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Dirofilaria immitis: Incidência, fatores associados à infecção e concomitância com Leishmaniose e Tumor Venéreo Transmissível em cães no semiárido do Nordeste, Brasil

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Dissertação submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Mestre em Ciência e Saúde Animal.

Dr. Antônio Flávio Medeiros Dantas

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ISABELA CALIXTO MATIAS

***DIROFILARIA IMMITIS*: INCIDÊNCIA, FATORES ASSOCIADOS À INFECÇÃO CONCOMITÂNCIA COM LEISHMANIOSE E TUMOR VENÉREO TRANSMISSÍVEL EM CÃES NO SEMIÁRIDO DO NORDESTE, BRASIL**

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SUMÁRIO

RESUMO

Esta dissertação é constituída por dois capítulos compostos por um artigo científico cada, produzidos através de pesquisas sobre o nematódeo *Dirofilaria immitis* em cães na região semiárida da Paraíba. O primeiro artigo, intitulado "Incidence and factors associated with *Dirofilaria immitis* in dogs in the semiarid region of Northeastern Brazil", foi submetido à revista *Veterinary Parasitology: Regional Studies and Reports.* Objetivou-se com este estudo, determinar a incidência, fatores de risco e a eficácia de testes de diagnóstico para *D. immitis* em cães do Canil Municipal de Sousa, Paraíba. Realizou-se teste imunocromatográfico, Knott modificado, esfregaço sanguíneo periférico e esfregaço sanguíneo capilar e exames de necropsia em 100 cães, além da coleta de dados como idade, sexo, raça, comprimento e cor da pelagem, e se o animal era reagente para Leishmaniose Visceral Canina (LVC). Para interpretação dos dados obtidos, foram realizadas as análises univariada e multivariada, além do coeficiente kappa ($p \leq 0.05$), sensibilidade, especificidade, valor preditivo positivo e valor preditivo negativo para avaliação da eficácia dos exames. A necropsia foi considerada como padrão ouro, e obteve-se uma incidência de 27% de cães com parasita adulto de *D. immitis*. Em 13% dos animais foram detectados antígenos de *D. immitis* através do teste rápido imunocromatográfico. A análise do coeficiente Kappa resultou em concordância moderada ($k=0,576$; $p>0,001$) entre os exames de necropsia e teste rápido imunocromatográfico. Além disso, os valores de sensibilidade (0,4814) e VPN (0,8390) foram maiores quando comparados aos outros exames, confirmando que o teste imunocromatográfico foi considerado o mais eficaz para ser utilizado no diagnóstico em animal vivo. A LVC foi considerada um fator estatisticamente significante ($p=0,03$), sendo um fator de risco para a parasitose. O segundo artigo, intitulado "Dirofilariasis, Leishmaniasis and Transmissible Venereal Tumor: concomitant affections in dogs in the Paraíba backwoods", a ser submetido para a revista *Acta Scientiae Veterinariae,* teve como objetivo relatar casos de *D. immitis,* LVC e TVT sob apresentação concomitante em cães necropsiados no Laboratório de Patologia Animal (LPA) da Universidade Federal de Campina Grande (UFCG). Para isso, foram revisadas as fichas de necropsia realizadas em cães nos anos de 2003 a 2019 e selecionado os casos de *D. immitis* e doenças associadas, para coleta de dados como sexo, raça, idade, procedência, sinais clínicos e achados anatomopatológicos. De 2465 cães necropsiados no período de estudo, quatro apresentavam *D. immitis* e leishmaniose (4/4) e dois apresentavam também TVT (2/4). No exame macroscópico, os principais achados foram linfonodos reativos, esplenomegalia com hiperplasia dos folículos linfoides e presença de *D. immitis* no ventrículo direito do coração e tronco pulmonar. Na avaliação histopatológica foram observadas figuras amastigotas de *Leishmania* spp. na medula óssea, linfonodos, baço e pele, além de glomerulonefrite membranoproliferativa nos rins e neoformação densamente celular e expansiva de células redondas no pênis de dois cães, característica de TVT. Através desses estudos, pode-se determinar a incidência de *D. immitis* na região semiárida da Paraíba, além de descrever a ocorrência de doenças concomitantes importantes, visto que são transmissíveis, e de interesse médico veterinário e de saúde única.

PALAVRAS-CHAVE: caninos; necropsia; diagnóstico anatomopatológico; dirofilariose; saúde única.

ABSTRACT

This dissertation consists of two chapters composed of one scientific article each, produced through research on the nematode *Dirofilaria immitis* in dogs in the semiarid region of Paraíba. The first article, entitled "Incidence and factors associated with *Dirofilaria immitis* in dogs in the semiarid region of Northeastern Brazil", was submitted to the journal Veterinary Parasitology: Regional Studies and Reports. The objective of this study was to determine the incidence, risk factors and efficacy of diagnostic tests for *D. immitis* in dogs from the Municipal Kennel of Sousa, Paraíba. Immunochromatographic test, modified Knott test, peripheral blood smear and capillary blood smear, and necropsy tests were performed in 100 dogs, in addition to data collection such as age, sex, breed, length and coat color, and whether the animal was reactive for Canine Visceral Leishmaniasis (CVL). Univariate and multivariate analyzes were performed to interpret the data obtained, in addition to the kappa coefficient ($p \leq 0.05$), sensitivity, specificity, positive predictive value, and negative predictive value to assess the effectiveness of the tests. Necropsy was considered the gold standard, and an incidence of 27% of dogs with adult *D. immitis* parasite was obtained. In 13% of the animals antigens of *D. immitis* were detected through the rapid immunochromatographic test. Kappa coefficient analysis resulted in moderate agreement ($k=0.576$; $p>0.001$) between necropsy and rapid immunochromatographic tests. In addition, the sensitivity values (0.4814) and NPV (0.8390) were higher when compared to other tests, confirming that the immunochromatographic test was considered the most effective to be used in the diagnosis in live animals. CVL was considered a statistically significant factor ($p=0.03$), being a risk factor for parasitosis. The second article, entitled "Dirofilariasis, Leishmaniasis and Transmissible Venereal Tumor: concomitant affections in dogs in the Paraíba backwoods", to be submitted to the journal Acta Scientiae Veterinariae, aimed to report cases of *D. immitis,* CVL and TVT under concomitant presentation in dogs necropsied at the Animal Pathology Laboratory (LPA) of the Federal University of Campina Grande (UFCG). For this, we reviewed the necropsy records performed on dogs in the years 2003 to 2019, and selected the cases of *D. immitis* and associated diseases, for data collection such as sex, breed, age, origin, clinical signs and pathological findings. Of 2465 dogs necropsied during the study period, four had *D. immitis* and leishmaniasis (4/4) and two also had TVT (2/4). On macroscopic examination, the main findings were reactive lymph nodes, splenomegaly with lymphoid follicle hyperplasia, and the presence of *D. immitis* in the right ventricle of the heart and pulmonary trunk. Histopathological evaluation showed amastigote figures of *Leishmania* spp. in bone marrow, lymph nodes, spleen and skin, as well as membranoproliferative glomerulonephritis in the kidneys and densely cellular and expansive round cell neoformation in the penis of two dogs, characteristic of TVT. Through these studies, it is possible to determine the incidence of *D. immitis* in the semiarid region of Paraíba, in addition to describing the occurrence of important concomitant diseases, as they are transmissible, and of veterinary medical interest and unique health.

KEYWORDS: canines; necropsy; anatomic-pathologic diagnosis; heartworm disease; cardiovascular system.

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CBS	Capillary Blood Smear
CEUA	Ethics Committee on the Use of Animals
CGDT	General Coordination of Transmissible Diseases
CGLAB	General Coordination of Laboratories of Public Health
CNPQ	National Council for Scientific and Technological Development
CVL	Canine Visceral Leishmaniasis
DPP	Dual Path Plataform
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
HE	Hematoxylin and Eosin
HV-ASA	Adílio Santos de Azevedo Veterinary Hospital
HVU	University Veterinary Hospital
IFPB	Federal Institute of Education, Science and Technology of Paraíba
LACEN	State Central Laboratory
MK	Modified Knott
NPV	Negative Predictive Value
Obj.	Objective
PB	Paraíba
PBS	Peripheral Blood Smear
PPV	Posite Predictive Value
sp.	Species (singular)
spp.	Species (plural)
TVT	Transmissible Venereal Tumor
UFCG	Federal University of Campina Grande

INTRODUÇÃO GERAL

A ocorrência de endoparasitoses transmitidas por artrópodes hematófagos nos cães tem chamado atenção pelo aumento do número de casos descritos na literatura e observados na rotina clínica. *Dirofilaria immitis* (LEIDY, 1856) é um exemplo de nematódeo transmitido por mosquitos (*Aedes* spp., *Culex* spp. e *Anopheles* spp.), que possui o cão como hospedeiro definitivo, e pode causar uma doença crônica e zoonótica chamada dirofilariose ou filariose canina (VIDAL, 2014).

