


# Serological and molecular investigation of *Leishmania* spp. infection in cats from an area endemic for canine and human leishmaniasis in Northeast Brazil

Investigação sorológica e molecular da infecção por *Leishmania* spp. em gatos provenientes de uma área endêmica para leishmaniose canina e humana no nordeste brasileiro

José Artur Brillhante Bezerra<sup>1</sup>; Ilanna Vanessa Pristo de Medeiros Oliveira<sup>1</sup>; Ana Carolina Yamakawa<sup>2</sup>; Mariana Guimarães Nilsson<sup>2</sup>; Klívio Loreno Raulino Tomaz<sup>1</sup>; Kalyne Danielly Silva de Oliveira<sup>1</sup>; Célio Souza da Rocha<sup>1</sup>; Cecília Irene Perez Calabuig<sup>1</sup>; Felipe Fornazari<sup>2</sup>; Helio Langoni<sup>2</sup>; João Marcelo Azevedo de Paula Antunes<sup>1\*</sup> 

<sup>1</sup> Hospital Veterinário Jerônimo Dix-Huit Rosado Maia, Universidade Federal Rural do Semi-Árido – UFERSA, Mossoró, RN, Brasil

<sup>2</sup> Núcleo de Pesquisas em Zoonoses – NUPEZO, Departamento de Higiene Veterinária e Saúde Pública, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista – UNESP, Botucatu, SP, Brasil

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## Abstract

The aim of this study was to investigate the occurrence of *Leishmania* spp. antibodies, and its association with feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV), in domestic cats from an area endemic for canine and human leishmaniasis in Rio Grande do Norte State, Brazil. Ninety-one cats were subjected to a complete clinical exam, and blood samples were collected. An epidemiological questionnaire was used to investigate the risk factors. IgG anti-*Leishmania* spp. antibodies were detected by immunofluorescence antibody test (IFAT), with a cut-off value of 1:40. Polymerase chain reaction (PCR) was performed to detect genetic material of *Leishmania* spp. in the blood samples. The presence of antibodies against FIV and antigens of FeLV was evaluated using an immunochromatographic test. Seropositivity for *Leishmania* spp., FIV, and FeLV was observed in 14/91 (15.38%), 26/91 (28.57%), and 3/91 (3.29%) cats, respectively. All samples gave negative results on PCR analysis. Based on these data, no significant statistical association was observed between seropositivity for *Leishmania* spp., and sex, age, presence of clinical signs, evaluated risk factors, and positivity for retroviruses. These findings demonstrated for the first time that cats from Mossoró, Rio Grande do Norte, are being exposed to this zoonosis and might be part of the epidemiological chain of transmission of visceral leishmaniasis.

**Keywords:** Leishmaniasis, *Felis catus*, zoonosis, epidemiology, IFAT.

## Resumo

O objetivo do presente estudo foi investigar a ocorrência de anticorpos contra *Leishmania* spp., e sua associação com o vírus da imunodeficiência felina (FIV) e o vírus da leucemia felina (FeLV), em felinos domésticos provenientes de uma área endêmica no estado do Rio Grande do Norte, para a leishmaniose visceral canina e humana. Noventa e um gatos foram submetidos a exame clínico completo e amostras de sangue foram coletadas. Um questionário epidemiológico foi feito para investigar fatores de risco. Anticorpos IgG anti-*Leishmania* spp. foram identificados por meio da imunofluorescência indireta (RIFI), adotando-se como ponto de corte a diluição de 1:40. A reação em cadeia da polimerase (PCR) foi executada visando detectar o material genético de *Leishmania* spp. a partir de amostras de sangue total. Para avaliar a presença de anticorpos contra o FIV e antígenos do FeLV foi utilizado um teste imunocromatográfico. Observou-se soropositividade em 14/91 (15,38%), 26/91 (28,57%) e 3/91 (3,29%) animais para *Leishmania* spp., FIV e FeLV, respectivamente. Nenhuma amostra foi positiva na PCR. Baseado nestes dados, não foi observada nenhuma associação estatística significativa entre a soropositividade para *Leishmania* spp. e gênero, idade, presença de sinais clínicos, fatores

