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DETECTION OF *Leishmania chagasi* IN CATS (*Felis catus*) FROM VISCERA LEISHMANIASIS ENDEMIC AREA IN BRAZIL

(Detecção de *Leishmania chagasi* em gatos de área endêmica de leishmaniose visceral no Brasil)

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ABSTRACT

The number of confirmed cases of feline leishmaniasis has increased in endemic areas in recent years, suggesting that the feline species must no longer be considered an uncommon host for *Leishmania* spp. Thus, the aim of this work was to investigate the occurrence of *Leishmania* infection in cats from Campo Grande Municipality, where visceral leishmaniasis is endemic. The 110 cats studied were subjected to clinical examinations and serological evaluation by IFA to determine anti-*Leishmania* antibody titers. The parasitological investigation of seropositive animals consisted in cytological examination, tissue culture, and PCR of bone marrow and popliteal lymph node aspirates. Seropositivity was detected for eight animals (7.27%). Amastigotes were not detected in the cytological examinations and promastigotes were not observed in the tissue culture. However, three (40.2%) of the seven processed samples were positive according to PCR, confirming the contact of cats with the parasite.

Keywords: *Leishmania chagasi*, PCR-RFLP, cat.

RESUMO

O número de casos confirmados de leishmaniose felina aumentou em áreas endêmicas nos últimos anos, o que sugere que a espécie felina não deve mais ser considerada um hospedeiro incomum para *Leishmania* spp. Assim, o objetivo deste trabalho foi investigar a ocorrência de infecção por *Leishmania* spp em gatos do Município de Campo Grande, onde a leishmaniose visceral é endêmica. Os 110 gatos estudados foram submetidos a exame clínico e sorológico por IFA para determinar a titulação de anticorpos anti-*Leishmania*. A investigação parasitológica de animais soropositivos consistiu em exame citológico, cultura de tecidos, e PCR de aspirados de medula óssea e linfonodos poplíteos. A soropositividade foi detectada em oito animais (7,27%). Amastigotas não foram detectados nos exames citológicos e promastigotas não foram observadas em cultura de tecidos. No entanto, três (40,2%) das sete amostras processadas foram positivas de acordo com o PCR, o que confirma o contato dos gatos com o parasita.

Palavras-chave: *Leishmania chagasi*, PCR-RFLP, gato.

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INTRODUCTION

Leishmaniasis, an infectious disease of public health importance, has an anthropozoonotic character and is caused by the protozoan *Leishmania* spp. (MURRAY et al, 2005).

The agent is transmitted by phlebotomine sand flies and its vertebrate hosts are wild animals such as rodents, skunks, anteaters, canids, primates and sloths, in addition to domestic animals, especially dogs, horses, cats and humans (GONTIJO & CARVALHO, 2003; GONTIJO & MELLO, 2004; MANCIANTI, 2004).

Infection by *Leishmania* is considered rare among felines (PENNISI, 2002; MANCIANTI, 2004), but the number of clinical cases has increased in recent years. Serological and parasitological examinations have detected this infection in a considerable number of cats. Species such as *Leishmania infantum*, *L. chagasi*, *L. donovani*, *L. braziliensis*, *L. amazonensis*, *L. mexicana*, *L. tropica* and *L. venezuelensis* have been found in domestic and wild cats with different clinical symptoms (MACHATTIE et al. 1931; MORSY et al. 1980; BARNES et al. 1993; BONFANTE-GARRIDO et al. 1996; OZON et al, 1998; SAVANI et al, 2004; MARTÍN-SÁNCHEZ et al, 2007; FIGUEIREDO et al, 2008, SOUZA et al, 2009).

Studies carried out in several countries have indicated that some *Leishmania* spp. vectors feed on felines (JOHNSON et al, 1993; OGOSUKU et al, 1995; COLMENARES et al, 1995; MAROLI et al, 2007).

