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Molecular diagnosis of *Dirofilaria immitis* in dogs in the eastern Amazon

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Abstract

Dirofilariasis, a heartworm disease, is an emerging anthropozoonosis that poses serious and imminent health risks to animals, in addition to affecting their quality of life and leading to death if not treated appropriately. Humans can also be infected through culicid vectors. Diagnosis is often performed by identifying microfilariae or by detecting antigens in blood samples. Therefore, the objective of this study was to diagnose animals positive for *D. immitis* using the Polymerase Chain Reaction (PCR) technique. Forty-nine dog samples were used for the agent research from veterinary clinics and laboratories located in Belém do Pará, Brazil, in 2022, of which 33% were positive for *D. immitis*, with a higher occurrence in males. However, there was no difference in infection when considering the sex of the animals. Considering that the state of Pará has a hot and humid climate, and some cities are bathed by rivers, it can be concluded that the general prevalence in dogs detected by PCR in this study was higher than expected and that this location can be considered an endemic area, and this is due to the presence of the mosquito species that are vectors of *D. immitis* being very frequent in this region.

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Introduction

Dirofilariasis has been considered a zoonosis by the World Health Organization since 1979 (1). Commonly called heartworm (2), the filariid is classified in the Order Spirurida, Family Onchocercidae, and Genus Dirofilaria; Herefore *Dirofilaria immitis* is the species of greatest veterinary medical interest (3,4). For the development of Parasite, an arthropod (intermediate host) and a vertebrate (definitive host) are necessary. Although the infection occurs mainly in domestic and wild canines, it can also occur in humans (5). Considering that these infectious conditions are vector-borne, the most important in the transmission of the nematode are the genera *Culex*, *Aedes*, *Anopheles*, and *Ochlerotatus* (6). Given the occurrence of cases in several countries, both within and outside the Americas, the disease mainly affects areas with temperate, tropical, and subtropical climates (7,8). Therefore, the State of Pará, being in a tropical zone, has a hot and humid climate with a stable thermal regime, which allows disease-transmitting mosquitoes to develop and reproduce more quickly (9). The prevalence in regions known as endemic is considered high in dogs, 40 to 70% (10). In places where animals test positive for *D. immitis*, the clinical routine for cases of humans with suspected lung neoplasms or fungal infections should consider human pulmonary heartworm disease as a differential diagnosis (11). Various laboratory techniques are used to diagnose the disease, such as microscopic, immunological, and molecular methods, with direct examination being the most used (12). However, polymerase

chain reaction has been proven to be a technique with high sensitivity and specificity, thus guaranteeing reliable results and applicability for diagnosing the infection (13).

This work aimed to use the technique to diagnose dogs naturally infected by *D. immitis* in the Eastern Amazon, considering the vulnerable and constant presence of vectors in this region.

Materials and methods

Ethical aspects

The samples used in the study were obtained following the standards recommended by the National Council for the Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee on the Use of Animals of the Federal Rural University of the Amazon (CEUA/UFRA) under the number 6531300620 (ID 000203).

Study and sampling areas

A total of 49 dogs were included in the study, and blood samples were made available by the Josyanne Christine veterinary clinic (Tamoios Street, Jurunas, Belém, Pará) and by the clinical analysis laboratories Vet Lab diagnostics (São Pedro, Campina, Belém, Pará) and Serology and Molecular Biology at the Institute of Animal Health and Production at the Federal Rural University of the Amazon, which provides services throughout the state of Pará - Brazil. Collections were carried out between June and August 2022.

Inclusion or exclusion criteria

The inclusion criteria were those who presented clinical symptoms such as circulatory disorders, cough, dysphagia, endocarditis, vomiting, bleeding in the oral cavity, lateralized mandibular enlargement, enlarged submandibular lymph nodes, weakness, anorexia, exercise intolerance, syncope, skin lesions and nodulations, hepatomegaly, and splenomegaly. In addition to these, animals that lived with dogs that had already tested positive at some stage of their lives for *D. immitis* were included in the research. Animals that did not show clinical signs or were being treated for the disease were excluded.

DNA extraction and purification

Blood samples treated with EDTA were subjected to DNA extraction using the commercial Bio Gene gDNA Extraction kit (Bioclin, Quibasa, Santa Branca, BH, Minas Gerais), according to the manufacturer's instructions. The quality and purity of the extracted DNA were assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with subsequent use.