\*Corresponding author: João Marcelo Azevedo de Paula Antunes. Hospital Veterinário Jerônimo Dix-Huit Rosado Maia, Universidade Federal Rural do Semi-Árido – UFERSA, Av. Francisco Mota, 572, Bairro Costa e Silva, CEP 59625-900, Mossoró, RN, Brasil. e-mail: [joao.antunes@ufersa.edu.br](mailto:joao.antunes@ufersa.edu.br)



de risco avaliados e positividade para as retrovírus. Esses achados demonstram pela primeira vez que felinos da cidade Mossoró, Rio Grande do Norte, estão sendo expostos a esta zoonose, sugerindo que os mesmos podem estar participando da cadeia epidemiológica de transmissão da leishmaniose visceral.

**Palavras-chave:** Leishmaniose, *Felis catus*, zoonose, epidemiologia, RIFI.

Feline leishmaniasis (FeL), which is mainly caused by *Leishmania infantum*, is considered to be an emerging disease (PENNISI & PERSICHETTI, 2018). In the last two decades, there has been a considerable increase in epidemiological studies and reports of FeL, especially in areas endemic for canine and human leishmaniasis (PENNISI et al., 2015). However, there is limited knowledge about its immunological and pathophysiological aspects, making the diagnosis challenging (SILVEIRA et al., 2015).

FeL has some particularities compared to dogs. Cats are considered to be more resistant to *Leishmania* infection, remaining asymptomatic, with parasitemia, even 16 weeks after *L. infantum* inoculation at a dose that usually results in clinical disease in dogs (AKHTARDANESH et al., 2018). Experimentally infected cats are capable of transmitting the protozoan to sand flies (MAROLI et al., 2007; SILVA et al., 2010). Nevertheless, studies are not consistent in affirming the precise role of cats in the epidemiology of this disease under natural conditions (PENNISI & PERSICHETTI, 2018).

Some authors have hypothesized that feline retroviruses, the feline immunodeficiency virus (FIV) and the feline leukemia virus (FeLV), are predisposing factors for FeL in endemic areas, due to its immunosuppressant characteristics, but only a few studies found a significant association between these infections (PENNISI et al., 1998; AYLLÓN et al., 2012; SOBRINHO et al., 2012; SPADA et al., 2013).

In Brazil, studies on FeL using serological, parasitological, and molecular methods revealed that infection frequencies varied from 0% to 54% (BRESCHIANI et al., 2010; VIDES et al., 2011; CARDIA et al., 2013; BRAGA et al., 2014; SILVA et al., 2014; SOUSA et al., 2014; OLIVEIRA et al., 2015; GODOI et al., 2016; MENDONÇA et al., 2017; COURA et al., 2018; MATOS et al., 2018). Only a small fraction of these studies was performed in the Northeast region that was home to 82.5% of all reported cases of human leishmaniasis in Brazil between 1980 and 2005, making this region an area of epidemiological relevance for this disease in Brazil (MAIA-ELKHOURY et al., 2008; SILVA et al., 2014; MENDONÇA et al., 2017). Because of the high prevalence of leishmaniasis in Northeast Brazil and due to the lack of scientific data about FeL in Rio Grande do Norte State, the aim of this study was to investigate *Leishmania* spp. infection using serological and molecular methods, and its association with FIV and FeLV, in domestic cats in Mossoró city—a region that is endemic for human and canine leishmaniasis.

The study was performed from August 2017 to September 2018. The Ethics Committee on Animals' Use (CEUA) of the Universidade Federal Rural do Semi-Árido (UFERSA) approved the experimental protocols and the procedures used for animal care (Process n° 23091.008147/2017-28).