Cases described in the literature show that both cutaneous and visceral types of leishmaniasis affect feline species. The general clinical symptoms, which appear to be related to disseminated leishmaniasis, include fever, lethargy, anorexia, vomiting, diarrhea, weight loss, emaciation, dehydration, polyuria, polydipsia, pale mucosa, local or generalized lymphadenomegaly, hepatomegaly, splenomegaly and respiratory disorders (OZON et al, 1998; HERVÁS et al, 1999; POLI et al, 2002; PENNISI et al, 2004; SAVANI et al, 2004; LEIVA et al, 2005). Cutaneous damage is reported for every leishmaniasis case, and the head is most affected, with lesions mainly located in the face, nose, nasal mucosa, lips, eyelids and ears (CRAIG et al, 1986; COSTA-DURÃO et al, 1994; PASSOS et al, 1996; OZON et al, 1998; HERVÁS et al, 1999; POLI et al, 2000; SAVANI et al, 2004; MARTÍN-SÁNCHEZ et al, 2007; SCHUBACH et al, 2004).

The main techniques used for leishmaniasis diagnosis are parasitological tests (direct visualization of the parasite in tissue aspirate, imprinting and culture); serological tests (especially indirect immunofluorescence assay - IFA and enzyme-linked immunosorbent assay - ELISA); and molecular tests (polymerase chain reaction - PCR) (PENNISI, 2002; MARTÍN-SÁNCHEZ et al, 2007; BANEHT et al, 2008; SOUZA et al, 2009).

Protocols for feline leishmaniasis treatment have not been established, and those applied for dogs have been ineffective in promoting a definitive cure, providing only temporary clinical improvement (LEIVA et al, 2005). There is no specific procedure for leishmaniasis control and prophylaxis in domestic cats because these animals are not included in the disease transmission chain.

Domestic cats are considered occasional hosts of *Leishmania*, and reports establishing any relationship between them are scarce. However, more studies should be devoted to cats living in leishmaniasis endemic areas, especially considering that domestic cats have a close relationship with humans and that the vectors have a high capacity to adapt to different environments and vertebrate hosts, in addition to constant and continuous ecosystem transformations. The aim of the present study

was to investigate infection by *Leishmania* spp. in domestic cats from Campo Grande Municipality, Mato Grosso do Sul State, Brazil.

MATERIAL AND METHODS

Studied area

Campo Grande is located in the central region of Mato Grosso do Sul state, in a plateau (between 500-675 meters high). The city is in a neotropical zone, and the predominant vegetation is cerrado. Estimated population in 2007 was 786,524 people according to Instituto Brasileiro de Geografia e Estatistica (IBGE), in 2010 was 786,797 people and the estimate for 2015 is 853,622. It is a visceral leishmaniasis endemic city, having registered canine and humane cases.It is a visceral leishmaniasis endemic city, having registered canine and humane cases.

Animals

According to the local Center for Zoonosis Control (CZC), the estimated cat population in 2007 was 24.450 cats. The study population was composed of 110 domestic cats of both sexes and different ages, living in their owner's houses. Animals from the four regions (north, south, east and west) of the metropolitan area of Campo Grande were sampled. They were randomly selected, and the sampling was carried out only after obtaining the owner's consent. The study followed the ethics protocol recommended by the Brazilian College of Animal Experimentation (COBEA). Comitê de Ética de Uso de Animais (CEUA/UFMS): protocol number 189/2008.

The cats were clinically evaluated during domiciliary visits for overall health conditions and presence of dermatosis. General information about the owners and the cats were also recorded on individual datasheets. Biological materials were collected from May to November 2007.

Biological sample collection

The animals were subcutaneously anesthetized with 5 mg/kg ketamine 50% (Vetanarcol®-König/Brazil) associated with 2 mg/kg xylazine 2% (Anasedan®-Vetbrands/Brazil). Anesthetized cats had 5 mL blood collected from the jugular vein with a 25x7 needle in a 5mL syringe. For blood count, 1mL blood was stored in a glass tube containing ethylenediaminetetraacetic acid (EDTA). The remaining blood was transferred to a glass tube, heated in water bath for 15min at 37°C and centrifuged for serum separation. Serum samples were stored in microfuge tubes at -20°C.

Bone marrow (tibial tuberosity) and popliteal lymph node aspirates were collected from seropositive animals (except cat nº 25, whose owner did not authorize anesthesia or sample collection). The animals were anesthetized before aspirate collection, as described for blood collection but using the endovenous route.