DNA amplification

A sequence from the mitochondrial gene encoding the cytochrome oxidase I (COI) gene was used, which amplifies a region of 203bp, with COI F 5'-AGTGTAGAGGGTCAGCCTGAGTTA-3' and COI R 5'-ACAGGCACTGACAATACCAAT-3 as primers' (14,15). Each reaction contained a final volume of 25µL, including 12.5µL of Taq DNA Polymerase Master mix Red (Ampliqon A/S, Stenhuggervej, Odense M, Denmark), 1µL of each primer (at a concentration of 10 pmol), 3µL of the DNA sample and 7.5µL of DEPC ultrapure water (Uniscience, Osasco, São Paulo, Brazil). The thermal cycling conditions were established as follows: Initial denaturation at 94 ºC for 2 minutes, followed by 39 cycles at 94ºC for 30 seconds, 63ºC for 30 seconds, and 72ºC for 30 seconds, and a final extension step at 72ºC for 7 minutes. DNA extracted from the blood of a dog infected with *D. immitis* was used as a positive control, and a Master mix without DNA was used as a negative control. All conventional PCR assays were performed on a CFX96 Touch™ Real-Time Detection System thermocycler (Bio-Rad, Hercules, CA, USA), and amplicons were analyzed by 2% agarose gel electrophoresis and visualized under ultraviolet light.

DNA sequencing

The PCR amplicons were purified using a commercial kit (Qiagen, QIAquick PCR Purification Kit, Germantown, USA) and commercially sequenced (ACTGene, Alvorada, RS, Brazil) from the primers used in the PCR; a research study was then carried out to confirm the identities of the species of the sequenced amplicons.

Statistical analysis

The results obtained were tabulated in Excel 2016 spreadsheets, and simple percentage statistics were used to create the graph. To verify significance, the BioEstat 5.0 program used the chi-square and p-value tests together with Yates' continuity correction.

Phylogenetic analysis

Phylogenetic inference was performed from the nucleotide sequences of different regions of the COX1 protein of the mitochondrial DNA of different filarids containing 205 nucleotides available in the National Center for Biotechnology Information database. The dataset generated, together with the samples from that study, was submitted to Multiple Sequence Alignment (MSA) using the Mafft v.7 program (16). The alignment result was manually inspected to carry out manual alignment corrections, when necessary, using the Geneious v.9.1.8 program. Initially, the aligned dataset was subjected to analysis to identify the best nucleotide substitution model. Then, the construction of phylogenetic trees was performed using the Maximum Likelihood (ML) methodology (17). Both methodologies were employed using the IQ-TREE v.1.6.12 program (18). In conjunction with these analyses, the bootstrap test was used, setting 1000 replicates to provide greater reliability to the grouping values (19). The phylogeny visualization was performed using the FigTree v.1.4.4 program. For the dataset

used, it was decided not to use a root sequence; for this reason, the midpoint rooting methodology was used, a tool available in the phylogeny visualization program. After evaluating and editing the phylogeny, a file with the extension ". svg" (Scalable Vector Graphics) was generated for editing and manipulating the image using the Inkscape v.1.1 program (https://inkscape.org/release/inkscape-1.1/).

Results

49 samples of dogs were analyzed, 29 (59.18%) males and 20 (40.81%) females. Regarding age, the prevalence was higher in animals with an age range of 1-3 years at 67%, followed by 6-9 years at 43%, with a higher occurrence in males at 38%. However, there was no difference in infection when considering the sex of the animals (Figure 1). The total number of animals that tested positive for *D. immitis* by the PCR technique was 33%. When the age range was analyzed, there was a significant difference in the age groups of 1-3 years and >9 years; however, the sampling was low (Table 1). In PCR, 16/49 (33%) of the samples demonstrated positivity, where it was possible to detect the mitochondrial region of the gene - COI, resulting in a single band of 203 base pairs (Figure 2). The positive samples submitted for sequencing identified as *D. immitis* demonstrated the phylogenetic inference analyses and allowed us to understand more clearly the molecular characteristics of the samples and their evolutionary relationships established with other findings, including those from Brazil. The Phylogram prepared from the COX1 protein gene generated by the Maximum Likelihood (ML) method revealed that the species identified in this study were grouped in 91% with the sequences of samples from Brazil, specifically from the state of Rio de Janeiro and Australia, which also investigated *D. immitis* (Figure 3).

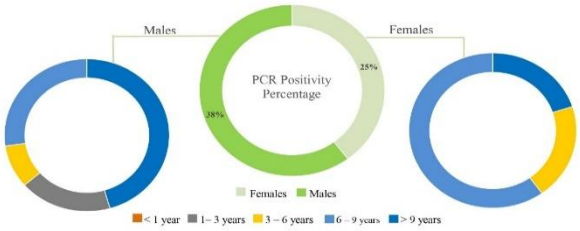

Figure 1: Percentage of positivity in males and females by Polymerase Chain Reaction.

