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Molecular detection of visceral leishmaniasis in dogs from Barão de Melgaço, Pantanal region of Mato Grosso, Brazil[1]

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ABSTRACT.- Dias A.F.L.R., Almeida A.B.P.F., Nakazato L. & Sousa V.R.F. 2021. **Molecular detection of visceral leishmaniasis in dogs from Barão de Melgaço, Pantanal region of Mato Grosso, Brazil.** *Pesquisa Veterinária Brasileira 41:e06485, 2021*. Laboratório de Leishmanioses, Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Campus de Cuiabá, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: valeriaregia27@gmail.com

The increasing expansion of visceral leishmaniasis (VL) in the Brazilian territory evidences the need for studies focused on the main reservoir of this parasite: the dog. This study aimed to conduct an epidemiological survey in the municipality of Barão de Melgaço, Pantanal region of the state of Mato Grosso (MT), Brazil. Conventional polymerase chain reaction (PCR) and qualitative SYBR®Green real-time PCR (qPCR) were used to diagnose canine VL (CVL) and characterize the factors associated with this infection. Of the 402 dogs that had blood samples collected, 31 presented the parasite DNA, representing a prevalence of 7.71% in the population studied. Positivity indices for PCR and qPCR were 3.48 (14/402) and 7.21% (29/402), respectively. Comparison of the results obtained by both techniques showed moderate agreement (Kappa = 0.5364). Of the independent variables analyzed, presence of clinical signs ($p \leq 0.05$) was the only one associated with CVL. Based on this study, we conclude that VL is a circulating disease, with relatively low prevalence, in dogs of Barão de Melgaço/MT, and that the presence of clinical signs is the only variable associated with canine infection.

INDEX TERMS: Molecular detection, visceral leishmaniasis, dogs, Pantanal region, Brazil, zoonoses, neglected disease, public health, PCR.

RESUMO.- [**Detecção molecular de leishmaniose visceral em cães do município de Barão de Melgaço, Pantanal Mato-Grossense, Brasil.**] A crescente expansão da leishmaniose visceral (LV) no território brasileiro evidencia a necessidade de estudos voltados ao principal reservatório doméstico do parasito: o cão. Sendo assim, o objetivo deste estudo foi realizar um inquérito epidemiológico no município de Barão de Melgaço, região do Pantanal Mato-grossense, utilizando as técnicas de reação em cadeia pela polimerase convencional (PCR) e teste qualitativo SYBR®Green real-time PCR (qPCR) para o diagnóstico da LV canina (LVC), além de caracterizar os fatores associados a infecção. Do total de 402 cães que tiveram amostras sanguíneas coletadas, 31 apresentaram o DNA do parasito, perfazendo uma prevalência de 7,71% na população estudada. Os índices de positividade para a PCR e qPCR foram de 3,48% (14/402) e 7,21% (29/402), respectivamente. A comparação dos resultados obtidos por ambas técnicas apresentou moderada concordância (*Kappa* = 0,5364). Das variáveis independentes analisadas, a presença de sinais clínicos ($p \leq 0,05$) foi a única associada a ocorrência de LVC. Com base neste estudo, concluímos que a LV está circulando, com prevalência relativamente baixa, em cães de Barão de Melgaço/MT, sendo a presença de sinais clínicos a única variável associada à infecção canina.

TERMOS DE INDEXAÇÃO: Detecção molecular, leishmaniose visceral, cães, caninos, Pantanal, Brasil, zoonoses, doença negligenciada, saúde pública, PCR.

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INTRODUCTION

Visceral leishmaniasis (VL) is a neglected severe public health problem worldwide. This disease has been classified by the World Health Organization (WHO) and the Pan American Health Organization (PAHO) as a priority infection (category 1: reemerging or uncontrolled infections) (WHO 2018). In the Americas, Brazil presents the highest occurrence of VL, corresponding to 96% of all reported cases, with highlight to the Midwest, Northeast and Southeast regions (OPAS 2018).