Popliteal lymph node was aspirated by using a 25G ⅝ needle in a 5mL sterile syringe for further culture, direct parasitological visualization and polymerase chain reaction (PCR). Bone marrow was aspirated through a 30x8 needle in a 10mL sterile syringe for further direct parasitological observation and PCR. To prevent contamination during tissue culture, a second aspirate collection from the bone marrow was performed in a closed system using a 30x8 needle in a sterile heparinized vacuum tube. All samples were processed immediately after collection. For PCR, the aspirates were stored in microfuge tubes at -20°C.

Laboratorial analyses

Indirect immunofluorescence antibody test (IFAT)

Indirect immunofluorescence antibody Test (IFAT) was carried out at the Immunology Section of the Central Public Health Laboratory-LACEN, Campo Grande.

The materials used for IFA were: 110 serum samples; *Leishmania* antigen (commercial kit, Bio-Manguinhos, Fiocruz, lot number 070LC007Z, validity Mar/08); positive control: female feline sera with 1:160 anti-*Leishmania* antibody titer and male feline sera with 1:40 antibody titer, both tested by the Laboratory of Zoonoses and Diseases Transmitted by Vectors of the Center for Zoonosis Control (CCZ), São Paulo Municipality, São Paulo State, Brazil; negative control: two feline serum samples tested in the same CCZ lab; cat anti-IgG conjugate produced and kindly donated by CCZ of São Paulo, lot number 106/00, at 1:300 dilution.

The initial dilution of test samples was 1:40. Thereafter, reactive sera were titrated (1:80; 1:160 and 1:320). IFA protocol followed the manufacturer's recommendations (Bio-Manguinhos, Fiocruz).

Hematological assay

A complete evaluation (erythrocyte count, leukocyte count and hematozoa examination) was performed for 108 animals (samples no. 53 and 107 were lost and could not be collected again). Blood smears were prepared, air dried, fixed and stained with May-Grünwald-Giemsa (MGG) to allow examination under an optical microscope (40x).

Serum biochemical tests

Total serum protein and albumin were determined for seropositive animals, and globulin levels were calculated from these values.

Parasitological investigations

To investigate amastigotes, thin smears of bone marrow and lymph node aspirates were fixed and stained with May Grunwald Giemsa (MGG) to allow examination under an optical microscope (100x).

To investigate promastigote forms, lymph node aspirates and 0.5mL bone marrow were cultured in a biphasic medium: a solid Novy-MacNeal-Nicolle (NNN) phase added to a Schneider's medium liquid phase. These were supplemented with 20% fetal bovine serum (Sigma®) and antibiotic, incubated at 24°C and weekly examined for seven weeks under an optical microscope.

DNA extraction and polymerase chain reaction (PCR)

The bone marrow and lymph node aspirates from seropositive animals were subjected to PCR for detection of *Leishmania* minicircle kinetoplast DNA (kDNA), according to the already described protocol (PIRMEZ et al, 1999). The adopted molecular weight marker was Promega® (Madison, Wiss, USA) with 50 bp (base pairs).

Restriction fragment length polymorphism (RFLP)

To identify *Leishmania* species, the PCR-restriction fragment length polymorphism (PCR-RFLP) assay was performed on previous PCR-amplified products, according to a modified methodology (VOLPINI et al. 2004; ANDRADE et al. 2006). In this case, the restriction enzime utilized was MspI (Fermentas International Inc., Ontario, Canada), which specifically digests *L. chagasi*.

RESULTS

Cats descriptions

Of the 110 cats evaluated, 56 were males and 54 females, with ages ranging from 5 months to 13 years. Two cats were Persian, 15 Siamese and 93 crossbred. The animals were domestic, living in their owners' houses, and had hunting habits.

Nine (8.18%) of the 110 cats had clinical alterations such as fever, weight loss, stomatitis, enteritis, jaundice and body underdevelopment; eight (7.27%) had dermatoses characterized by alopecia, rarefied hair, laceration and ulcerative dermatitis.