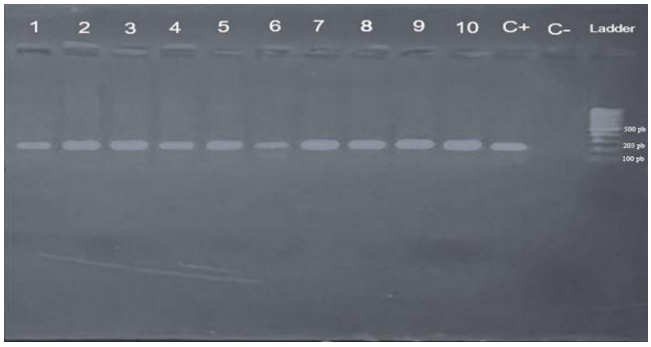

Figure 2: Electrophoresis of dogs that tested positive for *D. immitis*. Wells 1-10 refer to positive samples, well 11 positive controls with DNA from an animal previously positive for *D. immitis*, well 12 negative control, and well 13 DNA ladder (100 bp).

Table 1: Detection of *D. immitis* using the Polymerase Chain Reaction technique

Age	Male Positives/total (38%)	Female / Positives/total (25%)	Total / Positives/total (33%)	P value
< 1 year	0/2	0/0	0/2 (0%)	-
1 a 3 years	2/2 (100%)	0/1 (0%)	2/3 (67%)	< 0.0001
3 a 6 years	1/6 (17%)	1/4 (25%)	2/10 (20%)	0,217
6 a 9 years	3/8 (38%)	3/6 (50%)	6/14 (43%)	0,2008
> 9 years	5/11(45%)	1/9 (11%)	6/20 (30%)	< 0.0001
Total	11/29	5/20	16/49	0,1015

Discussion

The canine population in Brazil has accumulated growth of 3.5% per year (20), which is why dirofilariasis in dogs is alarming. It is caused by both the larval and adult life stages of *D. immitis*. The disease commonly occurs in endemic areas and where the vector is present, especially those of the Culex and Aedes genera, which are common in regions bathed by rivers and seas (21). Thus, the incidence may be higher or lower over time, depending on environmental and socioeconomic conditions (22).

In this study, the results of the diagnostics using the PCR technique allowed the identification of the bioagent. Of the 49 samples analyzed, 16 were positive for *D. immitis.* The prevalence rate of 33% of animals that tested positive for the parasite resembled studies carried out in the same state, in the Marajo archipelago in 2009 at 32.45% (23) and on Algodoal Island in 2019 at 35.8% (24). The relationship between the data and the investigations is a consequence of the density of the vectors in the region, such as the response to microclimatic elements (9) and the specificity and sensitivity of the PCR test (25).

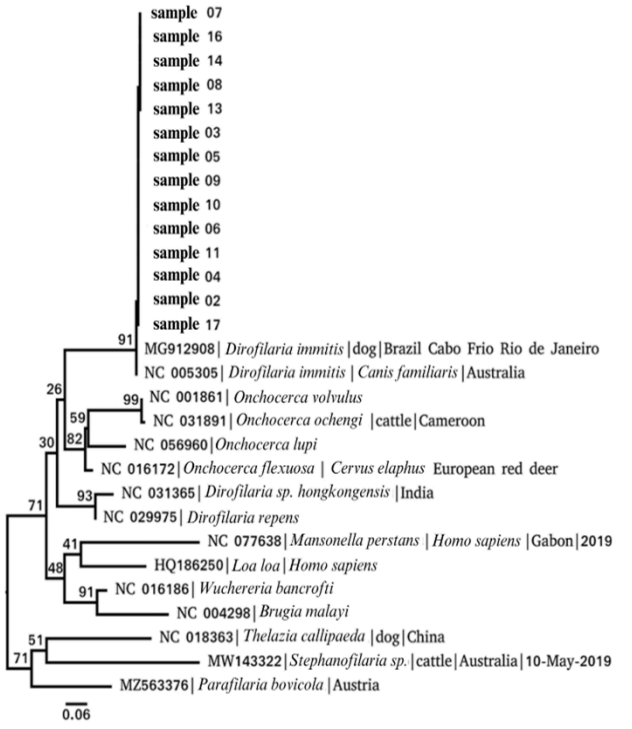

Figure 3: Phylogenetic tree generated by the Maximum Likelihood (ML) method based on the nucleotide sequences of a region of the COX1 protein of the mitochondrial DNA of different filariids containing 205 nucleotides, using TIM3+F+G4 as the best nucleotide substitution model. The numbers in each main node of the tree correspond to the bootstrap values in percentage (1000 replicates). The scale bar depicts the nucleotide divergence per site between the sequences.