Cats older than 6 months in age ( $n = 91$ ) treated at Hospital Veterinário Jerônimo Dix-Huit Rosado Maia from UFERSA were selected (Mossoró, RN, Brazil, 5°100'S, 37°100'W) for

this study. The cats were clinically examined, and data related to each animal and its clinical status were noted. Blood samples (5 mL) were collected by jugular venipuncture. Whole blood and serum samples were stored at -20°C prior to performing serological and molecular tests at the Núcleo de Pesquisas em Zoonoses (NUPEZO), Departamento de Higiene Veterinária e Saúde Pública da Faculdade de Medicina Veterinária e Zootecnia da UNESP, Campus of Botucatu, SP, Brazil.

Epidemiological questionnaires were used to identify risk factors related to *Leishmania* infection. The variables analyzed and the respective categories were as follows: sex, age (young, older than 6 months, but lesser than 1 year in age; adult, older than 1 year, but lesser than 8 years in age; senior, older than 8 years), access to streets (yes or no), contact with dogs (yes or no), living near water sources (yes or no), health status (healthy or sick), vaccination (yes or no), deworming (yes or no), and castration (yes or no). Specific clinical signs were selected to evaluate the association with FeL—cutaneous signs, lymphadenomegaly, ophthalmic signs, gingivostomatitis, weight loss, and pale mucosa.

IgG anti-FIV antibodies and p27 FeLV antigens were investigated using a rapid immunochromatographic test (Alere S.A., São Paulo, SP, Brazil). The test was performed as per manufacturers' recommendations, using serum samples.

IgG anti-*Leishmania* spp. antibodies were detected by immunofluorescence antibody test (IFAT), according to the method described by Camargo (1974). Immunofluorescence slides were sensitized with *Leishmania major* promastigotes obtained from cultures maintained in liver infusion tryptose (LIT) and Neal, Novy, Nicolle (NNN) media. Positive and negative controls were used for all slides. Serial serum dilutions of 1:40, 1:80, 1:160, 1:320, and 1:640 were prepared in phosphate buffered saline (PBS, pH 7.2), and a dilution of 1:40 was adopted as the cut-off value. A commercial anti-IgG antibody specific to cats, conjugated with the fluorescein isothiocyanate (Sigma-Aldrich, St. Louis, MO, USA), was used as a secondary antibody. Slides were examined using a fluorescence microscope at magnification of 40× (Scope.A1; ZEISS, Oberkochen, Germany). After checking the control slides, the highest dilution of the serum for which complete fluorescence occurred at the border of at least 50% of the promastigotes was considered.

DNA extraction from blood samples was performed using Illustra™ blood GenomicPrep Mini Spin kit (GE Healthcare, Chicago, IL, USA), following manufacturers' recommendations. Extracted samples were stored in DNase- and RNase-free micro tubes at -20°C prior to PCR. DNA samples were quantified in a spectrophotometer (NanoVue Plus; GE Healthcare, Chicago, IL, USA).

Primers 150, 5'-GGG(G/T)AGGGGCGTTCT(C/G)CGAA-3', and 152, 5'-(C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTACACCAACCCC-3', were used as described by Volpini et al. (2004). All reactions were performed using GoTaq® Green Master

Mix (Promega Corporation, Madison, WI, USA), a premixed solution containing Taq DNA polymerase, dNTPs, MgCl<sub>2</sub> and reaction buffers, following manufacturers' recommendations. For each reaction was used a total of 12.5µL of GoTaq® Green Master Mix, 1µL of each primer, 7.5µL of nuclease-free water and 3µL of the extracted samples, obtained a total volume of 25µL. DNA sample extracted from *L. major* (MHOM/SU/1973/5-ASKH strain) was used as a positive control. Ultrapure DNase/RNase-Free distilled water was used as a negative control.

