinarian. This fact has ensured the representativeness of the susceptibility of the studied animals to infection. All samples were collected from animals able to undergo castration surgery (Figures 1C, and D). Of the 52 (100%) animals analyzed, 42 were females (80.8%) and 10 were males (19.2%), with a margin of error of 10%.

Figure 1 − Algodoal Island (Maiandeua) is in the state of Pará, northern Brazil. A-B. Presence of dogs in the center of the island and on the beach C. Application of the questionnaire; C. Application of a questionnaire on the day of the dog and cat sterilization campaign.

Ectoparasite	Mission 1 (n = 32)	Mission 2 (n = 20)	Overall (n = 52)
	Presence (%)	Presence (%)	Presence (%)
Tick (only)	4 (12.50)	0	4 (7.7)
Flea (only)	6 (18.75)	7 (35)	13 (25)
Mite (only)	0	0	0
Tick + Flea	7 (21.88)	0	7 (13.5)
Tick + Mite	0	2 (10)	2 (3.85)
Flea + Mite	3 (9.38)	0	3 (5.77)
Tick + Flea + Mite	2 (6.25)	1 (5)	3 (5.77)

Table 1 − Absolute and relative frequencies of ectoparasites in domestic dogs of Algodoal village/Maiandeua island, Pará, Brazil. n = sample size.

Sequencing of the second-round PCR amplicons of E. *canis* and A. *platys* yielded sequences, including primers, that were 389 and 212 bp long, respectively. Alignment of all sequences resulted in one haplotype to E. *canis* and as well as one to A. *platys*. The respective Blast search of these two haplotypes resulted in 100% nucleotide identity in comparison to sequences KC109445 of E. *canis* and KC109446 of A. *platys*, which were detected from dogs of Belém, Pará (see Rufino et al. 2013).

The prevalence of positive samples for E. *canis*, A. *platys* and B. *vogeli* are shown in table 2. Overall, E. *canis* DNA was detected in 6 (11.5%) dogs, while A. *platys* DNA was also detected in 6 (11.5%) dogs. Coinfections by E. *canis* and A. *platys* were detected in 4 (7.7%) of all dogs analyzed. Considering only dogs with PCR positive for E. *canis*, A. *platys* or both, i.e., 8 dogs (15.4%), coinfection rate for both missions was 50% (4/8). Tick-borne pathogens were not detected in 44 (84.6%) of all dogs. *Babesia* DNA was not detected in any sample of our study.

TBP	Mission 1 (n = 32)	Mission 2 (n = 20)	Overall (n = 52)
	Positive (%)	Positive (%)	Positive (%)
E. canis (only)	1 (3.13)	1 (5.00)	2 (3.85)
A. platys (only)	2 (6.25)	0	2 (3.85)
Babesia spp.	0	0	0
E. canis + *A. platys*	4 (12.5)	0	4 (7.70)
Overall	7 (21.88)	1 (5.00)	8 (15.40)

Table 2 – Absolute and relative frequencies of tick-borne pathogens (TBP) in dogs of Algodoal village/Maiandeua island, Pará, Brazil. n = sample size.

Reference	Locality (n)	Prevalence (%)
Diniz et al. (2007)[1]	Botucatu – SP (198)	77.7
Ueno et al. (2009)[1]	Botucatu – SP (70)	40,0
Macieira et al. (2005)[1]	Rio de Janeiro – RJ (226)	15.0
Dagnone et al. (2003)[1]	Londrina – PR (129)	22.0
Dagnone et al. (2009)[2]	Jaboticabal – SP (25)	88.0
Nakaghi et al. (2008)[2]	Jaboticabal – SP (30)	53.3
Faria et al. (2010)[2]	Jaboticabal – SP (40)	72.5
Bulla et al. (2004)[2]	Botucatu – SP (217)	30.9
Santos et al. (2009)[2]	Ribeirão Preto – SP (221)	38.9
Carvalho et al. (2008)[2]	Ilhéus-Itabuna – BA (153)	7.8
Dagnone et al. (2009)[2]	Campo Grande – MS (26)	38.4
Soares et al. (2017)[2]	Campo Grande – MS (181)	55.75
Ramos et al. (2009)[2]	Recife - PE (100)	57.0
Sousa (2006)[2]	Cuiabá – MT (60)	20.0
Rufino (2013)[1]	Belém – PA (200)	24.0
Costa Jr (2007)[3]	Lavras – MG (97)	1.03
Costa Jr (2007)[3]	Belo Horizonte – MG (49)	24.49
Costa Jr (2007)[3]	Nanuque – MG (102)	26.47