In Brazil, VL is caused by the etiologic agent *Leishmania* (*Leishmania*) *infantum* [synonym: *Leishmania* (*L.*) *chagasi*], whose vast clinical, biological and epidemiological magnitude and complexity resulted in its dispersion and growing expansion throughout the country (Brasil 2014, WHO 2018). In the state of Mato Grosso (MT), this agent is endemic. In addition, indigenous cases in humans have already been reported in Barão de Melgaço, a tourist municipality in the Pantanal region (Information System for Notifiable Diseases - Sinan). These reports highlight the need and importance of conducting studies that can assist in better understanding the epidemiology of this disease in the region, providing data so that the health services can adopt efficient prevention and control measures.

In this context, studies addressing dogs, the main domestic reservoirs of *L. infantum*, are essential because the high population density and intense cutaneous parasitism in these animals allow maintenance of the disease transmission cycle in the urban environment (Belo et al. 2013). Among the diagnostic methods used in canine studies, serological tests are widely employed because of their high sensitivity and specificity, easy handling, and low cost. These characteristics have led the Brazilian Ministry of Health (MS), through its Visceral Leishmaniasis Surveillance and Control Program (PCLV), to adopt the rapid immunochromatographic test (DPP/Biomanguinhos®) and the immunoenzymatic assay (EIE/Biomanguinhos®) for the diagnosis of canine LV (CVL) (Brasil 2014). However, despite their advantages, these methods present some limitations, especially in the diagnosis of asymptomatic dogs, which may eventually underestimate the actual prevalence of the disease (Pessoa-e-Silva et al. 2019).

Due to the importance of dogs in the disease cycle and the ethical and legal discussions resulting from the euthanasia of seroreagent dogs - the primary strategy of the PCLV (Brasil 2014), complementary methods that assist with the early and definitive diagnosis of CVL are desirable. In this respect, molecular tests such as the polymerase chain reaction (PCR) and its variations have obtained good performance (Monteiro et al. 2019). They can detect small amounts of parasite DNA, characterize the circulating species, and diagnose the subclinical canine infection (Pessoa-e-Silva et al. 2019). Thus, the use of molecular techniques in canine studies can assist health services in carrying out control measures, which enable reduction of the exposure of reservoirs to vectors and minimization of disease dispersion (Coura-Vital et al. 2011).

Therefore, the present study aimed to evaluate the molecular prevalence of CVL and characterize the factors associated with infection in the municipality of Barão de Melgaço, Pantanal region of Mato Grosso, using the conventional PCR and qualitative SYBR®Green qPCR techniques.

MATERIALS AND METHODS

Ethical approval. The study was designed according to the Ethical Principles in Animal Experimentation (COBEA) and all procedures were previously approved by the Ethics Committee on the Use of Animals (CEUA) of the "Universidade Federal do Mato Grosso" (UFMT) under protocol no. 23108.018929/14-9. Furthermore, prior to performing any procedure on an animal, its owner was asked to sign an Informed Consent Form (ICF).

Study area. This study was conducted in the municipality of Barão de Melgaço (16°11'40" S; 55°58'03" W), in the central-south mesoregion of the state of Mato Grosso (MT) and microregion of Alto Pantanal, Brazil. Located in the Pantanal biome, the municipality is located 102 km from the state capital Cuiabá, comprises an area of 11,174,500 km^2 (IBGE 2017), and has a tropical climate. The minimum sample size was estimated at 283 dogs, considering the 1:7 ratio of dogs to human, expected prevalence of 50%, confidence level of 95%, and acceptable error of 5% (Brasil 2014). Household collections occurred in a census form in the urban area and 11 rural communities.

Animals and biological samples. The dogs included in the study were of both sexes, different breeds, and aged ≥6 months. They were examined and clinically classified as symptomatic or asymptomatic (Solano-Gallego et al. 2011). To describe the general characteristics of the canine population and the living environment and researching factors associated with CVL, an epidemiological questionnaire was applied to each household. Concerning the dogs, the following variables were analyzed: breed, sex, coat, clinical signs, access to the street, and place of residence in the house. Regarding the demographic aspects, the following variables were considered: proximity to forests, rivers or dams, presence of other domestic animals, and presence of cultivated crops near the residence.