Indirect immunofluorescence antibody test (IFAT)

Eight animals (7.27%) were positive in this test, one with titers of 1:80 and seven with titers of 1:40.

Hematologicla assay

Hemoparasites were not found in the hematological evaluation, but the following alterations were detected: anemia (23 cats; 21.29%); leukopenia (8 cats; 7.40%); leukocytosis (8 cats; 7.40%).

Serum biochemical test

It was observed an increase of total plasma proteins in 14(12,96%) animals, and a decrease in 15 (13,88%) of them. Hyperfibrinogenemia was seen in 10 (9,26%) cats. Concentrations of serum protein, globulin and albumin of the seroposotive animals are shown in Table 1.

Parasitological tests

Amastigote forms of the parasite were not detected in the cytological examination; similarly, promastigote forms were not found in the tissue cultures.

PCR and RFLP

Three of seven cats were positive according to PCR (Figure 1).

In the PCR-RFLP test, the enzyme MspI digested PCR products in three restriction fragments of 30, 90 and 120 bp, respectively, indicating that the incriminated species was *Leishmania chagasi* (Figure 2).

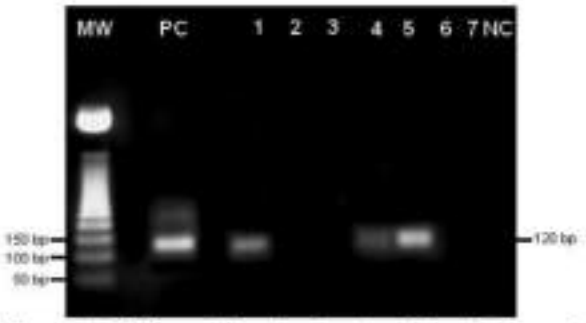

Figure 1. PCR-amplified products of the Leishmania minicircle conserved region in 1% agarose gel electrophoresis. **MW**: molecular weight marker, 50 bp (Promega, Madison, WI). **PC**: positive control, DNA from L. braziliensis (L566). **1**: cat number 85, lymph node sample; **2**: cat number 12; **3**: cat number 17; **4**: cat number 89, bone marrow sample; **5**: cat number 106, lymph node sample; **6**: cat number 20; 7: cat number 90; **NC**: negative control.

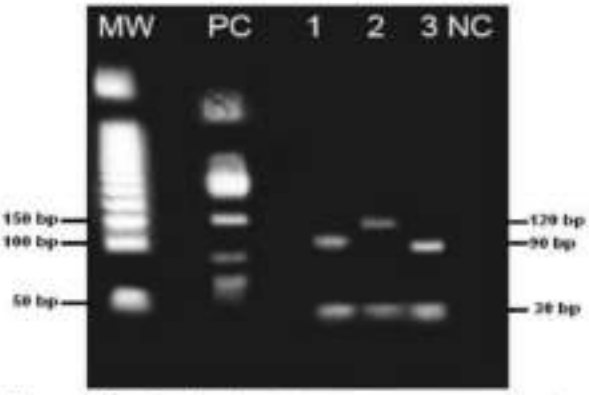

Figure 2. Restriction fragments generated after digestion with MspI of the conserved region of minicircle kDNA amplicons, in 10% polyacrylamide gel electrophoresis. **MW**: molecular weight marker, 50 bp (Promega, Madison, WI). **PC**: positive control, DNA L. braziliensis (L566); **1**: cat number 85; **2**: cat number 89; **3**: cat number 106; **NC**: negative control.

Clinical signs, hematological features, and PCR results for seropositive animals are shown in Table 1.

Table 1. Clinical signs, hematological features, concentrations of serum protein, globulin and albumin, and PCR results for seropositive cats. TPP: total plasma protein. IFA: indirect immunofluorescence assay. BM: bone marrow; LN: lymph node; -: negative; +: positive. Normal values for red blood cells: 5.0-10 $x10^6/mm^3$; leukocytes: 5.5-19.5$x10^3/mm^3$; TPP: 6.0-8.0 g/dl (CLINKEBEARD & MEINKOTH, 2000); a) 5.4-7.8 g/dl; b) 2.6-5.1 g/dl and c) 2.1-3.3 g/dl (KANEKO et al, 1997).