Regarding the sex of these dogs, the most infected were males, 38%, and overall, there were statistically significant associations between males and females, although the infection can occur in both sexes. Similar results were obtained by several authors in Brazil of different cities, such as Maceio (26), Cabo Frio (27) and Marajo Island (23), and abroad, in countries such as South Korea (28), Dominican Republic (29), Spain (30) and Thailand (31). The data obtained corroborates other studies that mention a higher incidence of infection in males and that there is also a significant difference when considering sex (24,32). For some researchers, the lower prevalence of infection in females is justified by factors such as physiological state, age, and exposure to vectors (33). The presence of estrogenic hormonal activity can make females more resistant since estrogens can activate the immune system and thus inhibit the development of the worm (34).

On the other hand, when analyzing age, the incidence was higher in animals with an age range of 1 to 3 years at 67% (24m27); in general, a significant difference was observed, and when considering the value ($P<0.01$) the highest incidence was in animals whose ages were 1 to 3 years and > 9 years. This means that the period of existence of these dogs is a possibility of danger, so it can determine the time of exposure in places where *D. immitis* is endemic and the chances of transmission through blood meals of contaminated vectors to hosts, and depending on the age the level of microfilaremic animals increases progressively (22,35), which explains the negativity of dogs whose age was < 1 year. In addition to harming canine health, *D. immitis* poses risks to human health, as it is a zoonosis, and the importance of identifying infected animals is fundamental to minimizing the risk of transmission and treating the animals. Therefore, it can be inferred that the use of PCR to diagnose and/or confirm infection in clinical routine is interesting, especially in those cases in which microscopy is inconclusive.

Conclusion

The specific molecular technique allowed the detection of *D. immitis* in 16 dogs, which made it possible to obtain a positive result for dirofilariasis using only a small fraction of the DNA. The sequencing of the samples provided substantial information for the confirmation of the species. With this, the diagnosis through the Polymerase chain reaction is concluded as valid for the identification of canine microfilariae. It can be used in clinical routines to reduce diagnostic errors and favor assertive treatments in infected animals, as it is a more sensitive tool.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

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التشخيص الجزيئي لالتهاب دودة قلب الكلب الخيطية في الكلاب في شرق الأمازون

كارلا سيبيل باروس براغا[1]، إليم كريستينا ماسيدو بارا دي سوزا[1]، بيدرو هنريكي ماركيز باروزو[2]، جيزيل جيرمانا جايا تيكسيرا[3]، جوزيان كريستين سيلفا سواريس[4]، لوسيليا مارتينز دي أندرادي[4]، ليفيا ميديروس نيفيس كاسيب[5]، ساندرو باتروكا دا سيلفا[5]، إيجور غيريرو هاموي[6]، و ألكسندر دو روزاريو كاسيب[7]

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الخلاصة

الداء الدوراني، وهو مرض الديدان القلبية، هو مرض مشترك بين الإنسان والحيوان يمثل مخاطر صحية خطيرة ووشيكة على الحيوانات، بالإضافة إلى التأثير على جودة حياتها والوفاة إذا لم يتم علاجها بشكل مناسب. يمكن أيضًا أن يصاب البشر عن طريق نواقل البعوض. غالبًا ما يتم التشخيص عن طريق تحديد الميكروفيلاريا أو الكشف عن المستضدات في عينات الدم. لذلك، كان الهدف من هذه الدراسة هو تشخيص الحيوانات الإيجابية لـ D. immitis باستخدام تقنية تفاعل البوليميراز المتسلسل (PCR). تم استخدام تسعة وأربعين عينة من الكلاب للبحث عن العامل من العيادات البيطرية والمختبرات الموجودة في بليم دو بارا، البرازيل، في عام ٢٠٢٢، منها ٣٣٪ كانت إيجابية لـ D. immitis، مع حدوث أعلى في الذكور. ومع ذلك، لم يكن هناك فرق في العدوى عند النظر في جنس الحيوانات. نظرًا لأن ولاية بارا تتمتع بمناخ حار ورطب، وبعض المدن تغمرها الأنهار، يمكن استنتاج أن الانتشار العام في الكلاب الذي تم اكتشافه بواسطة PCR في هذه الدراسة كان أعلى مما هو متوقع وأن هذا الموقع يمكن اعتباره منطقة موبوءة، وهذا يرجع إلى وجود أنواع البعوض التي تنقل D. immitis بكثرة في هذه المنطقة.