Blood samples were collected by puncturing the external or cephalic jugular vein and placed in tubes containing EDTA. Subsequently, they were centrifuged to obtain the leukocyte layer and stored in microtubes at -80 ˚C.

DNA extraction and molecular tests. The molecular methods were performed in the "Laboratório de Biologia Molecular do Hospital Veterinário" (HOVET-UFMT). Extraction of DNA from the leukocyte covered samples was performed by the phenol/chloroform/isoamyl alcohol method according to Sambrook & Russel (2001). The extraction product was eluted in Milli-Q water and stored at -80 °C until use in molecular tests.

For molecular detection by PCR, primers 150 (sense) 5'-GGCCCACTATATTACACCAACCCC-3 'and 152 (antisense) 5'-GGGGTAGGGGCGTTCTGCGAA-3' were used, amplifying a fragment of 120 base pairs of the conserved region of the kDNA minicircle of all *Leishmania* species (Degrave et al. 1994). Subsequently, primers RV1 (sense) 5'-CTTTTCTGGTCCCGCGGGTAGG-'3 and RV2 (antisense) 5'-CCACCTGGCCTATTTTACACCA-'3 were used to identify the parasite at the complex level, amplifying a fragment of 145 base pairs from the conserved region of the kDNA minicircle of the *Leishmania donovani* complex (Lachaud et al. 2002). The reactions and conditions of the two PCR protocols were modified and adapted as proposed by Almeida et al. (2013). The amplification products, stained with Gel Red (Biotium), were electrophoresed on 2% agarose gel at 100 V for 90 min and visualized on ChemiDocTM XRS using the ImageLabTM® software.

The qPCR technique was performed on the StepOne™ Real-Time PCR (Applied Biosystem®) sequence detection system using the RV1 and RV2 primers. The protocol described and optimized by Torres et al. (2013) was adopted to guarantee robust and accurate

amplification. The qPCR data were collected and processed using the ABI Sequence Detection System1.4 (Applied Biosystems®). For interpretation of the results, the two criteria proposed by Barbau-Piednoir et al. (2013) were applied: value of the quantification cycle (C_q) and amplicon melting temperature (T_m). Thus, the sample reactions were considered positive when exhibiting an amplification (exponential) above the threshold level, with a single peak in the fusion analysis, presenting a single value of T_m, and they were considered negative in the absence of the C_q value.

In all PCR and qPCR assays, a reference strain of *Leishmania infantum* DNA (MHOM/BR/1974/PP75) was used as positive control, and a free DNA reaction was used as negative control.

Statistical analysis. Statistical analyses were performed using the R 3.3.0 software in R Commander (Fox 2005). Association between the presence of *Leishmania* spp. DNA in dogs and the qualitative variables was determined using the Chi-squared (X^2) or Fisher's exact tests. Agreement between the diagnostic tests was determined by the Kappa test (Cohen 1960).

RESULTS

Samples from 402 dogs were collected and analyzed: 248 (61.69%, 95% CI: 56.85-66.31) in urban area and 154 (38.31%, 95% CI: 33.69-43.15) in the rural area. In general, males (56.97%, 95% CI: 52.08-61.72), mongrels (90.05%, 95% CI: 86.73-92.61), and short coats (89.8%, 95% CI: 96.46-92.39) were predominant in this study.