The amplification steps were carried out in a thermal cycler (Mastercycler pro; Eppendorf, Hamburg, Germany) as follows: initial denaturation at 95°C for 5 min, followed by 29 cycles at 95°C for 1 min, 55°C for 30 s, and 72°C for 10 s, and final extension of 72°C for 5 min. Electrophoresis was performed in a 2% agarose gel stained with Nancy-520® (Sigma-Aldrich, St. Louis, MO, USA) in 1x TBE buffer, at 80 Volts (V) for 20 min followed by 100 V for 20 min, with DNA MW Marker 100-bp Ladder® (Sinapse Biotecnologia, Tatuapé, SP, Brazil). The result was visualized at an ultraviolet transilluminator. The amplified products had an expected molecular weight of 120 bp; this corresponded to the molecular weight of the *Leishmania* minicircle kinetoplast DNA (kDNA).

For statistical analysis, contingency tables were prepared to identify the differences in infected animal proportions with respect to each variable assessed. Chi-square test was used to compare proportion of infected animals with respect to the risk factors, using a significance value of  $p < 0.05$ . To perform these analyses, the software IBM SPSS Statistics Version 22 was used.

Of the 91 cats studied, 51 (56%) were male, and 40 (44%) were female. All animals were crossbred. Using IFAT, IgG anti-*Leishmania*

spp. antibodies were observed in 14 (15.38%), with titers varying from 1:40 to 1:320 (Table 1 and Table 2). None of the cats were found to be positive for *Leishmania* infection using blood PCR. With respect to retrovirus infection, 26 (28.57%) cats tested positive for FIV and 3 (3.29%) cats tested positive for FeLV.

No statistical association was observed between seropositivity for *Leishmania* spp., and sex, age, presence of specific clinical signs, and evaluated risk factors. An association with retroviral infection was not observed, and only five cats had coinfection of *Leishmania* spp. and FIV ( $p = 0.052$ ). Of these coinfecting animals, two presented a titer of 1:40, two had a titer of 1:80, and one had titer of 1:160. Clinical data for cats seropositive for *Leishmania* spp. are shown in Table 1, Figure 1, and Figure 2. No coinfection between *Leishmania* spp. and FeLV was found.

Several studies have reported *Leishmania* infection in cats in regions endemic for canine and human leishmaniasis (VIDES et al., 2011; SHERRY et al., 2011; CHATZIS et al., 2014; PENNISI et al., 2015; SPADA et al., 2016). In the present study, the occurrence of antibodies against *Leishmania* spp. was observed in 15.38% of the cats evaluated from Mossoró—a city endemic for this disease (AMÓRA et al., 2006; BARBOSA, 2013). To the best of our knowledge, this is the first study to report FeL in Rio Grande do Norte State.

The seropositivity here observed was lower than that found in a study with dogs from Mossoró. Using IFAT and ELISA, these authors found frequencies of *Leishmania* spp. seropositivity of 34% and 45% for dogs living in urban and rural zones, respectively (AMÓRA et al., 2006). The low number of cats seropositive for *Leishmania* spp. in comparison with dogs has been shown

**Table 1.** Clinical manifestations of domestic cats seropositive for *Leishmania* spp. on IFAT from Mossoró city (n = 14), Rio Grande do Norte state, Brazil.

Animal	Age (years)	Sex	IFAT titer	FIV-status	Clinical signs
#2	7	F	1:80	N	Asymptomatic
#27	10	M	1:80	P	Gingivostomatitis
#40	12	M	1:80	P	Bronchopneumonia, gingivostomatitis, lymphadenomegaly
#43	9	F	1:40	P	Squamous cell carcinoma in nasal and palpebral border, chronic rhinitis
#48	3	M	1:40	N	Bilateral blepharitis
#51	1	F	1:320	N	Ulcerated plaque lesions with difficult healing, lymphadenomegaly
#55	2	M	1:80	N	Chronic kidney disease
#56	8	M	1:160	P	Alopecia, erythema and desquamation in ear borders, chronic kidney disease
#72	3	M	1:40	N	Cutaneous abscess
#73	6	M	1:40	P	Gingivostomatitis
#80	11	M	1:80	N	Bronchopneumonia
#85	3	F	1:40	N	Asymptomatic
#86	9	F	1:40	N	Asymptomatic
#91	10	F	1:40	N	Asymptomatic

# - number of the animal; F- female; M- male; N- negative; P- positive.

**Table 2.** Distribution of IFAT titers (IgG) for *Leishmania* spp. in domestic cats from Mossoró city, Rio Grande do Norte State, Brazil (n = 91).

