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ISOLATION OF *Leishmania (Leishmania) infantum chagasi* IN DOG WITH POLYARTHRITIS IN THE CITY OF ARAGUAÍNA, TOCANTINS: CASE REPORT

Taiã Mairon Peixoto Ribeiro[1], Samara Rocha Galvão[1], Sebastiana Adriana Pereira Sousa[2], Thássia Silva Reis[1], Helcileia Dias Santos[1]

[1]Escola de Medicina Veterinária, Universidade Federal do Tocantins, Araguaína, Tocantins.
[2]Instituto de Estudos do Trópico Úmido, Universidade Federal do Sul e Sudeste do Pará, Xinguara, Pará.
Author for correspondence: ribeiro.vet@uft.edu.br

Recebido em: 15/08/2021 – Aprovado em: 15/09/2021 – Publicado em: 30/09/2021
DOI: 10.18677/EnciBio_2021C27

ABSTRACT

Leishmaniasis are diseases with zoonotic potential caused by the protozoan *Leishmania* spp., which are transmitted by infected female phlebotomines. The present study aimed to describe the clinical condition and laboratory of visceral leishmaniasis and to report the isolation of *Leishmania* spp. from spleen and liver of a canine specimen admitted to the University Veterinary Clinic of the School of Veterinary Medicine and Zootechnics of the Federal University of Tocantins in 2015. The animal presented a condition of lameness and pain in the right and left calcaneal joints, in the metacarpal joint of the right forelimb and in the femoral-tibio-patellar joint. Fine-needle aspiration puncture of the animal's popliteal and pre-scapular lymph nodes was performed for direct parasitological examination for *Leishmania* spp. After confirming the presence of amastigote forms of *Leishmania* spp. in the parasitological examination, the animal was submitted to euthanasia and necropsy. Molecular diagnosis was also performed using the polymerase chain reaction technique, verifying an infection by *Leishmania* (*Leishmania*) *infantum chagasi*. Samples collected from spleen and liver were inoculated in Grace medium, with a significant increase in promastigote forms of *Leishmania* sp. after four days of incubation. Therefore, it was observed the presence of polyarthritis in a canine patient in the city of Araguaína and that parasitological, molecular and microbiological techniques can be used in the diagnosis of visceral leishmaniasis in dogs.

KEYWORDS: leishmaniasis, synovial fluid, immune complexes.

ISOLAMENTO DE *Leishmania (Leishmania) infantum chagasi* EM CÃO COM POLIARTRITE NO MUNICÍPIO DE ARAGUAÍNA, TOCANTINS: RELATO DE CASO

RESUMO

As leishmanioses são enfermidades de potencial zoonótico causadas pelos protozoários *Leishmania* spp., que são transmitidos por fêmeas de flebotomíneos infectadas. O presente estudo teve por objetivo descrever o quadro clínico e laboratorial de leishmaniose visceral e relatar o isolamento de *Leishmania* spp. de baço e fígado em um espécime canino admitido na Clínica Veterinária Universitária da Escola de Medicina Veterinária e Zootecnia da Universidade Federal do Tocantins no ano de 2015. O animal apresentou quadro de claudicação e dor nas articulações calcânea direito e esquerda, na articulação metacarpiana do membro anterior direito e na articulação fêmoro-tíbio-patelar. Foi realizada a punção aspirativa por agulha fina do linfonodo poplíteo e pré-escapular do animal para exame parasitológico direto para *Leishmania* spp. Após a confirmação da presença de formas amastigotas de *Leishmania* spp. no exame parasitológico, o animal foi submetido à eutanásia e necropsia. Foi ainda realizado o diagnóstico molecular pela técnica de reação em cadeia da polimerase, constatando a infecção por *Leishmania (Leishmania) infantum chagasi.* Amostras coletadas de baço e fígado foram inoculadas em Meio Grace, apresentando crescimento significativo de formas promastigotas de *Leishmania* sp. após quatro dias de incubação. Portanto, observou-se a presença de quadro de poliartrite em paciente canino no município de Araguaína e que as técnicas parasitológicas, moleculares e microbiológicas podem ser utilizadas no diagnóstico da leishmaniose visceral em cães.

