



***Dirofilaria immitis* infection in dogs in Algodual Island, Brazilian Amazon¹**

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ABSTRACT.- Moreira H.R., Madeira E.A.O., Lima Cunha D.N., Scofield A., Góes-Cavalcante G., Abel I., Guimarães R.J.P.S. & Fernandes J.I. 2019. ***Dirofilaria immitis* infection in dogs in Algodual Island, Brazilian Amazon.** *Pesquisa Veterinária Brasileira* 39(7):510-515. Instituto de Medicina Veterinária, Universidade Federal do Pará, Rodovia BR-316 Km 61, Bairro Saudade, Castanhal, PA 68740-970, Brazil. E-mail: vetjulio@yahoo.com.br

Dirofilaria immitis, a parasite that mainly infects domestic or wild canids, but can infect felines or humans as well, is frequent in many Brazilian areas. The main objective of this research was to determine the prevalence of natural canine infection at the Algodual-Maiandeuá Island complex, in the coastal region of the state of Pará, Brazil. A total of 67 dogs were sampled for blood microfilariae detection and for *D. immitis* DNA detection. Microfilaria and *D. immitis* DNA could be detected in 35.8% (24/67) of the animals. In one dog's sample no microfilariae were detected, but the PCR was positive, suggesting that either larvae recently were eliminated or adults died shortly before sample collecting. Therefore, it can be concluded that the occurrence of *D. immitis* is a health threat for domestic and wild canids at the Island of Algodual, as well as for feline or human health.

INDEX TERMS: Occurrence, canids, *Dirofilaria immitis*, infection, dogs, Algodual Island, Brazilian Amazon, heartworm, PCR.

RESUMO.- [Infecção por *Dirofilaria immitis* em cães na Ilha de Algodual, Amazônia brasileira.] *Dirofilaria immitis*, um parasito que infecta principalmente canídeos domésticos ou selvagens, embora também possa infectar felinos e humanos, é frequente em muitas áreas do Brasil. O objetivo deste estudo foi determinar a ocorrência da infecção natural em cães provenientes do complexo da Ilha de Algodual-Maiandeuá, região litorânea do estado do Pará, Brasil. Um total de 67 cães tiveram o sangue coletado para detecção de microfíliarias de *D. immitis* e seu DNA. Microfíliarias e o DNA de *D. immitis* foram detectados em 35,8% (24/67) dos animais. Na amostra de um animal, não foram observadas microfíliarias, mas o seu DNA foi detectado, sugerindo que as larvas tenham sido recentemente eliminadas ou os adultos tenham morrido

antes da coleta da amostra. Portanto, pode-se concluir que a ocorrência de *D. immitis* é uma ameaça à saúde de canídeos domésticos no complexo da Ilha de Algodual-Maiandeuá, bem como para felinos e seres humanos.

TERMOS DE INDEXAÇÃO: Ocorrência, cães, infecção, *Dirofilaria immitis*, verme do coração, PCR, Ilha de Algodual, Amazônia brasileira.

INTRODUCTION

Dirofilariasis is a parasitic disease caused by filarial worm *Dirofilaria immitis* (Leidy 1856), also known as the heartworm (Bolio-Gonzalez et al. 2007), affecting mainly domestic and wild canids (Batista et al. 2008). Humans and felines, which may be accidental hosts, are more susceptible to infection in areas with high prevalence of the canine disease (Garcez et al. 2006, Mirahmadi et al. 2017), which is distributed all over the globe (Bolio-Gonzalez et al. 2007, Bowman & Atkins 2009, Hou et al. 2011, Simsek et al. 2011, Montoya-Alonso et al. 2015, Bendas et al. 2017). It has been found in every geographical region of Brazil, with prevalence rates over the last decade ranging from 2.1% in Florianópolis, in the Southern state of Santa Catarina (SC), to 62.2% in Armação de Búzios, in the Southeastern state of Rio de Janeiro (RJ) (Silva et al. 2008,

¹ Received on December 23, 2018.

Accepted for publication on February 12, 2019.

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Furtado et al. 2009, Ogawa et al. 2013, Labarthe et al. 2014, Soares et al. 2014, Ramos et al. 2015).