ANIMAL NUMBER	CLINICAL SYMPTOMS	IFAT	BLOOD COUNT/SERUM PROTEIN[a] GLOBULIN[b]/ALBUMIN[c]	PCR-RFLP
12	General alopecia, pregnancy beginning	1:80	Hyperglobulinemia (6.5)[b] Hypoalbuminemia (0.5)[c]	BM - / LN -
17	Lymphadenomegaly, weight loss	1:40	Anemia($4.5x10^6$)/leukopenia($1.1x10^3$) TPP↑(8.2)/hyperproteinemia(7.9)[a] Hyperglobulinemia (7.2)[b] Hypoalbuminemia(0.7)[c]	BM - / LN -
20	absent	1:40	Anemia($4.8x10^6$)/leukopenia($4.5x10^3$) Hyperglobulinemia(7,0)[b] Hypoalbuminemia(0.8)[c]	BM - / LN -
25	absent	1:40	TPP ↑(9.4)/Hyperproteinemia(8.4)[a] Hyperglobulinemia(7.6)[b] Hypoalbuminemia(0.8)[c]	Not sampled
85	absent	1:40	Leukopenia($7.7x10^3$) Hyperglobulinemia(6.8)[b] Hypoalbuminemia(0.8)[c]	BM - / LN +
89	absent	1:40	Anemia($5.1x10^6$) Hyperglobulinemia(6.5)[b] Hypoalbuminemia(0.5)[c]	BM +/ LN -
90	absent	1:40	Leukopenia($5x10^3$) Hyperglobulinemia(6.1)[b] Hypoalbuminemia(0.7)[c]	BM - / LN -
106	constant bilateral epiphora	1:40	Hyperglobulinemia(6.6)[b] Hypoalbuminemia(0.6)[c]	BM - / LN +

DISCUSSION

The one hundred and ten cats evaluated represent 0,45% of the estimated cat population of Campo Grande.

All cats evaluated had hunting habits. This behavior may facilitate the vector biting and also the contact with other agents that cause many cat diseases since they used to

walk long distances from their home to hunt, especially at night which is the period of highest fly activity. In surrounding metropolitan areas, cats hunted mainly in forest areas where the vector occurs.

In the present study, serum samples from 110 cats were analyzed by IFA for anti-*Leishmania* antibody, which was found in eight animals (7.27%), one with titers of 1:80 and seven with titers of 1:40. Serological investigations in cats have indicated a wide variation in the number of sero-reactive animals, ranging from 0 to 68% (SHERLOCK, 1996; PENNISI et al, 2000). When detected, antibodies are frequently at low titers such as 1:10 and 1:20 (PENNISI, 2000; PENNISI, 2002; MARTÍN-SÁNCHEZ, 2007). Cats experimentally infected with *L. brasiliensis* showed low serology titers and later on suffered progressive decreasing titers levels until become seronegative (SIMÕES-MATTOS et al., 2005). Another study detected that cats naturally infected with *L. infantum* also had low antibody titration, which decreased until cats become seronegative within time (VITA et al, 2005). This may be related to the resistance of these animals. Furthermore, feline leukemia and immunodeficiency virus infections may compromise the production of anti-*Leishmania* spp. antibodies in cats (MARTÍN-SÁNCHEZ et al, 2007), possibly accounting for the low titers found in infected felines. A standard minimal titer value for anti-*Leishmania* spp. antibody has not been determined for cats; thus, the initial dilution of 1:40 was based on canine serology.

According to some authors, canine serum subjected to IFA can show cross-reactions with Chagas disease, ehrlichiosis and toxoplasmosis (GOMES et al, 2006), thereby indicating a false-positive result. Thus, cross-reactions with *Toxoplasma* spp., *Trypanosoma* spp. and *Ehrlichia* spp. cannot be discarded in IFA of cats. Therefore it is mandatory to associate other diagnostic tools such as PCR.