A dirofilariose ocorre em cães intensamente parasitados, onde os vermes adultos alojam-se no ventrículo direito e vasos pulmonares, gerando manifestações clínicas como tosse, dispneia, intolerância a exercício e apatia, que podem ser atribuídas a outras patologias de etiologia cardíaca, dificultando assim o seu diagnóstico. Animais que apresentam carga parasitária baixa são assintomáticos, servindo apenas como fonte de infecção para outros cães domésticos, canídeos silvestres, felinos e o homem (VIDAL, 2014).

Em humanos, os vermes adultos causam nódulos granulomatosos nos pulmões que são frequentemente confundidos por neoplasias, contribuindo para a negligência da doença (SILVA & LANGONI, 2008).

Índices de ocorrência de *D. immitis* encontram-se em todo o território brasileiro, mais especificamente em regiões costeiras (GARCEZ et al., 2006) onde há uma maior prevalência e as condições edafoclimáticas que favorecem o desenvolvimento e a proliferação dos vetores (LABARTHE, 2014).

A prevalência de *D. immitis* em regiões costeiras do Nordeste brasileiro tem sido determinada principalmente através de exames sanguíneos e parasitológicos, com um resultado de 0,33% (1/301) em João Pessoa, Paraíba (VIDAL, 2014), 10,4% (64/613) em Salvador e Lauro de Freitas, na Bahia, (ALMEIDA et al., 2001), 1,36% (15/1097) em Maceió, Alagoas (BRITO et al., 2001), 2,3% (14/611) em Recife, Pernambuco (ALVES et al., 1999) e de aproximadamente 15% (224/1495) na Ilha de São Luís, Maranhão (AHID et al., 1999).

Entretanto, no município de Sousa, no alto Sertão da Paraíba, nos últimos quatro anos tem chamado a atenção a ocorrência da parasitose, identificadas através da necropsia ou pela visualização de microfilárias em esfregaços sanguíneos. Adicionalmente, notou-se em necropsias de cães com outras doenças, a exemplo da Leishmaniose Visceral Canina, que, de forma incidental, havia concomitante a presença de *D. immitis*. Desta forma, destaca-se a importância da realização da necropsia de cães eutanasiados com leishmaniose, tendo em

vista que estes podem ser considerados sentinelas para a ocorrência do parasito ou da doença em uma determinada região.

Portanto, essa dissertação teve como objetivo estudar a incidência de *D. immitis* em cães do Canil Municipal de Sousa, Paraíba, mediante pesquisas de antígenos, microfilárias e parasitas adultos, determinar fatores associados à infecção pelo parasito e avaliar a eficácia dos exames de diagnóstico. Além disso, relatar casos da ocorrência concomitante de *D. immitis*, LVC e TVT em cães necropsiados no LPA do Hospital Veterinário Universitário Prof. Dr. Ivon Macêdo Tabosa da Universidade Federal de Campina Grande (HVU/UFCG), campus Patos, Paraíba.

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CAPÍTULO I:

Incidence and factors associated with *Dirofilaria immitis* infection in dogs in the semi-arid Northeast, Brazil

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Incidence and factors associated with *Dirofilaria immitis* infection in dogs in the semi-arid Northeast, Brazil

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ABSTRACT

Dirofilaria immitis is a zoonotic filarial nematode that parasitizes the dog's right ventricle of the heart and pulmonary trunk, causing a chronic disease called heartworm. Although *D. immitis* is considered in the literature as prevalent in coastal regions, the occurrence in necropsied dogs of the semi-arid region of Brazil aroused the need for studies to confirm the parasitism, in addition to identifying factors associated with its occurrence. The objectives of this study were to determine the incidence of *D. immitis* and factors associated with its infection in dogs from the semi-arid region of Paraíba, as well as to compare the effectiveness of diagnostic tests. One hundred dogs from the municipal kennel of Sousa, Paraíba, older than six months of age, which euthanized according to the protocol established by the establishment were evaluated. Data such as age, sex, race, and coat size and color were collected for univariate and multivariate analysis according to the results of the exams. For the diagnosis of *D. immitis* were performed the rapid immunochromatographic test, modified Knott (MK), peripheral blood smear (PBS), capillary blood smear (CBS), and necropsy examination. For the evaluation of diagnostic tests, the necropsy was considered the gold standard. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Kappa coefficient ($p \leq 0.05$) were calculated. There was an incidence of 27% (27/100) of dogs with adult forms of *D. immitis* found during necropsy in the right atrium and ventricle, pulmonary arteries, and bronchi lumen. In 13% of the animals, antigens of *D. immiti*s were detected through the rapid immunochromatographic test. The analysis of negative and positive results by the Kappa coefficient resulted in moderate agreement ($k=0.576$; $p>0.001$) between the necropsy and rapid immunochromatographic test. In addition, the sensitivity (0.4814) and NPV (0.8390) values of the latter were higher when compared to the other exams. The diagnosis of

canine visceral leishmaniasis was statistically significant (p=0.03), being considered a factor associated with *D. immitis* infection. This study confirmed the high incidence of *D. immitis* in the semiarid region of Paraíba. Among the exams performed, necropsy and immunochromatographic test allowed the diagnosis of occult infections. The use of immunochromatographic and MK tests is recommended to detect *D. immitis* in live animals in regions that have no reports of its occurrence since most animals are asymptomatic and possibly underdiagnosed.

Keywords: Nematode. Cardiovascular system. Necropsy. Paraíba.

1 Introduction

Dirofilaria immitis is a nematode of great veterinary importance, which parasitizes the circulatory (heart and large vessels), lymphatic and subcutaneous tissue of dogs, and less frequently of cats (Acha and Szyfres, 2003). Mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles* are the main intermediate hosts involved in third-stage larvae (L3) transmission of the parasite to dogs (Cruz, 2017).

Considered a parasite of tropical and subtropical climate regions, its prevalence has been higher in coastal areas, where the climate is hot and humid (Garcez et al., 2006; Labarthe et al., 2014). The first report of *D. immitis* infection in a dog in South America was described in Bahia state, Brazil, in 1878 (Pinto, 1944). Since then, Brazil has had a higher number of studies on the prevalence of the parasite when compared to other countries on the continent (Bendas et al., 2017).

Among the diagnostic methods, microfilaria concentration tests, such as the modified Knott test, are more accurate and indicated when compared to blood visualization tests, due to the low use of materials and quick execution (Bowman et al.,

2006). For the detection of antigens in the adult female, the Enzyme-Linked Immunosorbent Assay (ELISA) and immunochromatographic tests can be used (Genchi et al., 2018).

In animals that died and it was not possible to diagnose *D. immitis* infection in life, through necropsy it is possible to visualize the adult worms in the right ventricle of the heart, and pulmonary vessels and bronchi, diagnosing occult infections when the animal presents adults parasites, but still do not have circulating microfilariae (Almeida, 2010; Almeida, 2014; AHS, 2014). Necropsy has also been used as a parameter to check disease status in dogs in studies aimed at investigating the sensitivity and specificities of other tests (Henry et al., 2018).

Surveys using necropsy as the gold standard for evaluating other diagnostic tests and determining prevalence were carried out in Florida, United States of America (Henry et al., 2018); Yucatan, Mexico (Bolio-Gonzalez et al., 2007); Dandong, China (Hou et al., 2011) and Recife, Brazil (Alves et al., 1999).

In the semiarid region of Paraíba, in a retrospective study carried out at the Animal Pathology Laboratory of the Federal University of Campina Grande (UFCG), city of Patos, in the period 2003 to 2012, two cases of *D. immitis* (adult parasite) were found as necropsy finding in dogs (Almeida, 2014). More recently, *D. immitis* was also identified in the right ventricle of a dog's heart in the city of Sousa (Matias et al., 2016).