Methods and techniques with different analytical sensitivities are employed to diagnose infection by *D. immitis*. The most common diagnosis techniques are the identification of microfilariae on light microscopy, PCR and variations of this method for DNA detection, anti-*D. immitis* antibody detection by means of the enzyme-linked immunosorbent assay (ELISA) and the detection of antigens by immunochromatography (Traversa et al. 2010, Simsek et al. 2011, Rojas et al. 2015, Shalini et al. 2018).

Infection in a given region can be influenced by different factors, such as the diversity and quantity of mosquitoes in the area, climate changes, anthropic action and the presence of animals not receiving chemoprophylaxis (Bowman & Atkins 2009, Genchi et al. 2011, Labarthe et al. 2014). In the North region of Brazil, epidemiological studies on canine dirofilariasis are still incipient. In the state of Pará, *D. immitis* infection has been reported in dogs living in coastal areas, such as the islands of Algodoal (Castro et al. 2012, Furtado et al. 2012) and Marajó (Soares et al. 2014, Argôlo et al. 2018).

The Algodoal-Maiandeuá island, part of the municipality of Maracanã, in the northeastern mesoregion of the state of Pará and part of the Northeastern Coastal Plain of Pará (João et al. 2013), has approximately 23.78km² in area and its environment is influenced by the geographical proximity to the Amazon River. For its regional biota and fragile ecosystem - composed of sandbanks, dunes, mangroves and lakes - the island entered the status of Environmentally Protected Area (APA - State Law of Pará No. 5621, Pará 1990). The climate is warm and humid, and its population is distributed among a complex of four villages (Algodoal, Camboinha, Mocooca and Fortalezinha). The island's economy relies strongly on tourism, with an intense flow of people and domestic animals. This calls for health regulation measures, especially with regard to infections transmitted by arthropods, including *D. immitis*. The island houses a great number of free-ranging or stray dogs who do not get proper veterinary care, which favors a high incidence of these infections (Castro et al. 2012, Furtado et al. 2012).

This work aimed to identify the frequency of natural infection with *D. immitis* in dogs in the villages composing the island of Algodoal-Maiandeuá.

MATERIALS AND METHODS

Study area. Algodoal or Maiandeuá island (0°34'45" to 0°37'30" S and 47°32'05" to 47°34'12" W), with 3,100.34 ha, is located in the municipality of Maracanã, microregion Salgado and mesoregion Northeast of the state of Pará. The island is composed of four villages: Vila de Algodoal, Vila de Camboinha, Vila de Fortalezinha and Vila de Mocooca. The city of Maracanã is located at 165km by road from the state capital. Algodoal is accessed by river from Marudá port (city of Marapanim), a crossing which lasts approximately 40 minutes to Algodoal village (Ideflor-bio 2018).

Sample size and sampling. The communities composing the Algodoal island complex were visited from July to September 2013. The sample size was calculated with the aid of the Statcalc tool of software EpiInfo 3.5.3 considering an estimated human population of 2,050 inhabitants (Algodoal 2013), an estimated canine population of 293 dogs, 176 households with at least one dog, expected frequency of 40%, β error of 20% and α error of 0.01,

which amounts to a total of 50 households for sampling. At the first stage, the proportionate stratified sampling, which considered each community as a stratum, led to the visit of 29 households in Vila de Algodoal, five in Camboinha, ten in Fortalezinha and six in Mocooca. The second stage involved systematic sampling, where households with at least one dog were included in the sample. However, in cases where these households had more than one dog, all animals were submitted to the tests.

Georeferencing and spatial distribution map. All households included in the study have been georeferenced by a GPS MAP (60CSX, Garmin, EUA) for subsequent spatial distribution of *D. immitis* infected and non-infected animals. The maps were drawn on software ArcGis 10.3 using the Copernicus Sentinel-2 images (<http://open.esa.int/>) from 9 August, 2017 and the 2012 soil use and cover map by the State Secretary for the Environment (SEMA). The coordinates of residences without infected animals, as per the techniques employed in this study, were represented as green dots on the map, whereas those with at least one infected animal were represented as red dots.