All samples positive for *Leishmania* spp. were identified as *Leishmania infantum*, belonging to the *Leishmania donovani* complex in PCR. Of the 402 samples tested, 31 presented the parasite DNA in at least one of the molecular tests, with a prevalence of 7.71% (95% CI: 5.49-10.74). When analyzed individually, PCR detected the parasite DNA in 14 samples - prevalence of 3.48% (95% CI: 2.09-5.76), while qPCR detected the parasite DNA in 29 samples - prevalence of 7.21% (95% CI: 5.07-10.17%), with statistically significant difference ($p<0.0001$). When the result was considered in series, 12 samples were positive in both tests, with a prevalence of 2.99% (95% CI: 1.72-5.14). A Kappa index of 0.5364 (53.64%, 95% CI: 44.54-62.73) was obtained in the concordance analysis, thus showing a moderate agreement.

Of the 31 positive dogs, seven (22.58%, 95% CI: 9.59-41.10) were asymptomatic and 24 were symptomatic (77.42%, 95% CI: 58.90-90.41) ($p=0.05$). Among the clinical signs observed, the most common were lymphadenomegaly (14/24, 58.33%), dermatopathy (12/24, 50%), onychogryphosis (9/24, 37.5%), splenomegaly (6/24, 25%), and ophthalmopathy (3/24, 12.5%).

The statistical analysis between the independent variables (characteristics of animals and environment) and infection showed that only the variable presence of clinical signs was significant ($p\leq0.05$). Regarding the animals' habitat, no statistical difference was observed between prevalence of urban (6.05%) (15/248, 95% CI: 3.42-9.78) and rural (10.39%) (16/154, 95% CI: 6.06-16.32) areas ($p=0.112$); the environmental characteristics were not statistically significant ($p>0.05$).

DISCUSSION

The present study reports the molecular detection of CVL in the municipality of Barão de Melgaço/MT. The results confirm that the disease is present in dogs in the Pantanal region. Of the seven municipalities (Barão de Melgaço, Cáceres, Curvelândia, Itiquira, Nossa Senhora do Livramento, Poconé, and Santo Antônio do Leverger) that comprise the Mato Grosso Pantanal region, six have previous reports of CVL (except Curvelândia) and five of autochthonous cases of human LV (except Curvelândia and Santo Antônio do Leverger) (Brito et al. 2019). These data serve as a warning for the public health system because of the possibility of expanding the etiologic agent to neighboring municipalities and emphasizes the importance of adopting the strategic measures established by the PCLV, which recommend the diagnosis and treatment of human cases, monitoring and euthanasia of infected dogs, and reduction of the number of vectors (sandflies) through environmental management in public and residential areas (Brasil 2014). In addition, educational action programs that allow the population to engage effectively in prevention are strongly recommended, as knowledge of the disease can be relevant in controlling endemic diseases.

The molecular prevalence of CVL observed in Barão de Melgaço (7.71%) is within the values described in Brazil: 1.9-75% (Araujo et al. 2016). These values are higher than those reported by Almeida et al. (2013) in the capital of Mato Grosso, Cuiabá - an area of high endemicity for VL, who found the parasite DNA in 4.6% (20/430) of the leukocyte covered samples analyzed. In addition, the occurrence of molecular prevalence higher than the serological findings of Dias et al. (2017) is justified and described in the literature (Coura-Vital et al. 2011, Leite et al. 2011) since, due to the greater sensitivity and specificity of molecular tests, they could detect the infection before seroconversion (Strauss-Ayali et al. 2004). Furthermore, Nunes et al. (2015), in a longitudinal approach of a canine population, found that detection of CVL through PCR in blood samples showed greater sensitivity than that in the tests recommended by the MS in the first six months of follow-up. However, the authors found that this difference in sensitivity decreased with the evolution of the disease. Thus, the association of molecular tests in diagnostic protocols can contribute as a complementary method in the control and monitoring of the disease through detection of subclinical infections in epidemiological surveys.

Regarding the biological material analyzed, the choice of leukocyte pellet was based on the results of Mary et al. (2004). They associated the sensitivity of molecular tests to the DNA extraction method and identified greater effectiveness of the tests when the nucleated cells were isolated from whole blood before digestion with Proteinase K. Although blood may not be the best option for detection of DNA by PCR (Lombardo et al. 2012, Almeida et al. 2013, Aschar et al. 2016, Coiro et al. 2017), the collection of this material is less invasive than bone marrow/lymph node. Furthermore, it facilitates the process, since optimizing collection time in canine epidemiological surveys has better acceptance and collaboration by the owners.