	Number of positive cats	Titer	(%)
<i>Leishmania</i> spp.	7	1:40	7.69
	5	1:80	5.49
	1	1:160	1.09
	1	1:320	1.09





**Figure 1.** Clinical manifestations of *Leishmania* spp. seropositive cats from Mossoró city, Rio Grande do Norte State, Brazil. (A) Animal #48 (Male, 3-year-old, FIV-negative, IFAT titer of 1:40) with bilateral blepharitis; (B) Animal #56 (Male, 8-year-old, FIV-positive, IFAT titer of 1:160) with alopecia on ear borders associated with erythema and desquamation.



**Figure 2.** Clinical manifestations of *Leishmania* spp. seropositive cats from Mossoró city, Rio Grande do Norte State, Brazil. (A) Animal #27 (Male, 10-year-old, FIV-positive, IFAT titer of 1:80) presenting chronic gingivostomatitis; (B) Animal #43 (Female, 9-year-old, FIV-positive, IFAT titer of 1:40) with a squamous cell carcinoma ulcerative lesion involving nasal and palpebral borders; (C) Animal #51 (Female, 1-year-old, FIV-negative, IFAT titer of 1:320). Ulcerative lesions in plaque located in calcaneal region, with 5 months of evolution time and difficult healing.

by other research groups (SOLANO-GALLEGO et al., 2007; BRESCIANI et al., 2010; SOBRINHO et al., 2012; CARDIA et al., 2013), and this can be attributed to the natural resistance of the cats against the protozoan, due to a more effective cellular immune response (SOLANO-GALLEGO et al., 2007). Moreover, the use of different methodologies and non-standardized protocols could also be responsible for these frequencies (SOLANO-GALLEGO et al., 2011).

In dogs with leishmaniasis, coinfections with other pathogens such as *Ehrlichia canis* and *Dirofilaria immitis* can interfere with the parasite burden and affect disease progression because of the alterations in the immune response (TABAR et al., 2013; TOMMASI et al., 2013). In cats, the majority of leishmaniasis coinfections investigations are related to retroviruses, which cause immunosuppressive diseases (PENNISI & PERSICHETTI, 2018). However, only a few studies have found a significant association between *Leishmania* spp. seropositivity and FIV (PENNISI et al., 1998; AYLLÓN et al., 2012; SOBRINHO et al., 2012; SPADA et al., 2013) and FeLV infection (SHERRY et al., 2011).

During the course of feline retroviral infections, there is progressive immunosuppression due to the changes in the CD4 and CD8 lymphocyte count, which predisposes the animal to a variety of opportunistic pathogens (LACERDA et al., 2017). In this context, FIV-positive cats can represent a risk to *Leishmania* spp. infection, as humans infected by the human immunodeficiency virus (HIV) are known to be predisposed to leishmaniasis (PAGLIANO & ESPOSITO, 2017). In this study, in spite of the fact that no statistically significant association was found between FeL and FIV, five (37.5%) of the 14 cats seropositive for leishmaniasis presented coinfection with FIV and showed clinical signs suggestive of FeL (PENNISI et al., 2015). Further case-control studies are needed to assess the dynamics of these two contagious diseases in the evaluated region.