Sample collection. A total of 5mL of blood was collected from the cephalic vein of each animal and stored in tubes containing ethylenediamine tetra acetic acid (EDTA), with a final concentration of approximately 1.8mg/mL of blood.

Techniques for microfilaremia detection. All samples were submitted to three techniques for detection of microfilariae: fresh and thick blood smears and the Knott technique modified by Newton & Wright (1956), which was also used to identify the microfilariae found. In sum, the fresh blood smear technique consists in using a drop of blood with anticoagulant on a microscopy slide covered by a glass cover slip of 10×10mm. The thick blood smear technique uses two drops of blood on a microscope slide, spread with the aid of microhematocrit tubes in circular movements and air dried by manual movement. The slides were examined on an optical microscope with magnification of 400× (De Carli & Tasca 2007).

The Knott technique modified by Newton & Wright (1956) is performed by diluting 1mL of blood into 9mL of formalin 2% with subsequent centrifuging (1.500rpm for 5min). Then, the supernatant was discarded and the precipitate examined between slide and cover slip by light microscopy (magnification of 100 or 400×) for detection and morphological identification of microfilariae. Distinction of the microfilariae was based on the evaluation of their morphological characteristics, especially in the anterior and posterior ends, according to Genchi et al. (2007). The samples containing immature forms of *D. immitis* were registered as such.

Polymerase chain reaction (PCR). A commercial kit (AxyPrep™ Multisource Genomic DNA Miniprep Kit -Axygen-Biosciences, EUA) was used to extract the genomic DNA of the dogs' blood samples. The procedure was done according to the manufacturer's instructions. All samples were submitted to a PCR using primers 12SF (5'-GTTCCAGAATAATCGGCTA-3') and 12SRdeg (5'-ATTGACGGATG(AG)TTTGTAACC-3'), which amplify a product of 500 base pairs (bp) in the region of gene 12S rDNA, highly conserved for nematode species (Casiraghi et al. 2004). The samples tested positive in the first PCR were submitted to a second PCR using primers 12SF2B (5'-TTTTTACTTTTTGGTAATG-3') and 12SRdeg (5'-ATTGACGGATG(AG)TTTGTAACC-3'), which amplify a product of 204bp in the region of gene 12S rDNA of *D. immitis* (Gioia et al. 2010). The protocols were used according to Gioia et al. (2010) with modifications.

The amplification solution used for the reactions was made up of 2.5mM of Buffer (100mM of Tris-HCl, pH 8.5 and 500mM of KCl), 1.5mM of MgCl₂, 0.5mM of each dNTP (dATP, dGTP, dCTP and dTTP), 1pmol of each primer, 0.5 unit of Taq DNA polymerase

(Ludwig Biotec®) and 5µL of DNA (~43.4ng/µL), amounting a final volume of 25µL.

The reactions were performed in a thermocycler (Veriti 96 Well Thermal Cycler - Applied Biosystems®, USA) using a temperature protocol with an initial stage of denaturation at 92°C for 1min, followed by 40 cycles of 92°C for 30s, 49°C for 45 s and 72°C for 1min, with a final extension of 72°C for 10min.

All reaction products were analyzed by 2% agarose gel electrophoresis and staining with ethidium bromide (5mg/mL). The size of amplified products was estimated using a pattern of 100 base pairs (Ladder 50 pb-Ludwig Biotec®) and a transilluminator coupled to a photodocumentation system was used for visualization (Quantum-ST4 1000/26M).

Statistical analysis. The dogs were considered infected when the microfilariae species was identified using the modified Knott technique or when the *D. immitis*-specific PCR was positive. In order to evaluate the association of the variables used in this study with infection by *D. immitis*, the data obtained was submitted to an exploratory analysis and subsequently to chi-squared and Fisher's exact test (Sampaio 2002), using the Statcalc tool of software EpiInfo 3.5.2 with a level of significance of 95% ($p < 0.05$).