However, in the last four years, the occurrence of adult nematodes in the right ventricle of the heart, and pulmonary vessels and bronchi with morphology similar to *D. immitis* has been frequent in dogs necropsied at the Animal Pathology Laboratory of the Federal Institute of Education, Science and Technology of Paraíba (IFPB), Campus Sousa. Although *D. immitis* is considered in the literature as a nematode prevalent in

coastal regions (Manobe et al., 2016; Soares et al., 2014), the occurrence of parasites with similar morphology in necropsied dogs in the semi-arid region raises the need for studies that confirm the parasitism, in addition to identifying factors associated with its occurrence.

Therefore, the objectives of this study were to determine the incidence of *D. immitis* and factors associated with infection in dogs from the semi-arid region of Paraíba, as well as to compare the effectiveness of necropsy, immunochromatographic, modified Knott, peripheral blood smear, and capillary blood smears for the diagnosis of *D. immitis*.

2 Materials and Method

2.1 Characterization of the study area

The study area comprised the city of Sousa, located in Paraíba backwoods, Brazil, which has a territorial area of 738,547 km², with latitude 06º45'33" south and longitude 38º13'41" west (IBGE, 2017). It has 65,803 inhabitants, with a demographic density of 89.10 inhab./km², the majority coming from urban areas (IBGE, 2010). The climate is considered tropical semi-arid, with maximum rainfall of 1278.6 mm in 2019 (AESA, 2019) and vegetation predominantly composed of hyperxerophile caatinga and stretches of deciduous forest (Beltrão et al., 2005).

2.2 Selection of animals

The Municipal Kennel located in Sousa - PB was previously selected for the collection and analysis of blood from captured stray dogs, aiming to identify the presence of microfilariae of *D. immitis*, as well as observation of adult parasites in dogs

necropsied after euthanasia. The reasons for euthanasia of the animals varied between a positive diagnosis for Canine Visceral Leishmaniasis (CVL), as a preventive measure of disease control adopted by the municipality, in addition to an advanced state of some chronic disease, excessive weakness, and no therapeutic response, always carried out following the ethical precepts of animal welfare on euthanasia methods, as described in the Brazilian Guide of Good Practices in Animal Euthanasia - Recommended Concepts and Procedures (2013).

The diagnosis of CVL was performed at the State Central Laboratory (LACEN), in João Pessoa - PB, through ELISA, considered a confirmatory test by joint technical standard number 01 of the year 2011 developed by the General Coordination of Transmissible Diseases and General Coordination of Laboratories of Public Health (CGDT/CGLAB) (Brazil, 2011).

One hundred dogs, aged over six months, euthanized from April 2018 to November 2020 were evaluated. From these, data were collected from identification forms, such as age, sex, breed, size (short, medium, or long), and coat color (light or dark).

2.3 Blood collection and preparation of blood smear slides

Before euthanasia, 5 ml of blood from the cephalic vein was collected from each animal, and 2 ml was transferred to a sterile tube containing EDTA anticoagulant (Ethylenediaminetetraacetic acid) for subsequent performance of the modified Knott test (MK) and peripheral blood smear (PBS); and 3 ml transferred to a sterile tube without additives, for the subsequent rapid immunochromatographic test (ALERE Dirofilariasis Ag Test Kit®). Capillary blood smear (CBS) was also performed through a drop of blood obtained from the tip of the ear.

Two slides were made for each technique. The slides of the blood smears were fixed and stained with Rapid Panoptic®, identified, and then microscopically evaluated.

2.4 Research and identification of microfilariae

For the investigation of circulating microfilariae, three methods were performed: capillary blood smear (CBS), peripheral blood smear (PBS), and modified Knott test (MK) (Newton and Wright, 1956). The visualization of only one microfilaria classified the animal as positive. MK was performed as described by Bowman et al. (2006) using 1 ml of blood containing EDTA anticoagulant, and in positive animals, through this test, the morphometric diagnosis of microfilariae was performed, considering the values mentioned by CVBD (2006) to differentiate *D. immitis* microfilariae from other filariasis, as *Acanthocheilonema* (*Dipetalonema*) *reconditum*.

2.5 Immunochromatographic test

To perform the immunochromatographic test for detection of *D. immitis* antigens (ALERE Heartworm Ag Test Kit®) animal serum was used. This test has a specificity of 100% and a sensitivity of 98.6%. To obtain the serum, the tubes with blood collected without additive were centrifuged at 1500 rpm for 5 minutes, and the serum was collected and frozen at -20º C. Subsequently, the sera were thawed and homogenized, and with the aid of a pipette, two drops were added to the test sample well, waiting 10 to 15 minutes for result interpretation, according to the manufacturer's information.

2.6 Animals necropsy

After euthanasia, the necropsy was performed to search for adult parasites in the right ventricle of the heart, and pulmonary arteries and bronchi. The parasites were collected and fixed in 10% formalin, for later total counting of adults. The sexing of the parasites was not performed.

2.7 Statistical analysis

For data analysis and interpretation, dogs that were positive in any of the tests described in the methodology were considered infected with *D. immitis*. Based on the data obtained from the animal identification forms, it was possible to carry out an analysis to verify a possible association between the animal data (independent variable) and the necropsy result (dependent variable). The analysis was carried out in two steps.

Initially, an exploratory univariate analysis was performed to select variables that presented $p \leq 0.2$ using the chi-square test or Fisher's exact test. In the second stage, the selected variables were submitted to a multivariate analysis through multiple logistic regression at a significance level of 5% (Hosmer and Lemeshow, 2000). The adjustment of the final model was made using the coefficient of Hosmer and Lemeshow so that there was a good adjustment of the value of $p \leq 0.05$. The collinearity of the independent variables was performed by correlation analysis, which, if they presented a correlation coefficient <0.9, one of the variables would be eliminated according to biological plausibility (Dohoo et al., 1997).

For the evaluation of diagnostic tests, the necropsy was considered the gold standard, as for the animal to present circulating microfilariae and detectable antigens, it must be infected by at least two adult parasites of opposite sexes and by at least one mature female, respectively (AHS, 2014). By crossing the results, it was possible to calculate the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Furthermore, to verify the agreement between necropsy and MK, necropsy and PBS, necropsy and CBS, and necropsy and rapid immunochromatographic test, the Kappa test was performed ($p \leq 0.05$). Data analyzes were performed using SPSS 23.0 software for Windows.

To the data interpret, the classification described by Landis and Koch (1977) was adopted, where Kappa<0=no agreement, Kappa between 0 and 0.2=weak agreement, Kappa between 0.21 and 0.4= reasonable agreement, Kappa between 0.41 and 0.6=moderate agreement; Kappa between 0.61 and 0.8=strong agreement and kappa between 0.81 and 1=almost perfect agreement.

3 Results

The incidence of dogs with adult forms of *D. immitis* found during necropsy in the right atrium and ventricle (Figure 1 A and B), and pulmonary arteries and bronchi lumen was 27% (27/100) (Figure 1 C and D). The estimated number of adult worms ranged from 1 to 112 specimens. The general data of dogs positive for *D. immitis* and results of the necropsy, immunochromatographic test, MK, PBS, and CBS are summarized in table 1.

In 13% of the animals, *D. immitis* antigens were detected through the rapid immunochromatographic test. Among the methods for visualizing microfilariae in the blood (Figure 2), MK and PBS detected 7% of positive animals each. Among the 27% of dogs positive at necropsy, one was positive only in MK (dog 20). In two dogs there were differences in the pattern of results with the other animals, that is, they were positive in the necropsy, however, negative in the immunochromatographic test, presenting microfilariae only in the CBS and MK (dog 21) and PBS (dog 37).

Only two dogs were simultaneously positive for *D. immitis* in all tests performed. There were no dogs negatives on necropsy that were positive on other tests. All microfilariae were morphologically evaluated, being classified as *D. immitis*. The

analysis of diagnostic tests using the Kappa agreement index, sensitivity, specificity, PPV, and NPV are described in table 2.

The analysis of negative and positive results by the Kappa coefficient resulted in moderate agreement (k=0.576; p>0.001) between the necropsy and rapid immunochromatographic test. In addition, the sensitivity (0.4814) and NPV (0.8390) values were higher when compared to the other exams. The values of the Kappa coefficient, PPV, and NPV of the MK and PBS tests were equal (k=0.338; p>0.0001; NPV=0.7849), therefore, considered with reasonable agreement. For the CBS exam, the Kappa coefficient value obtained a weak agreement (k=0.202; p=0.001).