PALAVRAS-CHAVE:imunocomplexo, leishmaniose, líquido sinovial,

INTRODUCTION

Visceral leishmaniasis or kala-azar (VL) is classified as a neglected tropical disease. This zoonosis is considered highly lethal, without an early diagnosis, and with few treatment options (BEJANO *et al*., 2021; SILVEIRA *et al*., 2021). VL is caused by protozoa of the genus *Leishmania* spp. and transmitted mainly by female phlebotomine sandflies of the genus *Lutzomyia* spp. (ZIEMNICZAK *et al*., 2021). Most of these vectors have a dispersion radius of 100 meters. However, they can reach greater distances depending on environmental conditions and obstacles (GALVIS-OVALLOS *et al*., 2018). The canine species is considered the most important reservoir of leishmaniasis in the urban space, although wild animals have also been identified as reservoirs (TRAVI *et al*., 2018).

The methods recommended by the Ministry of Health in Brazil for the diagnosis of canine visceral leishmaniasis (CVL) include the immunochromatographic test (Dual Path Platform technology-DPP) as a screening test and the enzyme-linked immunosorbent assay (ELISA) as a confirmatory test (SILVA *et al*., 2016).

The parasitological method as a diagnostic resource offers a specificity of approximately 100% and is an appropriate option for places with less infrastructure to perform serological, molecular and microbiological tests (LAURENTI *et al*., 2009; BRAZ *et al*., 2014). However, the identification of species has its limitations, since the

members of the genus *Leishmania* spp. produce similar amastigote forms and differentiation is possible only by molecular methods.

Molecular methods have good sensitivity and specificity, especially when primers targeting the kinetoplast minicircle DNA (kDNA) are used, being useful in the diagnosis of CVL in early clinical stages and in dogs without clinical signs (FERNANDES *et al*., 2019). However, there is still no national and international consensus on the standardization of the molecular diagnosis protocol for CVL in dogs (GALLUZI *et al*., 2018).

Isolation of *Leishmania* spp. in culture media depends on sterile conditions during sample collection and the need for maintenance in microbiological incubators of biochemical oxygen demand (BOD) at an average of approximately 25 ºC. These protozoa can be cultivated in Grace, Roswell Park Memorial Institute (RMPI 1640) and Novy-MacNeal-Nicolle (NNN) media (SANTOS *et al*., 2018; SIRIPATTANAPIPONG *et al*., 2019).

Currently, VL control is based on active and passive surveillance measures focused on dog population control in urban spaces, euthanasia of serologically positive dogs, periodic sample surveys and vector monitoring (COSTA *et al*., 2020). CVL treatment with the active principle Miltefosine is already available in Brazil (ARAÚJO *et al*., 2018). The aim of the present study is to report a clinical case of canine visceral leishmaniasis with signs of polyarthritis, in addition to describing the isolation and molecular detection of *Leishmania* (*Leishmania*) *infantum chagasi*.

CASE REPORT

A female dog, mixed breed, five months old, seven kilos, medium size, from the municipality of Araguaína, Tocantins, Brazil was seen on November 5, 2015 at the University Veterinary Clinic of the School of Veterinary Medicine and Zootechnics of the Federal University of Tocantins (CVU-EMVZ/UFT). In the anamnesis, the tutor reported, as the main complaint, signs of constant pain in the front and back paws, lameness in two limbs and episodes of inability to stand up because of pain in the joints. The animal had been showing these signs for fifteen days, according to the tutor.

The animal had been adopted 45 days prior to attendance at the CVU-EMVZ/UFT in an adoption event promoted by an animal protection entity. The tutor was instructed to perform examinations to verify the presence of endemic diseases at the time of adoption, so as not to incur damage to his health and that of other animals.

On clinical examination, the animal showed normal colored mucosa; heart and lung rates according to the reference standards for the species and age; enlarged submandibular, pre-scapular and popliteal lymph nodes; enlarged spleen; dehydration; thinness; fur with opacity; difficulty in locomotion; vocalization of pain when walking, with sensitivity in the right and left calcaneus; increased volume and sensitivity in the metacarpal region of the right forelimb, in the right and left calcaneal joints, and in the femoral-tibio-patellar joint. These signs confirmed polyarthritis. Splenomegaly was also observed on abdominal palpation.

The northern region of Tocantins is an endemic area for leishmaniasis, and consequently, the animal underwent fine needle aspiration puncture of the popliteal and submandibular lymph nodes, followed by parasitological examination for *Leishmania* spp. Blood count and biochemical exams (aspartate aminotransferase,

alanine aminotransferase, urea, creatinine, total protein and fractions) were also requested.

The exams were received one day after the clinical consultation at the CVU-EMVZ/UFT, being administered during this period Tramadol hydrochloride (3 mg/Kg/PO/TID). The radiographic exam would only be requested if the animal was negative in the parasitological exam for *Leishmania* spp.