RESULTS

Sixty-seven (67) animals from six months of age were included in the study and stratified per village as follows: 40 dogs from Vila de Algodual, five from Camboinha, ten from Mocooca, and 12 from Fortalezinha (Fig.1), where 38 were male and 29 female. Almost all animals were mongrel (66/67) and one animal was a Pit Bull Terrier. The study included animals aged from six to 12 months old (21/67), one and three years old (32/67) and above three years old (14/67).

Of the 67 blood samples collected, 35.8% (24/67) were considered to have been taken from animals infected with *Dirofilaria immitis* (Table 1). Vila de Camboinha was the only study location where infected animals were not identified. Vila do Algodual presented the highest rate of infected animals, 47.5% (19/40), compared to the infection rate among dogs from Fortalezinha or Mocooca, 25% (3/12) and 20% (2/10) respectively, although there was no statistical difference ($\chi^2=3.73$, $p=0.1549$, $GL=2$).

Male dogs were more infected (47.4%, 18/38) than females (20.7%, 6/29) ($\chi^2=4$, $p=0.0455$, $GL=1$). Younger dogs, regardless of their gender, were less infected ($\chi^2=6.34$,

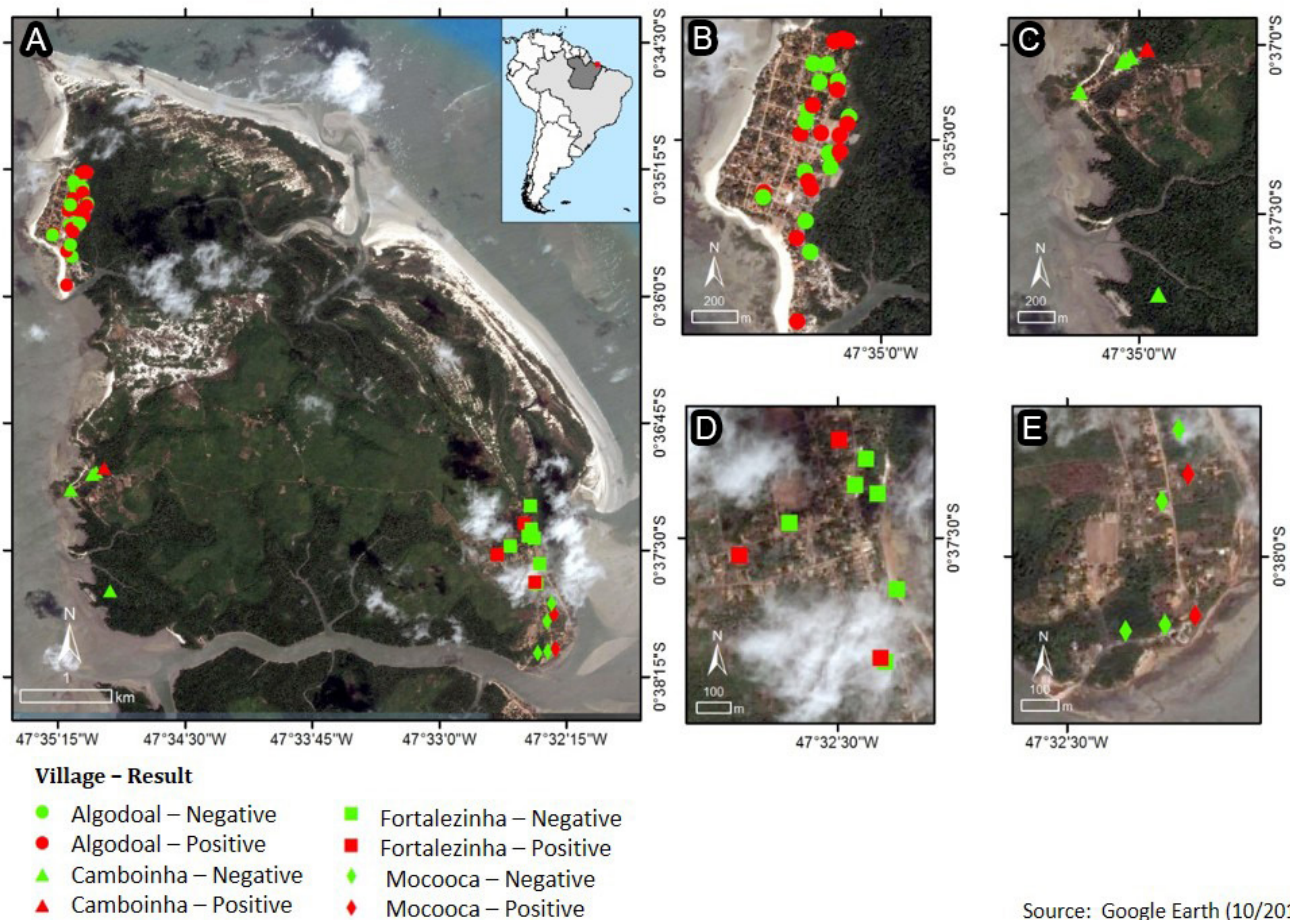


Fig.1. (A) Map of Algodual island using the images of Copernicus Sentinel-2 from August 9, 2017 and data on soil use and cover provided by SEMA. Each marker represents one household included in the study, according to the area studied. Red markers represent households with at least one infected animal; green markers represent households without infected animals in the villages of (B) Algodual, (C) Camboinha, (D) Fortalezinha and (E) Mocooca.

Table 1. Comparative results between microfilaria detection methods: modified Knott (K), thick bead (TB), fresh blood (FB), and detection of nucleic material (PCR) of *Dirofilaria immitis* microfilariae in dogs on Algodoal Island, Pará state, from July to September 2013

	Method			Total*
	K, TB, FB and PCR	K, TB, and PCR	K and PCR	
Positive	19	4	1	24
Negative	42	0	0	42
Total*	61	4	1	66

* One sample did not present microfilariae, but PCR was positive.

Table 2. Number of dogs ranked by gender and age regarding the occurrence of *Dirofilaria immitis* microfilariae on Algodoal Island, Pará state, from July to September 2013

Number of dogs	Female			Male			Total
	<1 year	>1 year and <3 years	>3 years	<1 year	>1 year and <3 years	>3 years	
Infected*	0	2	4	3	12	3	24
Free	8	10	5	10	8	2	43
Total**	8	12	9	13	20	5	67
% Infected		16.7 ^a	44.4	23.1	60 ^b	60	35.8

* Modified Knott technique, ** superscript different letters indicate $\chi^2=4.1$ and $p=0.04295$.