Of the 27 positive dogs, 81.48% (22/27) were of mixed breed, 7.41% (2/27) poodle, 7.41% (2/27) rottweiler, and 3.70% (1 /27) pinscher. The number of positive males (18/27) was higher than females (9/27), and the predominant coat was short and dark (20/27). There were no significant differences in the variables race, sex, and length and coat color of the animals (p>0.05). The variable age showed little statistical difference (p=0.07), where all positive dogs (27/100) were older than 12 months. The diagnosis of CVL was considered statistically significant (p=0.03) because 20 animals positive for the disease had the disease diagnosed through parasitological tests and/or ELISA.

4 Discussion

The incidence values of parasitism by *D. immitis* in dogs in this study were considered high, suggesting the possibility of underdiagnosed cases. Additionally, it is believed that the lack of knowledge on the part of tutors and veterinarians about its incidence in semi-arid regions, associated with the absence of clinical signs in animals

with low parasitemia, makes this diagnosis of *D. immitis* and consequently its prophylaxis difficult. It is important to emphasize that dogs are the main reservoirs of this zoonotic nematode (Manobe et al., 2016), therefore, considering the high incidence of *D. immitis* in dogs in the region, the need to include it in the list of differential diagnoses of lung diseases in humans is highlighted.

The increase in dog diseases transmitted by arthropod vectors in non-endemic areas is related to the increase in travel by tutors with their dogs around the world, relocation of animals from endemic to previously non-endemic areas, in addition to climate and environmental changes that favor the proliferation of vectors (Mendoza-Roldan et al., 2020; Wilke et al., 2019). Although these factors have not been evaluated in this study, it is believed that they may influence the high incidence of parasitism in the semi-arid region. It is also noteworthy that although the evaluated dogs came from the Municipal Kennel of Sousa, it is believed that the factors listed above may influence the incidence of parasitism in other cities surrounding the city of Sousa. Failure to perform tests in a clinical routine can lead to underdiagnosis since most dogs remain asymptomatic.

The superior efficacy of the immunochromatographic test concerning microfilariae research methods was confirmed by the moderate agreement obtained by the Kappa coefficient ($k=0.576$; $p>0.001$) and high sensitivity (0.4814) and NPV (0.8390), demonstrating that this is the most accurate and indicated method to perform the diagnosis in live animals, as in addition to being easy to perform and interpret, it is capable of detecting occult infections (AHS, 2014).

Recent studies comparing the sensitivity and specificity of immunochromatographic tests of other brands with the presence of adult *D. immitis* parasites have also demonstrated high efficacy of this antigen search test (Genchi et al.,

2018; Henry et al., 2018). To date, there have been no studies comparing the effectiveness of the ALERE Heartworm Ag Test Kit® with necropsy results.

It is believed that the non-detection of antigens in the immunochromatographic test in dogs that presented the adult parasite observed during the necropsy (dogs 04, 09, 10, 20, 21, 31, 33, 37, 40, 64, 65, 78, 95 and 99), may be associated with the use of unreported adulticidal drugs, or the presence of only immature male or female nematodes, as this test detects proteins secreted mainly by the mature female parasite (AHS, 2014; Courtney and Cornell, 1990). It is also suggested that interference with the immunochromatographic test result may occur due to the formation of antigen-antibody complexes that end up blocking the detection of *D. immitis* antigens by these commercial tests (AHS, 2014; Velasquez et al., 2014; Drake et al., 2015), which possibly justifies the cases that were positive for microfilariae in the blood tests and false-negative in the immunochromatographic exam (dogs 20, 21 and 37).

Studies demonstrate that the formation of antigen-antibody complexes in dogs with *D. immitis* is directly related to chronic inflammation caused by the presence of the agent, the individual characteristic of each dog's immune system, and the use of macrocyclic lactones, with or without the use of doxycycline, for the monthly treatment or prevention of the infection, causing the death and elimination of the parasites over a long period (AHS, 2014; Drake et al., 2015).

Additionally, the animals that were positive in the immunochromatographic test and negative for microfilariae test (dogs 22, 28, 61, 84, 92, and 97) possibly occurred due to the presence of only one sex of the parasite, making sexual reproduction and elimination of first-stage larvae into the bloodstream impossible; a little amount of circulating microfilariae; treatment with macrocyclic lactones, which ended up acting as

a microfilaricide; or pre-patent time of less than 6-7 months (AHS, 2014; Almeida, 2010).

The weak agreement (k=0.202; p=0.001) between the CBS and necropsy tests indicated that the CBS was less reliable, diverging from the idea suggested by Moreno (2015) that this test could increase the sensitivity of microfilariae detection, suggesting that they have a predilection for peripheral capillary zones. It is believed that CBS was less effective when compared to other tests, probably due to the small amount of blood used to make the slide, due to the collection site (the pinna) being irrigated by small vessels. In addition, the CBS and PBS had a lower NPV (0.07604 and 0.7849, respectively) when compared to the immunochromatographic test, indicating that they have a low-efficiency rate for identifying truly negative animals.

The occurrence of cases with a large number of adult stages of the parasite found during necropsy in the right ventricle and pulmonary arteries and bronchi is highlighted. However, some of these animals were negative for microfilariae research. Although it has not been reported, it is believed that these dogs were possibly treated with macrocyclic lactones, such as ivermectin, aiming at deworming other parasitic diseases, causing the unintentional death of microfilariae (Meireles et al., 2014). Furthermore, this fact confirms again that blood smear and concentration methods do not have a high sensitivity for detecting the truly negative.

The PPV value calculated in all tests have been considered 100%, as the detection of the antigen or the observation of only one microfilaria already classified the animal as positive, that is, both are capable of accurately diagnosing the truly positive.

There were no significant differences in the variables sex, race, and coat and coat color of the animals (p>0.05), in agreement with the studies carried out by Almeida

et al. (2001), who also did not demonstrate significant differences for sex, race, and hair color and length, in addition to the age of animals positive for *D. immitis*. Hou et al. (2011) found no significant difference only concerning the sex of the animals. However, there is a need to consider that most of the animals in the study were male, of no defined breed, and with short and dark coats, justifying the fact that they are the main positives for *D. immitis.*

Almeida et al. (2001) reported that male dogs are more prone to infection due to more frequent activities such as hunting, guarding and sports, which favor exposure to vectors; as well as the estrogen produced by females that generates an innate immunity, preventing the development of the filaria in this sex. Regarding breed, it is believed that small animals are less exposed to vectors, unlike larger breeds, which, due to their size, usually stay outdoors and develop outdoor activities (Almeida et al., 2001; Vieira et al., 2014). In this study, all dogs were strays, and most dogs that live in these conditions are of mixed breed, which favors their exposure to *D. immitis* vectors.

Age was a factor with little significant difference for dogs over 12 months of age. It is known that *D. immitis* infection is more incident in older dogs when compared to younger ones, due to longer lifetime exposure to the vectors (Vieira et al., 2014).

CVL was considered a statistically significant factor in this work. It is suggested that the consequent low immunity due to CVL may have favored the installation and rapid reproduction of the nematode in these animals, explaining the high parasite load found in some of the dogs. Recent works on the concomitance of CVL with *D. immitis* were carried out by Mendoza-Roldan et al. (2020) in Italy and by Ntais et al. (2016) in Greece. The concomitance of animals positive for CVL and *D. immitis* should draw the attention of public health agencies in the city to establish control and prevention measures, considering that *D. immitis* and *Leishmania* sp. are zoonotic pathogens.

Eliminating vectors is the main form of control. However, the use of repellent collars, deworming with drugs that act against *D. immitis*, the use of window screens, and avoidance of walks between the peak hours of the vector is also recommended.

5 Conclusion

This study revealed the high incidence of *D. immitis* in the semi-arid region of Paraíba. Necropsy examination and immunochromatographic test allowed the diagnosis of occult infections caused by the parasite.

Immunochromatographic and MK tests are recommended by veterinary clinicians for the diagnosis of *D. immitis* infection in live animals in regions that do not have reports of its occurrence, as it is believed that there may be an underdiagnosis, owing to most infected animals do not show clinical signs.

Considering that CVL was a factor associated with *D. immitis* infection, the importance of performing the necropsy examination is noted, since these animals end up acting as sentinels for infections, both considered zoonoses.

6 Ethical approval

This study was submitted to the Ethics Committee on the Use of Animals (CEUA) of the IFPB, Sousa campus, for the use of animals in teaching, research, and extension activities, and approved under registration number 01.0462.2017.

