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Short Communication

Serological, molecular, and microscopic detection of *Leishmania* in cats (*Felis catus*) in Belo Horizonte, Minas Gerais State, Brazil

Detecção sorológica, molecular e microscópica de *Leishmania* em gatos (*Felis catus*) em Belo Horizonte, Minas Gerais, Brasil

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Abstract

The role of cats in the epidemiological cycle of leishmaniasis remains unclear. To better understand the occurrence of leishmaniasis in cats, we studied the frequency of *Leishmania* in serum samples of 100 cats living in an endemic region for canine and human leishmaniasis by serological, parasitological, and molecular methods. Of the 100 cats, 54 were seropositive for *Leishmania* antibodies by immunofluorescence antibody test. None of the bone marrow aspirates collected from these cats tested positive for the parasite in culture or upon polymerase chain reaction (PCR) analysis. Biopsy samples of the ears also tested negative for *Leishmania* upon PCR analysis. These findings may indicate that the region is endemic for canine leishmaniasis and cats are infected by *Leishmania*; or that cross-reaction with antibodies against other parasites increases the frequency of seropositivity; or that cats respond to *Leishmania* infection by producing antibodies when few or no parasites are present in bone marrow and tissue samples. Overall, our results suggest that cats can be infected by *Leishmania*; however, we failed to demonstrate feline parasitosis. These findings highlight the need to study leishmaniasis in cats, since sandflies feed on cats, these animals may act as a reservoir for the parasite.

Keywords: Diagnosis, feline leishmaniasis, reservoir.

Resumo

O papel dos gatos no ciclo epidemiológico da leishmaniose ainda não está claro. Para entender melhor a ocorrência de leishmaniose em gatos, estudou-se a frequência de *Leishmania* em amostras de soro de 100 gatos, os quais vivem em uma região endêmica para leishmaniose canina e humana, por métodos sorológicos, parasitológicos e moleculares. Dos 100 gatos, 54 foram soropositivos para anticorpos de *Leishmania* por teste de anticorpos de imunofluorescência. Nenhum dos aspirados de medula óssea coletados desses gatos mostrou-se positivo para o parasita em cultura, ou após a realização da reação em cadeia da polimerase (PCR). Amostras de biópsia das orelhas também foram negativas para *Leishmania* submetidas a PCR. Esses achados indicam que na região estudada endêmica para leishmaniose canina, os gatos podem se infectar por *Leishmania*; ou que a reação cruzada com anticorpos contra outros parasitas aumenta a frequência de soropositividade; ou que os gatos respondem à infecção por *Leishmania* produzindo anticorpos quando poucos ou nenhum parasita estão presentes na medula óssea e em amostras de tecido. Em geral, os resultados sugerem que os gatos podem ser estar infectados por *Leishmania* spp. No entanto, não foi possível demonstrar parasitismo felino. Essas descobertas evidenciam a necessidade de estudar a leishmaniose em gatos, uma vez que, como os flebotomíneos se alimentam em gatos, e esses animais podem atuar como um reservatório para o parasita.

Palavras-chave: Diagnóstico, leishmaniose felina, reservatório.

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Introduction

Visceral leishmaniasis (VL) is a zoonosis caused by *Leishmania infantum*, an endemic parasite in over 70 countries worldwide (BANETH et al., 2008). Dogs are considered the main reservoir of the parasite in domestic and peridomestic areas; however, since recent studies have reported that cats may become infected with the protozoan, questions have been raised regarding the epidemiological role of cats in the zoonotic cycle and their potential as parasite reservoirs (MAIA & CAMPINO, 2011).

Leishmaniasis is transmitted by the bite of female phlebotomine sandflies, mainly *Lutzomyia longipalpis* and *Lutzomyia cruzi* in Brazil. Although rarely seen, the most frequent clinical signs of leishmaniasis in cats include skin changes such as papules, nodules, ulcers, and alopecia (SILVEIRA et al., 2015). *Leishmania* species identified in cats include *Leishmania mexicana, Leishmania venezuelensis, Leishmania braziliensis, Leishmania amazonensis,* and *L. infantum* (PENNISI et al., 2015).

Multiple studies have examined the seroprevalence of *Leishmania* (CARDIA et al., 2013; SILVA et al., 2014; BRAGA et al., 2014; NOÉ et al., 2015; OLIVEIRA et al., 2015; BALDINI-PERUCA et al., 2017) and the occurrence of natural cases of leishmaniasis (PASSOS et al., 1996; SCHUBACH et al., 2004; SOUZA et al., 2005) in cats in Brazil. However, experimental studies on leishmaniasis in cats are scarce (KIRKPATRICK et al., 1984; SIMÕES-MATTOS et al., 2005), and reports regarding the evaluation of the cellular immune response against the parasite are lacking (PENNISI et al., 2015). Such studies could help understand the rarity of clinical signs observed in cats infected with *Leishmania* spp. as well as the corresponding immune response, thereby helping to standardize diagnostic procedures.