The following were observed: normocytic hypochromic anemia, lymphocytosis and thrombocytopenia, and an increase in serum urea on biochemical examination. The parasitological examination showed a large number of amastigote forms characteristic of *Leishmania* spp. (Donovan corpuscles), classified by the EMVZ/UFT Veterinary Parasitology Laboratory as highly parasitized, which occurs when more than seven amastigote forms are found in a microscopic field.

The tutor was informed on the second visit to the clinic that canine leishmaniasis was a zoonosis, and that it constituted a risk to public and animal health. The dog's euthanasia was recommended to the tutor, as the treatment was made impossible because there was no registered product for the treatment of dogs in Brazil at the time of the service. After the consent of the tutor, the animal was euthanized in accordance with current legislation.

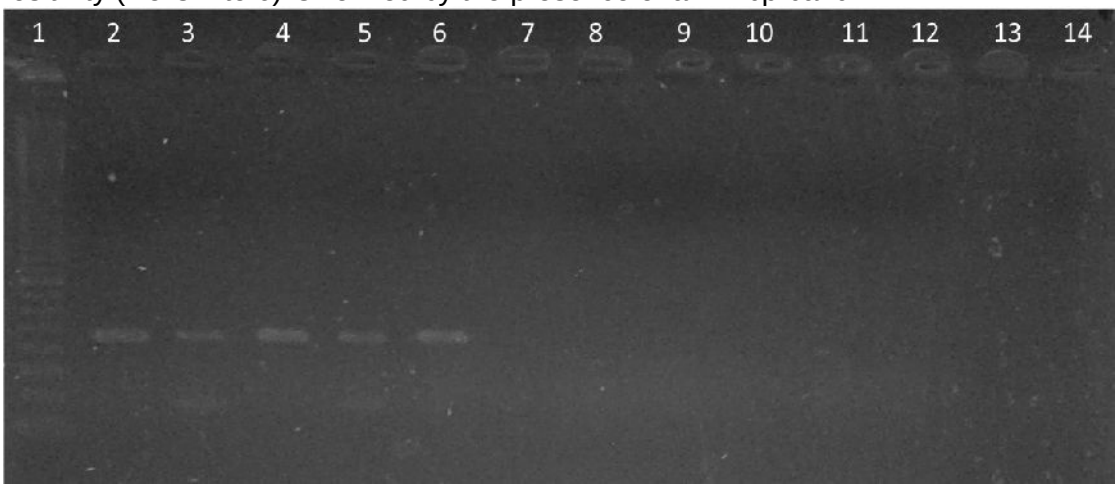
After euthanasia, necropsy was performed, and macroscopically it was found that the spleen was enlarged, in addition to polyarthritis in the fore and hind limbs. Microscopically, amastigote forms of *Leishmania* spp. were observed in an imprint of the splenic tissue, in a spinal smear obtained by puncturing the greater trochanter of the femur, and by arthrocentesis of the synovial fluid of the femoral-tibio-patellar joint.

Spleen, liver, bone marrow and synovial fluid samples were removed for inoculation in Grace Medium and incubation at 25 ºC. Promastigote forms were seen on the fourth day post-inoculation in the samples taken from the spleen and liver.

Liver tissue samples obtained during animal necropsy were submitted to DNA extraction using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich®, United States), according to the manufacturer's recommendations. Then, conventional PCR was performed using primers MC1 (5'-GTTAGCCGATGGTGGTCTTG-3') and MC2 (5'-CACCCATTTTTCCGATTTTG-3'), according to the methodology described by Cortes *et al.* (2004).

The PCR took place in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific®, United States) under the following conditions: an initial denaturation cycle of 95 °C for 5 minutes; 40 cycles of 95 °C for 30 seconds, 61 °C for 30 seconds, and 72 °C for one minute; and a final cycle of 72 °C for 10 minutes. Electrophoresis was performed in a specific vat (Bio-Rad®, United States) at 140V for five minutes, followed by 45 minutes at 110V. Each sample (10 µL) was homogenized with 2.5 µL of 0.25% bromophenol blue and applied to a 1.5% agarose gel (Agarose LE, Uniscience®, Brazil) containing 3.8 µL of ethidium bromide. After this process, the gel was visualized in a UV light transilluminator (Figure 1).