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Table 1 - General data of dogs positive for *Dirofilaria immitis* and results of necroscopic exams, immunochromatographic test, modified Knott, peripheral blood smear, and capillary blood smear.

Dog	Breed	Coat	Sex	Age	CVL	CBS	PBS	MK	Quick test	Necrop./nº heartworms
04	Mixed breed	Short Dark	M	4 years	Reag.	-	-	-	-	4 adults
09	Mixed breed	Short Dark	F	4 years	Reag.	-	-	-	-	1 adult
10	Mixed breed	Long Dark	M	4 years	Reag.	-	-	-	-	1 adult
13	Mixed breed	Short Dark	M	2 years	Reag.	-	+	+	+	2 adults
15	Mixed breed	Short Dark	M	2 years	Reag.	-	+	+	+	8 adults
18	Mixed breed	Short Dark	M	2 years	Reag.	-	-	-	+	112 adults
20	Mixed breed	Short Dark	M	> 1 year	Non-reag.	-	-	+	-	4 adults
21	Poodle	Medium Light	F	> 1 year	Reag.	+	-	+	-	1 adult
22	Pinscher	Short Dark	M	> 1 years	Reag.	-	-	-	+	1 adult
28	Mixed breed	Short Dark	F	5 years	Reag.	-	-	-	+	26 adults
31	Rottweiler	Short Dark	M	3 years	Reag.	-	-	-	-	20 adults
33	Mixed breed	Short Dark	M	4 years	Reag.	-	-	-	-	2 adults
37	Mixed breed	Short Light	M	> 1 year	Non-reag.	-	+	-	-	4 adults
40	Mixed breed	Short Light	F	> 1 year	Non-reag.	-	-	-	-	1 adult
43	Mixed breed	Short Dark	F	2 years	Reag.	+	+	-	+	1 adult
55	Mixed breed	Short Dark	F	7 years	Reag.	+	+	+	+	5 adults
61	Mixed breed	Short Dark	M	4 years	Reag.	-	-	-	+	8 adults
64	Mixed breed	Short Light	M	7 years	Reag.	-	-	-	-	7 adults

Continued

Table 1 - General data of dogs positive for *Dirofilaria immitis* and results of necroscopic exams, immunochromatographic test, modified Knott, peripheral blood smear, and capillary blood smear.

Dog	Breed	Coat	Sex	Age	VCL	CBS	PBS	MK	Quick test	Necrop./nº heartworms
65	Rottweiler	Short Dark	M	3 years	Reag.	-	-	-	-	1 adult
74	Mixed breed	Short Dark	M	2 years	Reag.	+	+	+	+	28 adults
78	Mixed breed	Short Light	F	> 1 year	Reag.	-	-	-	-	4 adults
84	Mixed breed	Short Dark	F	> 1 year	Non-reag.	-	-	-	+	8 adults
92	Poodle	Short Dark	M	> 1 year	Reag.	-	-	-	+	5 adults
95	Mixed breed	Short Dark	M	> 1 year	Non-reag.	-	-	-	-	2 adults
97	Mixed breed	Short Dark	M	> 1 year	Non-reag.	-	-	-	+	1 adult
99	Mixed breed	Short Dark	F	> 1 year	Reag.	-	-	-	-	1 adult
100	Mixed breed	Short Light	M	> 1 year	Non-reag.	-	+	+	+	2 adults

VCL - Canine Visceral Leishmaniosis; CBS – Capillary blood smear; PBS – Peripheral blood smear; MK – Modified Knott Test; Necrop./nº heartworms - Necropsy and number of heartworms found; M – male; F – female; Reag. – Reagent; Non-reag. – Non-reagent.

Table 2 – Kappa statistics between necropsy results and other tests, according to the classification between positive and negative dogs for *Dirofilaria immitis*.

	Necropsy results		
	Positive	Negative	
Immunoc. result			Sensit. = 0,4814 / Specif = 1
Positive	13	0	PPV = 1 / NPV = 0,8390
Negative	14	73	k = 0,576; p < 0.001
MK result			Sensit. = 0,2592 / Specif. = 1
Positive	7	0	PPV = 1 / NPV = 0,7849
Negative	20	73	k = 0,338; p > 0,0001
PBS result			Sensit. = 0,2592 / Specif. = 1
Positive	7	0	PPV = 1 / NPV = 0,7849
Negative	20	73	k = 0,338; p > 0,0001
CBS result			Sensit. = 0,1481 / Specif. = 1
Positive	4	0	PPV = 1 / NPV = 0,7604
Negative	23	73	k = 0,202; p = 0,001

Immunoc. - immunochromatographic test; PBS - peripheral blood smear; CBS - capillary blood smear; Sensit. – sensitivity; Specif. – specificity; PPV – positive predictive value; NPV – negative predictive value; k = Kappa coefficient.

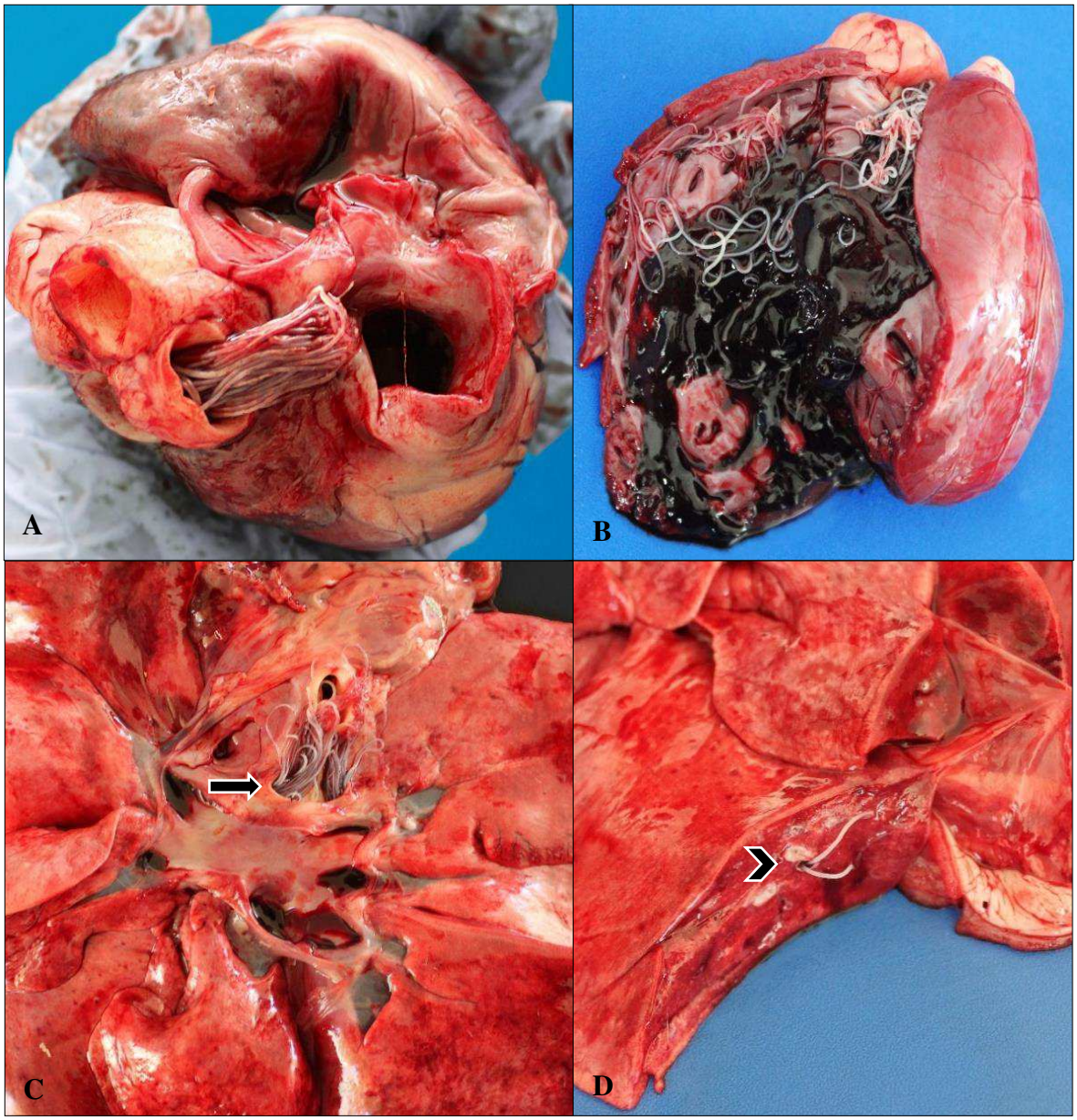

Fig. 1. Adult stages of *Dirofilaria immitis* found during necropsy in organs of dog number 18. **A.** Heart. Adults stages of *D. immitis* in the pulmonary trunk; **B.** Adult *D. immitis* worms associated with cruoric clot in the right ventricle; Lungs. **C** and **D.** Adult stages of the parasite in the pulmonary artery lumen (arrow); and in the lumen of the bronchi (arrowhead).