$p=0.0420$, $GL=2$). Although no female dog below one year old was infected among the study population, females were less infected than males only in the age range from one to three years old ($\chi^2=4.1$, $p=0.0429$, $GL=1$) (Table 2).

Of the 24 blood samples presenting microfilariae with *D. immitis* morphology, young forms with characteristics of *Acanthocheilonema reconditum* were also detected in 16 animals (66.7%). All animals with microfilariae also presented amplified DNA with primer pair 12SF and 12SRdeg and primers 12SF2B and 12SRdeg (24/24). On the other hand, in a sample without microfilariae, *D. immitis* DNA was detected by means of the same methodology. The fresh blood smear technique failed to detect microfilariae in five samples, whereas the thick blood technique failed to detect it in one sample (Table 1).

DISCUSSION

Canine infection with *Dirofilaria immitis* has been studied across the five regions of Brazil with different infection rates recorded in 14 out of 26 states (Bendas et al. 2017). Therefore, it is known that rates can vary over time and according to environmental or socioeconomic conditions (Labarthe et al. 2014). The most widely studied area in the state of Pará is the island of Algodoal, a touristic destination of the region where domestic animal health services are incipient. Vila do Algodoal is the village presenting the largest human resident population, in addition to being the gateway to the island; therefore, it concentrates the largest canine population. Most of the animals infected with *D. immitis* were found in this village (79,2%), which is likely to be due to the sample size. The prevalence rates show that infected dogs were found in all of the different villages of the island, except for the smallest one, Camboinha. The sample size was influenced by the size of each village, so that no difference was observed among prevalence rates. Nevertheless, it was

observed that 35.8% of the dogs in Algodoal island have microfilaremia of the *D. immitis* species, which indicates that all dogs residing or visiting the island should receive specific chemoprophylaxis. Additionally, previous reports have shown the need to use prophylaxis upon reporting that 46.8% (Furtado et al. 2012) and 42.4% (Castro et al. 2012) of the dogs in Algodoal village had microfilaremia in January and March 2012 respectively. As this study was carried out more than one year after the first records, in which prevalence rates for Algodoal village were similar to the findings of this study (47.5%), it may be inferred that animal welfare in the island has not improved in spite of the work done.