Hematological evaluation of seropositive cats detected anemia, leukopenia and hyperproteinemia associated with globulin increase and albumin decrease. These alterations corroborate other studies about feline visceral leishmaniasis describing anemia, leukopenia or leukocytosis and hyperproteinemia with hyperglobulinemia and hypoalbuminemia (CRAIG et al, 1986; COSTA-DURÃO et al, 1994; OZON et al, 1998; BRITTI et al, 2005; RUFENACHT et al, 2005; SOUZA et al, 2009), although these findings can also be present in other diseases.

Cytological analyses and tissue culture were negative. A study evaluated seven felines with positive PCR for *Leishmania*, of which three showed positive cytological results and none of them had positive result for tissue culture (MARTÍN-SÁNCHEZ et al, 2007). This may have occurred due to the low *Leishmania* parasitemia or because the studied felines were in a controlled disease stage, reinforcing the idea that these animals have a natural resistance to *Leishmania* infection.

In contrast, the possible association between feline leishmaniasis and immunosuppressive diseases indicates that *Leishmania*-infected felines affected by feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline infectious peritonitis (FIP) or stress could facilitate parasite multiplication and disease manifestation, thereby making these animals potential reservoirs (HERVÁS et al, 2001; POLI et al, 2002; SAVANI et al, 2004; GREVOT et al, 2005). The animals have not

been tested for FIVand FeLV. Thus, once there is no confirmation between the association of these viral infections with feline leishmaniasis (SOLANO-GALLEGO et al, 2007; SHERRY et al, 2011; SOBRINHO et al, 2012), besides budget restraint, tests were not performed on the studied animals.

PCR of bone marrow and lymph node samples from seropositive animals (except cat no. 25; Table 1) was positive for three clinically healthy animals (two lymph node and one bone marrow samples). Studies on seropositive and seronegative cats have shown that the parasite DNA is not necessarily detected in tissue samples subjected to PCR, whereas the genetic material of the parasite can be found in seronegative animals (PENNISI et al, 2000; MARTÍN-SÁNCHEZ et al, 2007). The absence of parasite DNA in seropositive animals may be associated with serological cross-reaction, persistence of antibody titers after parasite elimination, low parasitemia and/or infection control.

The PCR positive animals were clinically healthy. Epidemiological surveys have detected *L. infantum* by PCR, in asymptomatic cats (MARTÍN-SÁNCHEZ et al, 2007; MAIA et al, 2008; HATAM et al, 2010) and with high parasitic burden (TABAR et al, 2008). In Brazil, a epidemiologic survey detected 66 *Leishmania spp* infected cats, from which 26 were asymptomatic (SOBRINHO et al, 2012). Controlled infection, detection after parasite elimination or low parasitemia may justify the absence of clinical signs in PCR positive animals. These results demonstrate the natural resistence of cats to the infection by *Leishmania* spp. However, little is known about the real role of cats in the cycle of *Leishmania* spp.

Considering the cases of feline infection by *Leishmania* spp. in Brazil, seven cases which occurred in Cotia, Andradina and Araçatuba city-São Paulo State; Rio de Janeiro city-Rio de Janeiro State; Belo Horizonte city-Minas Gerais State, were caused by the species *Leishmania chagasi* (SAVANI et al, 2004; LINO et al, 2007; SILVA et al, 2008; COELHO et al, 2010; SILVA et al, 2010; VIDES et al, 2011; SOBRINHO et al, 2012;). In Campo Grande, *Leishmania amazonensis* was the only species identified in a study on feline leishmaniasis (SOUZA et al, 2005). Thus, the present work is the first report of the occurrence of *Leishmania chagasi* in domestic cats from Campo Grande.

CONCLUSIONS

Serological analyses and molecular confirmation and identification of *Leishmania chagasi* indicated the contact of cats with the protozoan. Since visceral leishmaniasis in dogs and humans is endemic to Campo Grande Municipality, further studies are needed on the role of the cat in the epidemiology of the disease.

Researchers, especially small animal practicers should also be advised to include leishmaniasis in the differential diagnosis of feline systemic diseases and dermatopathies.

This result should alert sanitary authorities about the epidemiological importance of this species, especially in visceral leishmaniasis endemic areas.

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