Source: Animal Pathology Laboratory – HV-ASA/IFPB.

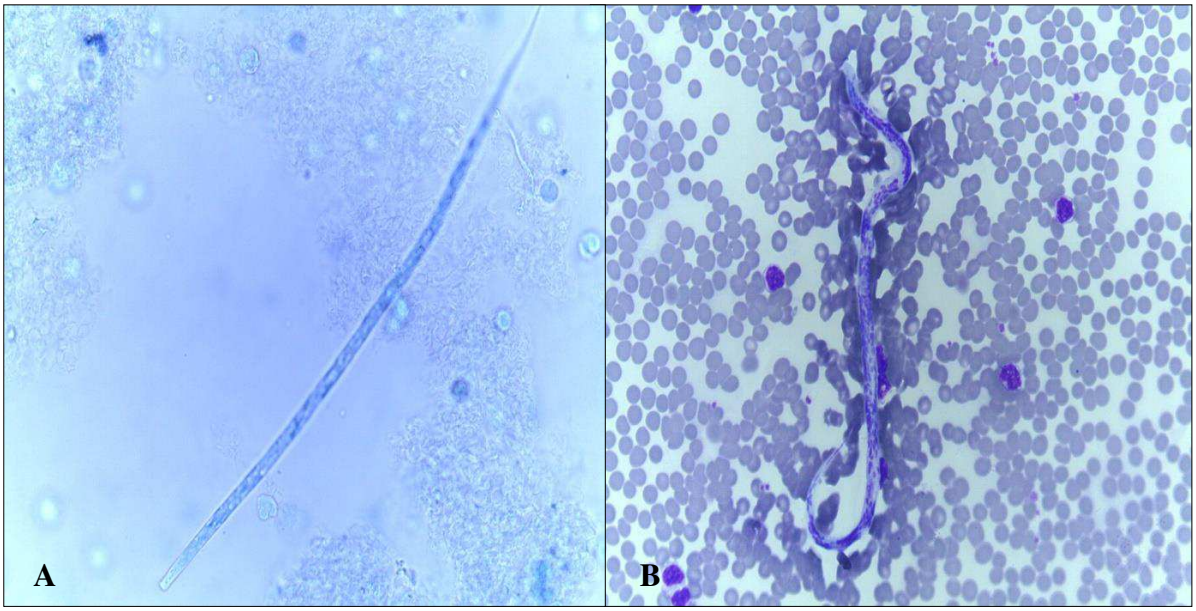

Figure 2 – *Dirofilaria immitis* microfilariae observed in blood tests of positive dogs. Obj. 40x. **A.** Distended microfilariae stained blue in MK, with a conical head and straight tail; **B.** Microfilaria stained basophilically in PBS.

Source: Animal Pathology Laboratory – HV-ASA/IFPB

Declarations of interest

None.

CAPÍTULO II:

Dirofilariasis, leishmaniasis and transmissible venereal tumor: concomitant affections in dogs in the Paraiba backwoods

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Trabalho a ser submetido à revista *Acta Scientiae Veterinariae*
(Qualis B1)

Dirofilariasis, leishmaniasis and transmissible venereal tumor: concomitant affections in dogs in the Paraiba backwoods

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ABSTRACT

Background: Several pathogens can often concurrently infect the same host and influence each other, directly or indirectly. Heartworm, Leishmaniasis, and Transmissible Venereal Tumor (TVT) are examples of diseases that can occur simultaneously in dogs and are mainly present in regions that have favorable environmental and socioeconomic factors, in addition to often being asymptomatic or not presenting characteristic clinical signs, which contributes to underdiagnosis. The objective of this study was to report cases of infection by *D. immitis* concomitant with other endemic diseases in dogs, such as Leishmaniasis and TVT, in the metropolitan region of Patos, in the interior of Paraíba.

Materials, methods & results: The records of necropsies of dogs at the Animal Pathology Laboratory of the University Veterinary Hospital of the Federal University of Campina Grande, Patos state, Paraíba, were reviewed, selecting the cases of incidental finding of *D. immitis* and associated diseases. From the clinical and necropsy protocols, information was obtained regarding epidemiological data (gender, race, age, and origin), clinical signs, and anatomopathological findings. Photographic records were also retrieved. During the study period, 2,465 dogs were necropsied, four of which had *D. immitis* infection and leishmaniasis (4/4), with this disease being considered the cause of death; and two also presented TVT (2/4). Four male, adult and mixed-breed dogs were affected. Of these, three were wanderers and one semi-domiciled. In the macroscopic examination, the main findings were reactive lymph nodes, splenomegaly with hyperplasia of the lymphoid follicles and the presence of *D. immitis* in the right ventricle of the heart and pulmonary trunk. In the histopathological evaluation, amastigote figures of *Leishmania* spp. in bone marrow, lymph nodes, spleen and skin, in addition to membranoproliferative glomerulonephritis in the kidneys and densely cellular and expansive round cell neoformation in the penis of two dogs, characteristic of TVT.

Discussion: Infection by *D. immitis* was identified at necropsy through the visualization of specimens in the right ventricle of the heart and pulmonary trunk. The diagnosis of leishmaniasis was established through laboratory tests and that of TVT based on pathological findings. The identification of heartworm cases in the semiarid region reinforces the importance of performing the necropsy in cases of euthanized animals due to a diagnosis of leishmaniasis, in addition to the need to perform tests on live animals, to verify the occurrence of the parasite in a certain region, once that canine heartworm disease was seen as an infection that typically occurred in coastal regions and hot climates. It is interesting to note that the occurrence of TVT in some of these dogs may reflect the epidemiological condition

to which these animals are exposed, since this pathology occurs predominantly in dogs with street access, and this condition was identified in animals in this study. The occurrence of zoonotic diseases such as leishmaniasis and heartworm disease, simultaneously in stray and semi-domiciled dogs in the Paraíba backwoods region, demonstrates the role of the epidemiological reservoir of these animals in the urban environment. The observation of *D. immitis* as an incidental finding in dogs with leishmaniasis reinforces the importance of performing a necropsy in these cases since not performing this exam can lead to an underdiagnosis. Furthermore, the concomitant occurrence of TVT further warns of the risk that dogs are exposed to. The management of the population of dogs, particularly the stray dogs, represents an important measure for the prevention and control of diseases of veterinary medical interest and unique health.

Keywords: *Dirofilaria immitis*, *Leishmania* sp., necropsy, zoonosis.

INTRODUCTION

Several pathogens can often concurrently infect the same host and influence each other, directly or indirectly. Heartworm, Leishmaniasis and Transmissible Venereal Tumor (TVT) are examples of diseases that can occur simultaneously in dogs and are present mainly in regions that have favorable environmental and socioeconomic factors [1,21], in addition to often being asymptomatic or not have characteristic clinical signs, which contributes to underdiagnosis [18].

Dirofilaria immitis is an example of a zoonotic filariform nematode, whose adult forms inhabit the pulmonary arteries and the right heart of dogs, causing a chronic disease called heartworm [16]. The parasite is broadcast by mosquitoes of the genera *Aedes, Culex,* and *Anopheles* that transmit microfilariae during the blood meal, mainly to dogs, felines, in

addition to humans, which are considered an accidental host in regions with high rates of the disease in dogs [12.15].

Canine Visceral Leishmaniasis (CVL) is an infectious disease caused by the protozoan *Leishmania infantum chagasi*, which also has arthropod vectors as intermediate hosts, in this case, sand fly mosquitoes of the genus *Lutzomyia* spp. With a recognized zoonotic potential, the disease is chronic and sometimes oligosymptomatic in dogs [7,3,19].

Transmissible Venereal Tumor (TVT) is a neoplasm that primarily develops in the external genitalia of dogs. The disease is more common in young, sexually active and free-living dogs in urban areas [11]. The objective of this study was to report cases of infection by D. immitis concomitant with other endemic diseases in dogs, such as Leishmaniasis and TVT, in the metropolitan region of Patos, in Paraíba backwoods.