Very few studies were performed to investigate FeL in Northeast Brazil. The FeL seropositivity here was higher than that observed in Recife and Teresina, where 3.9% and 4% of the cats were found to be seropositive for FeL using ELISA (SILVA et al., 2014; MENDONÇA et al., 2017). Sero-epidemiological investigations in other regions of the country found frequencies varying from 0.5% to 54%, using IFAT and the same cut-off value adopted here (1:40) (VIDES et al., 2011; CARDIA et al., 2013; BRAGA et al., 2014; SOUSA et al., 2014; OLIVEIRA et al., 2015; GODOI et al., 2016; COURA et al., 2018; MATOS et al., 2018). In canine leishmaniasis, high serological titers (four times the established cut-off value) with clinical signs compatible with leishmaniasis are diagnostic for the disease (SOLANO-GALLEGO et al., 2011). Combining this information with the results obtained in the present study, cats with IFAT titers equal to or above 1:160 could have the confirmation of FeL.

The serological methods used to diagnose FeL are still the main tools for epidemiological studies and for investigation of the infection because of the low costs, good sensibility, and ease of obtaining blood samples from animals (SILVEIRA et al., 2015). IFAT is the most widely used method and was implemented in this study. Nevertheless, ELISA seems to have higher sensitivity (SILVEIRA et al., 2011; COELHO et al., 2011). This difference between the sensitivity of the methods and frequency of

positive animals can be explained by the lack of studies on the standardization of diagnosis methods, associated with the little understanding of FeL immunology and pathophysiology, making the identification of infected animals a challenge (SPADA et al., 2013; SILVEIRA et al., 2015).

The molecular study of *Leishmania* spp. using PCR from blood samples did not give positive results for any of the animals. Similar results were observed by other authors, i.e., seropositive cats testing negative on PCR (BRAGA et al., 2014; CHATZIS et al., 2014; NOÉ et al., 2015; MONTOYA et al., 2018; COURA et al., 2018). This finding could be explained by the capability of the protozoan to compartmentalize in determined lymphoid organs such as bone marrow, lymph nodes, and spleen, by the absence of parasitemia at the moment of the blood collection, or because of a parasite burden lower than that of the detection limit of the test used (CHATZIS et al., 2014; COURA et al., 2018). Another hypothesis for the presence of seropositive animals with negative PCR result could be related to the possibility of cross-reactions with antibodies against other parasites, especially other species of *Leishmania* and *Trypanosoma* (ZANETTE et al., 2014; SILVEIRA et al., 2015; SOARES et al., 2016).

In spite of no statistical association was found between presence of antibodies against *Leishmania* spp. and specific clinical signs assessed (cutaneous signs, lymphadenomegaly, ophthalmic signs, gingivostomatitis, weight loss, and pale mucosa), the majority of cats seropositive for *Leishmania* spp. in this study presented clinical signs that associated with FeL (PENNISI et al., 2015; PENNISI & PERSICHETTI, 2018). Another important finding was a concurrent squamous cell carcinoma in a seropositive feline. Some studies have suggested a synergism between FeL and this neoplasia (GREVOT et al., 2005; POCHOLLE et al., 2012; MAIA et al., 2015; SOARES et al., 2016). It is important to emphasize that serological evidence and clinical signs of *Leishmania* spp. infection were found in this study, although infection was not reinforced by PCR.

The evidences are still insufficient to determine the real role of the cats in the epidemiological chain of leishmaniasis transmission (PENNISI & PERSICHETTI, 2018). It is believed that cats can act as a secondary reservoir for *Leishmania* spp. Under natural conditions, however, in the absence of the primary reservoir, cats alone would not be capable of transmitting the infection to sand flies (PENNISI et al., 2015). However, it has been suggested that hares and not dogs served as reservoirs of leishmaniasis during an outbreak of human leishmaniasis in Spain (MOLINA et al., 2012; MORENO et al., 2014). Therefore, as the population of domestic and stray cats is bigger than the dog population in some endemic areas, cats could have an important role in leishmaniasis transmission in these locations (PENNISI et al., 2015).

The presence of antibodies against *Leishmania* spp. suggests that these animals are being exposed to and may be infected by this protozoan. The cats might represent an important link in the epidemiological chain of leishmaniasis transmission in the studied region. As this disease has a great impact on public health, further studies should be performed to investigate the clinical, pathophysiological, and epidemiological aspects of this infection in domestic cats.



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