FIGURE 1. Polymerase Chain Reaction for *Leishmania* (*Leishmania*) *infantum chagasi* from biological samples from domestic animals in the municipality of Araguaína-TO. Observations: 1- 100bp molecular weight marker; 2- Positive control, strain P75; 3- Positive animal; 4- Positive animal; 5- Positive animal; 6- Liver biopsy of a canine patient (Meg); 7- Negative animal; 8- Negative animal; 9- Negative animal; 10- Negative animal; 11- Negative animal; 12- Negative animal; 13- Well without sample; 14- Negative control. Positivity (wells 2 to 6) is verified by the presence of a 447bp band.



Source: Authors' personal archive (2021).

DISCUSSION

Animals with arthritis caused by visceral leishmaniasis in dogs are not uncommon, and this clinical manifestation is a consequence of the presence of the agent in the joint, causing local inflammatory reaction or the deposition of immune complexes (GIZZARELLO *et al*., 2020). The use of arthrocentesis followed by cytological examination of the synovial fluid is an option for the parasitological diagnosis in animals that have died. When applied to live animals, the collection must always be done with the animal under general anesthesia or sedation (CHAVES *et al*., 2015). In dogs with laboratory-confirmed leishmaniasis, the presence of amastigotes in the synovial fluid can reach 87.5% (SILVA *et al*., 2014). Rennó *et al*. (2019), however, emphasize that sensitivity is better in animals in stage three of the disease, that is, in animals with severe conditions. Other species of *Leishmania* spp., such as *Leishmania* (*Leishmania*) *amazonensis* also cause clinical presentations with polyarthritis (HOFFMANN *et al*., 2012).

The presence of anemia and thrombocytopenia corroborated what was found by Braz *et al*. (2015). Normochromic anemia is also common in CVL, however hypochromia can be attributed to pro-inflammatory disorders in active visceral leishmaniasis that lead to alterations in the cytokine profile, resulting in hepcidin-mediated iron storage in macrophages, reducing its availability for erythropoiesis (SINGH *et al*., 2019). Thrombocytopenia is also a common finding in canine leishmaniasis and can occur because of disturbances in thrombocytopoiesis, increased by consumption or immune-mediated thrombocytopenic mechanisms (DOMINGUEZ; TORAÑO, 2001). The increase in serum urea may be caused by renal impairment (EBERT *et al*., 2021). Renal tubular and interstitial and disease-specific lesions are related to the deposition of immune complexes or immune cell mechanisms resulting from infection by *Leishmania* spp. (SALGADO-FILHO *et al*., 2003).

The parasitological diagnosis has high specificity, but there may be variation in sensitivity (30-85%), as the parasites are not homogeneously distributed in the tissues of the parasitized animal (LAURENTI *et al*., 2009; BRAZ *et al*., 2014). In dogs, with or without a clinical history of leishmaniasis, in the municipality of Araguaína, a prevalence of 40.7% has previously been observed using parasitological diagnosis by puncture of peripheral lymph nodes (SANTOS *et al*., 2017). Even though this method has a sensitivity limitation, it is a practical tool in laboratory diagnosis.

Isolation allows characterizing the species of *Leishmania* spp. that affected the animal, in addition to enabling the development of serological tests with antigens from strains circulating in the region. This action can increase the sensitivity in the development of serological tests with the crude antigen of the isolate, and, when associated with recombinant antigens, can improve the test (ROSÁRIO *et al*., 2005). However, there is the disadvantage of the need for infrastructure and of contamination during collection, after inoculation into the culture medium, or even after isolation, mainly caused by fungal or bacterial contamination.

Molecular diagnosis is useful for determining the species of *Leishmania* spp, but also for the phylogenetic study of the parasite. Fernandes *et al*. (2019) indicate greater capacity of the primers 13A/13B, developed by Rodgers *et al*. (1990), in detecting positive canine specimens at disease onset compared with other primers. Molecular diagnosis is promising, though, there is a need for standardization at an international or national level and the use of specialized equipment, which limit its application. Nevertheless, with the advent of processes that reduce inputs and equipment, the trend is that molecular techniques will be increasingly used in clinical-veterinary diagnosis and in laboratory routine.

CONCLUSION

Leishmania (Leishmania) infantum chagasi can cause clinical cases of polyarthritis in dogs in the municipality of Araguaína. Furthermore, isolation, parasitological and molecular diagnosis can provide good results in clinical conditions compatible with canine visceral leishmaniasis. This prospect demonstrates alternatives for the phylogenetic study of the parasite and helps the medical-veterinary diagnosis.

ACKNOWLEDGMENTS

The authors would like to thank the Research Support Foundation of Tocantins and the Research Program for the Brazilian Unified Health System (PPSUS) of the National Council for Scientific and Technological Development (CNPq).

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