Because of the suggestion that cats could serve as potential reservoirs of *Leishmania* spp., the objective of the present study was to investigate the occurrence of *L. infantum* infection in cats living in an endemic region for canine leishmaniasis (CL) and human VL, using serological, parasitological, and molecular methods.

Materials and Methods

The experimental group consisted of 100 cats (*Felis catus*) that were randomly selected from among 250 cats rescued from streets and housed in a shelter for animals in Belo Horizonte, Minas Gerais, Brazil. The city is endemic for CL and has registered cases of human VL. As the cats previously lived on the streets, the shelter could not provide any details regarding breed, sex, or age of the animals.

The cats were clinically evaluated before blood sample collection. Physical examination consisted of general appearance, body condition, weight, temperature, hydration status, mucous membrane color, capillary refill time, palpation of lymph nodes, heart and respiratory rates, abdominal palpation, and examination of the skin and haircoat. All cats were clinically regarded as asymptomatic. The experimental procedures were approved by the Ethics Committee on Animal Experimentation, Universidade Federal de Minas Gerais (CETEA 242/2014), prior to the start of this study.

Using disposable syringes and needles, blood samples were collected from the jugular or cephalic vein and transferred into tubes without ethylenediaminetetraacetic acid. These samples were centrifuged at 1500 x *g* for 15 min, and the separated serum was transferred into plastic microtubes and stored at -20 °C until serological analysis. The serum samples were tested for *Leishmania* antibodies via the indirect immunofluorescence antibody test (IFAT) (VIDES et al., 2011). Cats were categorized as seropositive on the basis of the cutoff point (40 UI) that was previously suggested for dogs (MINISTÉRIO DA SAÚDE, 2000).

Cats found to be seropositive according to the IFAT were further evaluated through parasitological analysis of bone marrow aspirates collected from the tibial tuberosity. Bone marrow samples were collected for cytological smear, culture, and polymerase chain reaction (PCR) analyses.

In order to investigate the presence of amastigotes, smears of bone marrow aspirates were fixed and stained with a panoptic stain (May-Grünwald Giemsa) and examined under an optical microscope (100×). To investigate the presence of promastigote forms, bone marrow samples were cultured in Novy–MacNeal–Nicolle (NNN) medium, supplemented with liver infusion tryptose medium (SCHUSTER & SULLIVAN, 2002). These samples were incubated at 25 °C and examined weekly for 8 weeks under an optical microscope. Bone marrow aspirates from seropositive animals were subjected to PCR analysis to detect *Leishmania* minicircle kinetoplast DNA (kDNA), as described by DEGRAVE et al. (1994) and PASSOS et al. (1996). Briefly, PCR was performed in a total volume of 25 µL of a mixture containing 5 µL of DNA template, 0.2 mM of DNTPs, 1.5 mM MgCl2, 1.5 µL of DMSO 5%, 0.4 µM of each primer, buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.0) and 1.25 U of Taq DNA polymerase (Invitrogen™; Carlsbad, CA, USA). PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 61 °C for 1 min, 72 °C for 3 min, and a final extension at 72 °C for 10 min. Negative and positive PCR controls were included in each reaction set. The negative control contained no DNA, whereas the positive controls contained 0.01 and 1.0 pg of standard *Leishmania* DNA (*L. infantum* strain MHOM/BR/74/PP75).

In addition, 1 cm^2 biopsy samples of ears were collected and stored in RNA*later*® storage solution (Invitrogen™; Carlsbad, CA, USA) in microtubes. This site was chosen because experimental models for *Leishmania* spp. transmission allow sandflies to feed in their ears (SECUNDINO et al., 2012). These samples were processed for DNA extraction in accordance with the manufacturer's instructions (Gentra® Puregene® Tissue Kit; Qiagen; Valencia, CA, USA). The DNA samples were then subjected to PCR to detect *Leishmania* minicircle kDNA.

Results

Of the 100 feline serum samples collected, 54 were found to be positive for *Leishmania* antibodies on the basis of IFAT findings.

Cytological smears of all bone marrow aspirates were negative for *Leishmania* amastigotes. Similarly, none of the culture samples were positive for promastigote forms during the 8-week period.

There were no instances of contamination of culture. The results of PCR analysis revealed the bone marrow and biopsy samples did not find for *Leishmania*.

Discussion

The maintenance and spread of vector-borne diseases depends on the availability of infected hosts to serve as parasite reservoirs. In the case of leishmaniasis, it is important to understand the epidemiological impact of asymptomatic hosts in the transmission of the parasite to new vectors (SADLOVA et al., 2015). Previous studies in Brazil and other countries have emphasized the importance of feline leishmaniasis. However, there remain many questions to be answered. The present study was conducted in Belo Horizonte, Minas Gerais. The prevalence of leishmaniasis in the canine and human populations in this state is worrying. In 2016, Minas Gerais registered 492 cases of human VL, with 2.3 cases per 100,000 inhabitants and 54 deaths. Belo Horizonte registered a high number of cases of VL in humans and CL in dogs (BRASIL, 2017). A study conducted in Belo Horizonte showed a highly significant correlation between the incidence of human VL and the prevalence rates of CL (OLIVEIRA et al., 2001). Although dogs are the main hosts and reservoir for *L. infantum* (MAIA & CAMPINO, 2011), our study found 54% of the evaluated cats to be seropositive, thus reinforcing previous findings that cats can be infected with *Leishmania* spp.