The findings of this study and the ones published in the islands of Algodoal (Castro et al. 2012, Furtado et al. 2012) and Marajó (Furtado et al. 2009) suggest that these islands in Pará have favorable environmental conditions for the transmission of *D. immitis* infection. It may be suggested that the most prominent of these conditions are, as in other regions of Brazil (Paiva 2009): high temperatures and relative humidity maintained throughout the year, an extensive area of preserved nature and the rare characteristic of sediment deposition along the coast in areas juxtaposed to the Amazon River, forming small sand strips.

In Brazil and elsewhere, filarial co-infection in dogs with microfilaremia has been diagnosed, where the species detected most often are *Acanthocheilonema reconditum* and *D. immitis* (Brito et al. 2001, Mar et al. 2002, Reifur et al. 2004, Argôlo et al. 2018). Co-infection with *A. reconditum* and *D. immitis* was diagnosed based on the morphological identification of microfilariae; however, due to the diversity and morphological similarity between immature forms of filarial species found in the Amazon, the morphological diagnosis must be interpreted with care, as it was not associated with molecular diagnosis and phylogenetic analysis (Argôlo et al. 2018). The fact that microfilariae were not detected by the

fresh and thick blood smear technique in five animals may reflect a low concentration of microfilariae, as the analytical sensitivity threshold of these tests is of 30Mf/mL (Bowman & Mannella 2011).

It is worth highlighting that microfilariae were not found in the blood sample of a five-year-old female dog from Algodual village, although the PCR detected the parasite's DNA. Given that finding *D. immitis* DNA in blood samples depends on the presence of microfilariae in the sample (Rossi et al. 2010), it is possible that: i) the dog did not have the disease and adult microfilariae had died, releasing DNA; ii) the microfilariae died as a result of sample manipulation; iii) the microfilariae died, freeing the dog from the disease months before the blood sample collection, as has been observed in animals treated with doxycycline (Rossi et al. 2010).

The infection was found more often among male dogs from one to three years old than among females within the same age range, which is not in line with other Brazilian studies that report not finding any differences between genders among infected animals (Almeida et al. 2001, Silva et al. 2008, Labarthe et al. 2014). It is likely that females are cared for differently in Algodual Island, which makes them less exposed to mosquitoes than males, or that they receive some protective treatment.

The chances of transmission via blood from infecting mosquitoes to susceptible hosts increase over time so much that young animals usually present lower infection rates (Hou et al. 2011, Labarthe et al. 2014), although in hyperenzootic areas, time of residence in the region has not influenced the prevalence rate (Labarthe et al. 2014). In addition, it must be considered that the prepatent period of the infection is of at least six months, which may disguise the results of animals under one year old (American Heartworm Society 2018).

The expressive prevalence rates of canine infection observed in the state of Pará, which vary from 32.4% to 46.8% (Castro et al. 2012, Furtado et al. 2009, Furtado et al. 2012), associated with the results of this study (35.8%), show that transmission of *D. immitis* is frequent, especially in the coastal and touristic areas of the state. Therefore, considering the importance of unified health care, especially since the parasite infects domestic or wild canids and human beings, all public health agents in the region must be committed to educating the population residing and visiting the region as to the risks of infection and prevention measures.

CONCLUSION

It may be concluded that the expressive prevalence rates of *Dirofilaria immitis* in the villages composing Algodual Island, associated to the animals' mobility, the island's environmental characteristics and the flow of inhabitants and visitors, points to the need to implement control and prophylaxis measures, which are responsibility of healthcare professionals acting in the corresponding fields of study.

Acknowledgements.- The authors thank CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil) for the financial support (scholarship) provided during this study.

Conflict of interest statement.- The authors declare no conflict of interest.

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