Thus, the different approaches to the diagnosis of CVL are a reflection of its complexity. Despite the many tests available for its diagnosis, parasitological methods are still the most chosen and considered the gold standard; however, the time and training required for their execution hinders their application in epidemiological surveys. On this premise, molecular methods (PCR and qPCR) have been considered more sensitive, as they are amenable to automation and have

the advantage of replicating the agent genome from minimal amounts of DNA (Galluzzi et al. 2018). However, their use for diagnosing CVL in public health services is still far away due to the difficulties inherent in its execution: acquisition of equipment, preparation of protocols, and standardization of diagnostic kits.

The molecular tests were performed with oligonucleotides belonging to the *Leishmania* kinetoplast DNA minicircle (kDNA). They have high specificity and sensitivity in different biological samples, representing an ideal target for molecular analyses (Vergel et al. 2005, Reis et al. 2013). Also, since they have many copies (10,000-20,000 per parasite) containing conserved and varied regions, it allows differentiation between *Leishmania* spp. (Sundar & Singh 2018). Although oligonucleotides were used by Lachaud et al. (2002) to amplify species belonging to the *Leishmania donovani* complex, *Leishmania infantum* is the only one described in Brazil. Despite the qualities of molecular tests, it is necessary to emphasize that they must be associated with clinical and laboratory evidence to diagnose the disease in an animal.

The Kappa test revealed moderate agreement (k=0.5364) between the diagnostic methods, a result explained by the high positivity presented by the qPCR (p<0.0001). Among the main characteristics that make qPCR superior is its ability to detect fluorescence, which allows identification of the agent at a DNA concentration a thousand times lower than in PCR (Mohammadiha et al. 2013). In addition, qPCR allows elimination of the various post-amplification manipulation steps, reducing the risk of contamination and the time required for analysis (Nicolas et al. 2002, Ferreira et al. 2014, Paşa et al. 2015), since amplification and detection of the target DNA occur as the reaction progresses (Ferreira et al. 2014).

Regarding the demographic aspects evaluated in this study, no environmental characteristics were associated with occurrence of the disease (p>0.05). However, it is necessary to emphasize that the Pantanal region presents, mainly in its rural area, a scenario favorable to VL development. It is the natural habitat of sandflies, and there is abundance of wild animals cohabiting with domestic species, which can be relevant in the maintenance of the parasite. Furthermore, the rainy season in the region, which floods 80% of the Pantanal (Alho 2008) and 98% of its area in Barão de Melgaço, promotes the migration of families, domestic and wild animals to dry areas. This migration results in increased animal and human population density, consequently increasing the risks of transmission and dissemination of VL.

As for the characteristics of the dogs assessed, no statistically significant difference (p>0.05) was observed concerning sex, breed, age, or coat, with animals being equally exposed to infection. The symptomatology variable, which describes the clinical status of dogs, was the only one associated with CVL: symptomatic dogs were 2.32 times more likely to have the disease (p≤0.05), corroborating the literature, which describes a positive correlation between *Leishmania* parasitic load and clinical manifestations in dogs with VL, since its main clinical signs are attributable to intense parasitism and, consequently, deposition of immune complexes (Manna et al. 2009, Manzillo et al. 2013).

CONCLUSIONS

The findings of present study confirm that canine visceral leishmaniasis (CVL) is present in the rural and urban regions of the municipality of Barão de Melgaço/MT, with relatively low molecular prevalence.

As for the independent variables, the information collected assists in understanding the epidemiology of this disease in the region, demonstrating that the municipality presents environmental conditions favorable to VL maintenance.

Presence of clinical signs is the only variable associated with canine infection.

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Conflict of interest statement.- The authors have no competing interests.

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