The frequency of leishmaniasis in cats determined using serological tests varies among regions. A study in Angola found none of the evaluated cats to be seropositive (MARTÍN-SÁNCHEZ et al., 2007) and 3.87% in Greece (DIAKOU et al., 2009). In Brazil, the frequency of leishmaniasis in cats has been reported as follows: 4.06% in Belém (OLIVEIRA et al., 2015); 3.9% in Pernambuco (SILVA et al., 2014); 7.27% (NOÉ et al., 2015) to 30% (BRAGA et al., 2014) in Mato Grosso do Sul and 0.52% (CARDIA et al., 2013) to 2.8% (BALDINI-PERUCA et al., 2017) in São Paulo.

The high frequency of feline leishmaniasis observed in the present study can be explained by the high prevalence of CL in the area of this study. In endemic areas for CL, cats can become infected with *Leishmania* spp. and act as secondary reservoirs in the presence of the primary reservoir, dogs (PENNISI et al., 2015). It is important to highlight that the cat flea *Ctenocephalides felis felis*, which can mechanically transmit *L. infantum,* can also infect dogs (PAZ et al., 2013).

Additionally, a previous study suggested that, at low serum dilutions, such as were used in the present study, remnants of the dye used in the IFAT might be misidentified as being fluorescent (CHATZIS et al., 2014b). Another hypothesis for the high frequency of feline leishmaniasis observed in the present study is the possibility of cross-reaction with antibodies against other parasites (ANDRADE et al., 2009; LUCIANO et al., 2009; SOARES et al., 2016).

All cats in the present study were asymptomatic and tested negative for *Leishmania* spp. upon parasitological and molecular analysis of bone marrow aspirates. Moreover, upon PCR screening, all tissue biopsy samples were found to be negative for *Leishmania* spp. These findings are of great importance. First, they demonstrate that cats are infected with and produce antibodies against *Leishmania* spp., although they do not host parasites at the body sites evaluated in this study. Second, the diagnosis of feline leishmaniasis solely on the basis of serological findings might lead to misdiagnosis of cats as positive carriers of the disease, especially in endemic areas.

Absence of *Leishmania* DNA or parasites in seropositive cats has been reported previously (BRAGA et al., 2014; CHATZIS et al., 2014a; NOÉ et al., 2015). In an experimental infection of cats with *L. braziliensis,* although all cats exhibited antibodies against the parasite, none exhibited amastigotes in the bone marrow (SIMÕES-MATTOS et al., 2005). Cats infected intradermally or intravenously with *L. chagasi* (KIRKPATRICK et al., 1984) have been reported as capable of responding to the infection by producing antibodies. Humoral immune response and antibody production may be important for protection against feline leishmaniasis. However, these responses are not observed in dogs (MARTÍN-SÁNCHEZ et al., 2007; MAIA & CAMPINO, 2011). Moreover, studies on the cellular immune response to *Leishmania* infection in cats, to demonstrate the balance between cellular and humoral response to the infection have not yet been performed as they have been in dogs (MAIA & CAMPINO, 2011; PENNISI et al., 2015).

There is no consensus regarding the ideal organ for cytological or molecular diagnosis of leishmaniasis, which could explain the absence of parasites in the bone marrow. Some authors have suggested lymph node aspiration (COSTA et al., 2010) or skin biopsy (CHATZIS et al., 2014a). It is necessary to identify the best lymphoid organ to target with aspiration for cytological diagnosis of leishmaniasis (SILVEIRA et al., 2015). Moreover, low parasitemia (below the PCR detection limit) or infection control by the immune response could contribute to the cytological results (CHATZIS et al., 2014a; NOÉ et al., 2015).

Conclusions

Our findings revealed a high seroprevalence of leishmaniasis in cats living in a city endemic for CL and with reported cases of human VL. However, the parasite was not detected in bone marrow aspirates or ear biopsy samples collected from seropositive cats. This indicates that cats can become infected with, and produce antibodies against, *Leishmania* spp. Moreover, the feline immune system might clear the infection or contain the parasites within an organ, thus maintaining low levels of parasitemia. Nevertheless, it is not clear whether these antibodies are protective or whether the cellular immune response is effective for infection control in cats. The present results highlight the need to continue standardization of diagnostic procedures for feline leishmaniasis, as well as to investigate the role of cats as reservoirs of *Leishmania* spp.

Conflict of Interest

The authors declare that they have no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethics Approval and Consent to Participate

All animal care and procedures as well as license for blood sampling were approved by the current national law on animal experimentation and ethics (CONCEA), according to the certificate number CETEA 242/2014 generated by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (UFMG).

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