Table 3 – Summary of prevalence of *Ehrlichia canis* in Brazilian domestic dogs based on molecular diagnosis. 1PCR, 2nested PCR, 3Real time PCR

Reference	Locality (n)	Prevalence (%)
Lasta et al. (2013)[2]	Porto Alegre – RS (199)	14.07
Soares et al. (2017)[2]	Campo Grande – MS (181)	16.96
Melo et al. (2016)[2]	Poconé – MT (320)	7.19
Costa (2015)[1,3]	Goiânia – GO (500)	6
Vieira (2017)[3]	Espírito Santo – ES (378)	6
Costa Jr (2007)[3]	Lavras – MG (97)	7.22
Costa Jr (2007)[3]	Belo Horizonte – MG (49)	4.08
Costa Jr (2007)[3]	Nanuque – MG (102)	19.61

Table 4 – Summary of prevalence of *Anaplasma platys* in Brazilian domestic dogs based on molecular diagnosis. 1PCR, 2nested PCR, 3Real time PCR.

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4. Discussion

Over the past years, there has been a significant increase in the number of cases of VBDs in several regions worldwide (Gubler 2010; Chala and Hamde 2021), and preventive actions such as (i) identifying the circulating pathogens, (ii) implementing effective measures for epidemiological surveillance, and (iii) establishing appropriate vector management strategies are essential in reducing the incidence and mitigating the impact of these diseases.

In this context, to the best of our knowledge, this is the first survey of vector-borne pathogens carried out in Algodoal village/Maiandeua island, on the northeast coast of Pará, Brazil, which has a domestic dog population living in very close contact with local vegetation, wild animals, migratory birds, and the local human population, as well as tourists. These factors, in addition to the environmental conditions, may favor the proliferation of ticks and, consequently, the dissemination of vector-borne pathogens (Temoche et al., 2022).

Regarding the results of the present study, the overall prevalence of E. *canis* in domestic dogs of Algodoal village/Maiandeua island was lower than that observed for most records from other Brazilian localities (Table 3), including Belém, Pará, Brazil, which is 140 km from our study area. On the other hand, the A. *platys* prevalence we found here is similar to that recorded elsewhere (Table 4). In general, higher frequencies of infection are expected in regions where the tick vector is more abundant, and differences in the prevalence of vector-borne pathogens can also be explained by the methodologies used for diagnosis, geographic regions evaluated, sampling period, vector, and host (Benavides-Arias and Soler-Tovar 2020). Costa Jr. (2007) provides an example where the overall prevalence of E. *canis* and A. *platys* was found to be lower in urban areas compared to rural areas in Minas Gerais, Brazil. In that study, infestation by R. *sanguineus* ticks was identified as the sole risk factor contributing to this difference, particularly for E. *canis*. In this sense, according to our observation of a low prevalence of tick infestation, this may be the case in Algodoal village or Maiandeua island.

Despite the prevalence of E. *canis* and A. *platys* in Algodoal village/Maiandeua island, one of the most notable results of this study is the high frequency of coinfections in comparison to individual infection rates. Among factors potentially contributing to this condition, we point out the unrestricted movement of dogs within Algodoal village/Maiandeua island, which has led to close contact between animals in a territorially limited area, as well as the lack of preventive measures to control tick infestation. According to Sousa et al. (2021), anaplasmosis and ehrlichiosis are recurrent diseases, and their coinfection has been growing among domestic animals. It is necessary to implement the best way of diagnosing these diseases by associating, in addition to clinical signs, molecular tests. In our research, animals coinfected by *Ehrlichia* and *Anaplasma* did not show significant clinical signs, although in some cases, oral and gingival mucous membranes were slightly pale, capillary refill time increased (> 3 seconds), and eye discharge was observed. Clinical signs are evidenced mainly in young animals (5–10 months old) and may also be related to endoparasites.