MATERIALS AND METHOD

Dog necropsy records were reviewed at the Animal Pathology Laboratory of the University Veterinary Hospital (HVU) of the Federal University of Campina Grande, Patos state, Paraíba, from January 2003 to October 2019, selecting the cases found incidental of *D. immitis* and the associated diseases.

From the clinical and necropsy protocols, information was obtained regarding epidemiological data (gender, race, age and origin), clinical signs and anatomopathological findings. Photographic records were also retrieved.

Dogs with a positive diagnosis of leishmaniasis were submitted to the rapid screening test for visceral leishmaniasis Dual Path Platform (DPP) by the Bio Manguinhos Institute of Technology in Immunobiologicals, recommended by the Canine Visceral Leishmaniasis Surveillance and Control Program of the Ministry of Health in Brazil. Euthanasia was carried

out according to the bioethical and animal welfare precepts recommended by Resolution No. 1,000, of May 11, 2012, of the Federal Council of Veterinary Medicine.

The collected tissues were fixed in 10% buffered formalin, cleaved, routinely processed, embedded in paraffin blocks, sectioned into 3µm sections, and stained with hematoxylin and eosin (HE). Subsequently, the slides were microscopically described.

RESULTS

During the study period, 2,465 dogs were necropsied, four of which had *D. immitis* infection and leishmaniasis (4/4), with this disease being considered the cause of death; and two also presented TVT (2/4). Epidemiological and clinical data for these animals are described in table 1.

Table 1. Epidemiological and clinical data of animals with *Dirofilaria immitis,* Canine Visceral Leishmaniasis (CVL) and Transmissible Venereal Tumor (TVT).

Dog	Diagnostic	Breed	Sex	Age	Situation	Clinical signs
1	*D. immitis*/ CVL/ TVT	Mixed breed	Male	Adult	Stray dog	Uninformed
2	*D. immitis*/ CVL	Mixed breed	Male	Adult	Stray dog	Uninformed
3	*D. immitis*/ CVL	Mixed breed	Male	Adult	Stray dog	Uninformed
4	*D. immitis*/ CVL/ TVT	Mixed breed	Male	Adult	Semi-domiciled	Weight loss, loss of appetite, diarrhea, skin lesions

Four male, adult, and mixed-breed dogs were affected. Of these, three were stray dogs and one semi-domiciled. The animals lived in the urban area of the Patos state (2/4) and

Piancó (2/4), belonging to the metropolitan region of Patos in the Paraíba backwoods, Northeastern Brazil.

On external examination of the cadavers, the animals presented body conditions ranging from cachectic (3/4) to regular (1/4); mucous membranes ranging from pale (3/4) to normal (1/4); palpable reactive lymph nodes (4/4); alopecic and scaly skin lesions, sometimes crusty, predominantly distributed in the face and ventral abdomen (3/4) (Figure 1A); presence of ectoparasites (fleas and ticks) (3/4); onychogryphosis (1/4); generalized muscle atrophy (1/4); dental tartar (1/4); bristly and opaque fur (1/4); and diarrheal stool adhered to fur in the perianal region (1/4). In two animals, upon retraction of the foreskin, multilobulated, white, and irregular nodules were observed at the base and body of the penis (Figure 1B).

At necropsy, enlarged submandibular, superficial cervical, axillary, popliteal and mesenteric lymph nodes were observed (4/4). Enlarged spleen, with rounded edges and irregular surface (splenomegaly). When cut, moderate hyperplasia of the lymphoid follicles and congestion (4/4). Slightly enlarged liver, with rounded edges and evidence of the lobular pattern (hepatomegaly) (2/4).

In the opening of the right ventricle of the heart and pulmonary trunk, there were white filariform nematode parasites measuring 10 to 20 cm in length, morphologically compatible with *D. immitis* (Figures 1C and D) in all four cases. Six to 15 specimens were identified among the animals.

In the histopathological evaluation of the bone marrow, numerous histiocytes were observed with ample cytoplasm and full of slightly basophilic structures, round or oval, delimited by a clear halo and measuring 2-4 µm, morphologically compatible with amastigote forms of *Leishmania* spp. In some areas, amastigotes were visualized free, individually, or forming groups (2/4) (Figure 1E).

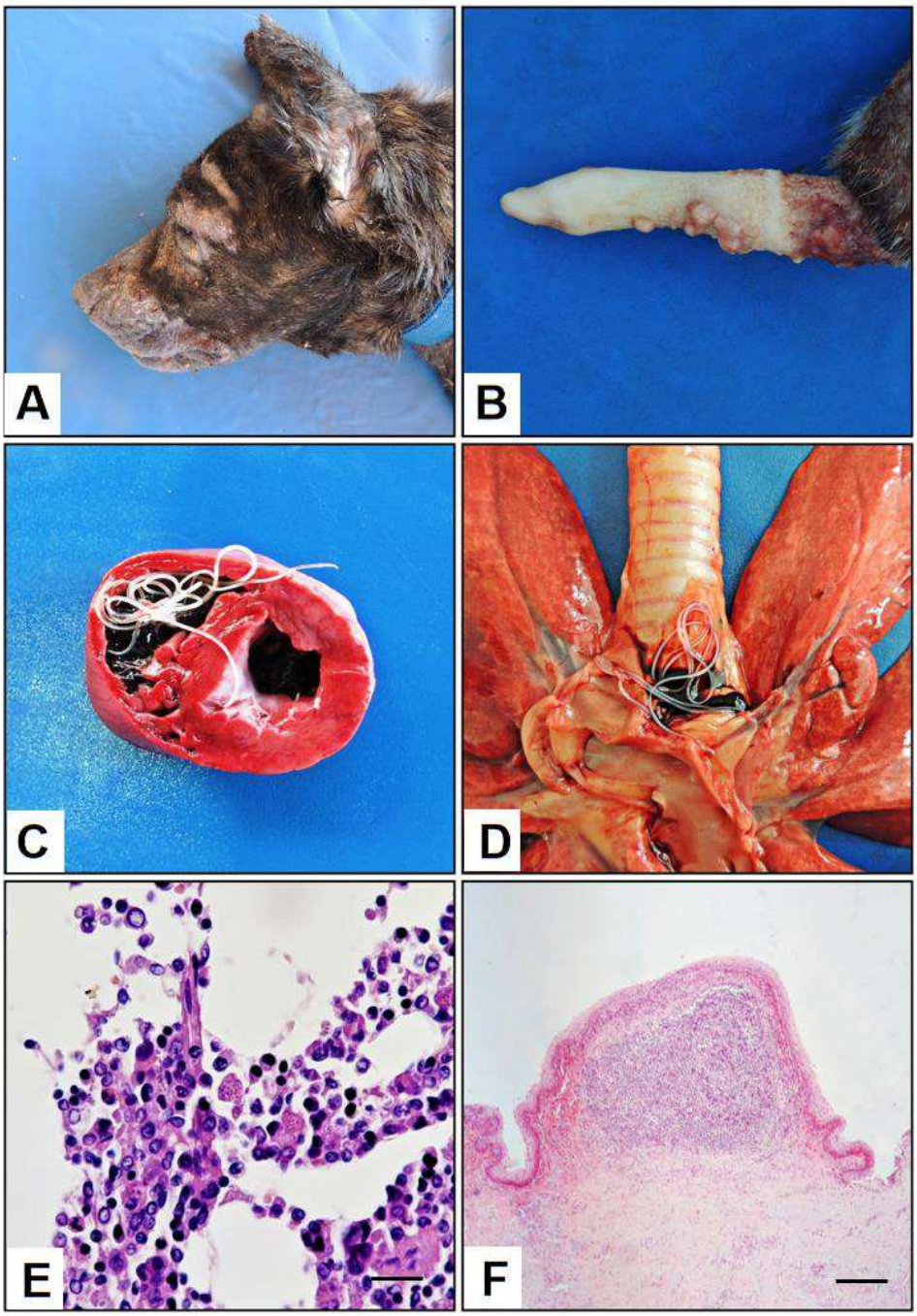

Figure. 1. Co-infections of leishmaniasis, heartworm and transmissible venereal tumor in dogs. **A)** Alopecic and desquamative skin lesions in the periocular region, nasal plane and face (Case 4). **B)** Penis, after removal of the foreskin. Multiple white and exophytic nodules (Case 4). **C)** Heart, right ventricle. Numerous specimens of *Dirofilaria immitis* (Case 1). **D)** Pulmonary trunk. Numerous specimens of *Dirofilaria immitis* (Case 1). **E)** Photomicrograph of the bone marrow. *Leishmania* spp. intrahistiocytic (Case 4) (HE. Obj. 40x). **F)** Photomicrograph of the penis. Densely cellular and expansive neoformation composed of round cells assuming a mantle arrangement (Case 4) (HE. Obj. 20x).