Another relevant result of this study is our failure to detect *Babesia* spp., which may reflect our small sample size (15% of the total population, i.e., domiciliated and non-domiciliated dogs), as well as a low prevalence of infection or even a complete absence of this pathogen in that locality. Unfortunately, data on canine babesiosis in northern Brazil is scarce. Panti-May and Rodriguez-Vivas (2020), investigating available data on canine babesiosis in Latin America and the Caribbean (from January 2000 to December 2019), reported only 34 published studies, which showed prevalence varying considerably based on parasite species and geographic location, with values close to zero to 26.2%. In northern Brazil, an exception is the study of Moraes et al. (2014), who, based on the same detection protocol used in the present study, reported a prevalence rate of B. *vogeli* in domestic dogs in Belém, Pará, Brazil, equal to 15.7% (27/172), which, in comparison to other studies (see Panti-May and Rodriguez-Vivas 2020), is moderately high. However, since it was based on a relatively smaller sample size, the contrast with our findings supports the hypothesis of a low prevalence or even the absence of *Babesia* in Algodoal village or Maiandeua island. Confirming the occurrence of *Babesia* spp. in this area requires extensive research, including long-term epidemiological surveillance.

It is worth highlighting that Algodoal village/Maiandeua island, located on the northern coast of Brazil, play a role as an important stopover and wintering site for a number of aquatic migratory birds (Gonçalves et al., 2007; Krietsch et al., 2017). The ecology of aquatic birds, such as their migratory behavior, diet, habitat use, and aggregation habits, has direct effects on the global distribution and diversity of vectors and pathogens (Morshed et al., 2005; Abdelbaset et al., 2023). Thus, this reinforces Panti-May and Rodriguez-Vivas (2020), who highlight the need for further research in order to enhance our understanding of the ecology and epidemiology of *Babesia* spp. in dogs in Latin America and the Caribbean.

In general, our results contradict the hypothesis that animals with an exclusively outdoor lifestyle, living virtually in sympatry with sylvatic species, and without regular controls and treatments, as in the case of the dogs studied here, are more exposed to the risk of infection. Based on our observations, especially during the second mission, the most coherent explanation for this is the low prevalence of tick infestation.

The transmission of VBDs requires an introduced and/or established vector population, a pathogen, and suitable environmental and climatic conditions across the full cycle of VBD transmission (Randolph and Rogers 2010). It is widely known that ticks are susceptible to climatic determinants, specifically humidity and temperature. Indeed, a high incidence of tick-borne disease has been reported to be linked with moderate winters and humid, warm summers, although incidence may also be affected by the influence of climate on recreational activities (Ostfeld and Brunner 2015). Since the temperature in Algodoal village/Maiandeua island is favorable, its low prevalence of ticks, as we observed here, seems to be influenced by other biotic and/or abiotic factors. Showler et al. (2019) studied the relationships between some abiotic and biotic factors that influence ixodid distribution. observed (i) exposure of lone star tick, *Amblyomma* americanum, and *Rhipicephalus* microplus eggs to hypersaline water is lethal; (ii) although intermittent hypersaline flooding kills ixodid eggs, saline soil was not particularly toxic; (iii) when relative humidity is relatively low, desiccation causes high egg mortality on dry soil, regardless of salinity; and (iv) substantial year-round populations of mud flat fiddler crabs, Uca rapax (Decapoda: Ocypodidae), on saline soil eliminated 80% of an *Amblyomma* americanum egg masses overnight. Since Algodoal village/Maiandeua island are situated in the "salgado paraense" region, it is reasonable to believe that at least the influence of saltwater toxicity and predation by crabs on survival and the abundance of ticks occurring there should not be overlooked.

Although the results of our study are primarily relevant to veterinary concerns, its significance for public health should not be disregarded because the literature has reported an increasing number of human anaplasmosis cases caused by A. *platys* and human ehrlichiosis cases caused by E. *canis* in recent years (Perez et al., 2006; Maggi et al., 2013; Arraga-Alvarado et al., 2014). *Anaplasma* and *Ehrlichia* are responsible for causing nonspecific febrile illnesses that are mostly self-limiting. However, older individuals, patients with underlying medical conditions, or those with weakened immune systems face a higher risk of experiencing severe illness or even death if prompt and appropriate treatment is not administered.

5. Conclusion

E. *canis* and A. *platys* were responsible for naturally infecting domestic dogs in Algodoal/Maiandeua island, north of Brazil. These preliminary data show the need for more comprehensive work with greater sampling effort and the collection of vectors for the diagnosis of infectious agents transmitted in the APA of Algodoal.

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