In the lymph nodes, hyperplastic cortical lymphoid follicles and distended germinal centers (lymphoid reactivity) were observed, with a marked inflammatory infiltrate consisting of macrophages, lymphocytes and plasma cells. In the medullary region, there was marked infiltration of plasmocytes, with occasional macrophages, lymphocytes and Mott cells, stretching the cords and filling the medullary sinuses; in addition to occasional macrophages with ample cytoplasm and containing granular and brownish pigment (hemosiderophages). In the subcapsular sinus, there were numerous macrophages with cytoplasm full of amastigote forms of *Leishmania* spp. (2/4).

In the spleen, subversion of tissue architecture was observed due to hyperplasia of the white pulp by infiltration of macrophages, plasmocytes and lymphocytes, which sometimes extended to the medullary sinuses. Macrophages were occasionally visualized with ample cytoplasm replete with amastigote forms of *Leishmania* spp. (2/4).

In the kidneys, a moderate interstitial lymphoplasmohistiocytic inflammatory infiltrate was observed, particularly in the periglomerular region. The renal glomeruli were enlarged, hypercellular, with thickening of the basement membrane and decreased urinary space, sometimes with formation of adhesions between the capillary tuft and Bowman's capsule, which was thickened, characterizing membranoproliferative glomerulonephritis. In the contorted tubules, there were numerous eosinophilic, homogeneous and proteinaceous casts (hyaline cylinders), from the cortical to the medullary region (3/4).In the spleen, subversion of tissue architecture was observed due to hyperplasia of the white pulp by infiltration of macrophages, plasmocytes and lymphocytes, which sometimes extended to the medullary sinuses. Macrophages were occasionally visualized with ample cytoplasm replete with amastigote forms of *Leishmania* spp. (2/4).

In the liver, there was an inflammatory infiltrate consisting of plasmocytes, lymphocytes and macrophages in the periportal regions and extending slightly to the

sinusoidal spaces, with occasional macrophages with ample cytoplasm and containing granular and brownish pigment (hemosiderophages) (3/4).

In the skin, there was a multifocal inflammatory infiltrate consisting of macrophages, neutrophils and rare lymphocytes and plasma cells irregularly distributed in the superficial dermis. In the overlying epidermis, there were multifocal areas of ulcerations, with deposition of serocellular crust, and sometimes interposed by areas of acanthosis and hyperkeratosis (3/4). In one case, amid the inflammatory infiltrate, there were macrophages with cytoplasm full of amastigote forms of *Leishmania* spp.

In the penis of two dogs, a densely cellular, well-delimited, non-encapsulated and expansive neoformation was observed, composed of round neoplastic cells, arranged in a mantle, and supported by a sparse fibrovascular stroma (Figure 1F). These neoplastic cells had abundant eosinophilic cytoplasm with well-defined limits. The nuclei were round to oval and peripherally displaced with loose chromatin and evident nucleoli, in addition to marked anisocytosis, anisocariosis and heterochromasia. There were also figures of mitosis present (1 to 3 per field at 400x magnification). In the areas of overlying ulcerations, extensive necrosis permeated by a neutrophilic inflammatory infiltrate, hemorrhage and myriads of basophilic coccobacillary bacteria were observed.

DISCUSSION

Infection by *D. immitis* was identified at necropsy through the visualization of specimens in the right ventricle of the heart and pulmonary trunk. The diagnosis of leishmaniasis was established through laboratory tests and that of TVT based on pathological findings.

In these animals, the main clinicopathological changes consisted of skin lesions, generalized lymphadenopathy, and hepatosplenomegaly. The clinical presentation of dogs

with leishmaniasis is very variable and can include skin lesions, splenomegaly, generalized lymphadenopathy, cachexia, anorexia, muscle wasting, polyuria, polydipsia, proteinuria, keratoconjunctivitis, nail overgrowth, and hematologic abnormalities [13].

Although in two dogs (cases 1 and 2) the amastigote forms of Leishmania spp. in none of the analyzed sections, these presented suggestive macroscopic lesions and were positive in the rapid Dual Path Platform (DPP) test. The diagnosis of canine leishmaniasis can be based on parasitological or serological examination. The parasitological diagnosis, through visualization of amastigote forms, although of high specificity, depends on the degree of parasitemia [3]. It has been proposed that animals with infections at an early stage or that have a low concentration of protozoa become difficult to visualize the agent, which could possibly have occurred in these cases.

In the present work, it is evident that stray dogs represent important reservoirs of diseases that can affect humans and are of medical and veterinary importance, such as infections by *Leishmania* spp. and *D. immitis*, as well as for its own species, as in the case of TVT.

The identification of heartworm cases in the semiarid region reinforces the importance of performing the necropsy in cases of euthanized animals due to a diagnosis of leishmaniasis, in addition to the need to perform tests on live animals, to verify the occurrence of the parasite in a certain region, once that canine heartworm disease was seen as an infection that typically occurred in coastal regions and hot climates [6,8,9].

Although humans are considered less susceptible hosts to infection by *D. immitis*, as they trigger a specific immune response that enables the destruction of the parasite in most cases, attention has been drawn to the increase in the number of cases of infections in this species in the last few two decades [10,17,20]. Leishmaniasis has a wide distribution, and in Latin America, for example, it has been described in at least 12 countries, with 90% of cases

occurring in Brazil, especially in the Northeast region [3]. Therefore, we emphasize the need to investigate the presence of infected dogs in leishmaniasis endemic areas with greater attention, considering their role as a reservoir in the transmission chain of *Leishmania* sp. in the home and peridomestic environments.

However, the transmission cycle has characteristics peculiar to each endemic region, so it is not always possible to obtain relative averages of the data from one location to another, as the various factors such as phytogeographic conditions, population control of stray dogs, animal health, urban constructions close to the forest tend to be distinct, and there may not be similarities between biotic factors such as the presence of the same species of parasite, wild reservoirs, and sandflies.

It is interesting to note that the occurrence of TVT in some of these dogs may reflect the epidemiological condition to which these animals are exposed since this pathology occurs predominantly in dogs with street access, whether this access is intermittent and in a reduced perimeter (peri-domiciled) or continuous and unrestricted (straying) [11], and this condition was identified in animals in this study.

The free access of these animals to the streets represents a great risk to the susceptible ones, as it allows the transmission of the disease through direct interaction. TVT is typically transmitted during intercourse, which determines the highest incidence of the tumor in the genital mucosa. Transmission can also occur through the normal social behavior of dogs when they lick, sniff, scratch, or bite their own genitals (self-implantation) or the genitals of other affected dogs (heteroimplantation) [14].

CONCLUSION

The occurrence of zoonotic diseases such as leishmaniasis and heartworm, simultaneously in stray and semi-domiciled dogs in the Paraíba backwoods region,

demonstrates the role of the epidemiological reservoir of these animals in the urban environment. The observation of *D. immitis* as an incidental finding in dogs with leishmaniasis reinforces the importance of performing a necropsy in these cases since not performing this exam can lead to an underdiagnosis. It is also recommended for veterinary clinicians to perform a diagnostic test on a live animal, as most animals remain asymptomatic. Furthermore, the concomitant occurrence of TVT further warns of the risk that dogs are exposed to. The management of the population of dogs, particularly the stray dogs, represents an important measure for the prevention and control of diseases of veterinary medical interest and unique health.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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CONCLUSÃO GERAL

Através desse estudo foi possível determinar que a incidência de *D. immitis* em cães na região semiárida da Paraíba é alta, principalmente porque esse local era anteriormente caracterizado como não endêmico para a parasitose. O exame de necropsia mostrou-se eficaz para detecção da infecção oculta de *D. immitis*, e em animais vivos recomenda-se a realização do teste imunocromatográfico rápido para detecção de antígenos da fêmea adulta associado ao teste de Knott modificado para concentração de microfilárias, afim de diminuir as chances de ocorrência de falsos-negativos. Além disso, a LVC foi considerada um fator associado a ocorrência de *D. immitis.*