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Serological Diagnosis of Feline Tegumentary Leishmaniasis by Indirect Immunofluorescence (IFI) and Enzyme-Linked Immunosorbent Assay (ELISA) in an Endemic Area in Brazil

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ABSTRACT

Background: American tegumentary leishmaniasis (ATL) is a serious public health problem, and the participation of domestic cats in its epidemiological process has not yet been fully elucidated. Therefore, the standardization of monitoring methodologies of cat populations becomes important for the generation of information on the disease. In Brazil, ATL presents a wide geographical distribution, being *Leishmania* (*Viannia*) *braziliensis* its etiologic agent of ATL in Rio de Janeiro. The main objective of the present study was investigate the presence of specific anti-*Leishmania* (*Viannia*) *braziliensis* Immunoglobulin G (IgG) in 34 cats from an ATL endemic area in this municipality.

Materials, Methods & Results: Sera from three cats from the study area naturally infected with *Leishmania* (*Viannia*) *braziliensis* were used as positive control. Analyses were performed with antigen preparations using indirect immunofluorescence (IFI) and enzyme-linked immunosorbent assay (ELISA). We found prevalence rates of 20.6% (7/34) in the IFI and 14.7% (5/34) in the ELISA. Specificity was 87.1% for the IFI and 93.5% for the ELISA, and both tests presented sensitivity of 100%. Concordance between the used tests was assessed as moderate.

Discussion: By conducting a feline serological survey in an endemic area for ATL we provide information on the involvement of this species in such epidemiological process. In this context, the participation of the cat in cycles of leishmaniases has been highlighted by several authors; infection of the phlebotomine by parasites originated from this vertebrate host can already be demonstrated by xenodiagnosis and several cases of feline infection have been reported in endemic regions of several countries such as Brazil, Spain, France, Portugal, Greece, Iran, Israel and Italy. Therefore, the standardization of diagnostic techniques that support the screening and monitoring of such populations becomes of utmost importance. In fact, the use of the IFI and ELISA serological techniques, showing sensitivity and NPV of 100%, confirm the use of such tools in epidemiological surveys for the detection of anti-*L.* (*V.*) *braziliensis* antibodies in domestic cats, and these methodologies are frequently employed in canine surveys showing good results. The serological titers found in this study for *Leishmania* (*V.*) *braziliensis* are similar to those of studies carried out with cats in endemic areas of visceral leishmaniasis in Spain. This similarity shows that the titers can be high even when *L.* (*V.*). *braziliensis* is the etiological agent. It is worth mentioning that these values are significant when compared with those of canine studies carried out in ATL endemic regions. Ultimately, the sample refers to an ATL endemic region located in Brazil, one of the countries with the highest endemicity levels of cutaneous leishmaniasis in the world and, therefore, an important area for the implementation and evaluation of diagnostic techniques for the generation of data on the disease and its possible hosts. Thus, the present study provides information on the occurrence of ATL by *Leishmania* (*V.*) *braziliensis* in domestic cats; demonstrates that the methodologies employed were favorable to the use of such techniques as tools in epidemiological surveys for the diagnosis of feline tegumentary leishmaniasis; and paves the way for further studies on the subject.

Keywords: *Leishmania* (*Viannia*) *braziliensis*, domestic cat, serological diagnosis.

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INTRODUCTION

American tegumentary leishmaniasis (ATL) is a serious public health problem that can cause destructive, occasionally disabling, deformities. In Brazil, ATL presents a wide geographical distribution [15], being *Leishmania* (*Viannia*) *braziliensis* is the etiologic agent of ATL in the municipality of Rio de Janeiro [5]. In this context, it is worth mentioning the presence of domestic hosts such as cats, which may act as reservoirs and amplifiers of cycles installed in the peridomicile [13,25].

The first case of feline leishmaniasis (FL) was described in Algeria [38]. Since then, authors have been notifying both the cutaneous and visceral forms, in felines from around the world [3,6,9,17,19,31,33,35].

In Brazil, the first report of FL occurred in 1939 [26]. Later, other cases were reported [9,32] with *L.* (*Viannia*) and *Leishmania* (*Leishmania*) *amazonensis*. In São Paulo state, it was suggested that the cat could be part of the transmission cycle as a leishmaniasis reservoir in endemic areas [36] and in Rio de Janeiro state, the presence of domestic cats infected with *L.* (*V.*) *braziliensis* is concurrently with canine and human infections [14,37].

In this context, this study aimed to evaluate the detection of specific IgG in samples of domestic cats using IFI and ELISA reactions with antigen preparations of *Leishmania* (*V.*) *braziliensis*. Based on the use of such methodology in an endemic area for ATL, we sought to demonstrate the feasibility of these techniques for the diagnosis and monitoring of cat populations susceptible to cutaneous leishmaniasis.

MATERIALS AND METHODS

Study design

The study consists of the serological evaluation of 34 domestic cats (*Felis catus*) from an ATL endemic area in the municipality of Rio de Janeiro using the IFI and ELISA methods with *Leishmania* (*Viannia*) *braziliensis* antigen preparations.

Three cats from the study area naturally infected with *Leishmania* (*V.*) *braziliensis*, diagnosed by culture isolation and characterization of isoenzymes, were used as positive control. Such isolation and characterization procedures were performed for all animals in the study which presented skin lesions, and co-infection with the viruses of Feline Immunodeficiency (FIV) and Feline Leukemia (FeLV) were also assessed by means of detection of antigens and antibodies, respectively (SNAP® FIV Antibody/FeLV Antigen Combo Test).

Parasitological culture and isoenzymatic characterization

Animals presenting skin lesions were submitted to parasitological diagnosis. Fragments were collected from the edge of the lesions using a 3-mm punch. This procedure was carried out after the animals were sedated, by intramuscular injection, with ketamine hydrochloride at 10% (10 mg/kg) associated with acepromazine at 1% (0.2 mg/kg) and local anesthesia with lidocaine hydrochloride at 2%. The fragments were stored in saline solution containing 1,200 UI of penicillin, 1,000 ug of streptomycin, and 100 ug of 5' fluorocytosine per milliliter; after 24 h, they were seeded in NNN (Novy, MacNeal, Nicolle)/ Schneider biphasic culture medium supplemented with 10% fetal bovine serum. The vials were incubated at room temperature (26-28ºC) and examined weekly. The isolates were characterized by electrophoresis of enzymes using a previously established protocol [7].

ELISA

The assays were performed according to the procedures previous described [29]. Polystyrene 96-well plates (Nunc immuno Maxisorp 96-well plates)[1] were sensitized with 100 uL of the total *Leishmania* (*V.*) *braziliensis* antigen partially soluble at a concentration of 2.5 µg/mL diluted in carbonate-bicarbonate pH 9.6 buffer solution; they were subsequently incubated overnight (for 18 h) at 4ºC. After four washes with PBS pH 7.2 with 0.05% of Tween 20 (PBS-T), 100 µL of the serum samples diluted at 1:40 in PBS-T containing 1% skim milk (PBS-TL) were added to each well; incubation for 45 minutes at 37°C in a humid chamber was then conducted.

After four new washes, 100 µL of Goat anti-cat IgG Fc conjugated with horseradish peroxidase (HRP A20-117P)[2] diluted 1:40,000 in PBS-TL were added. After 45 min of incubation at 37°C in a moist chamber and four new washes, we added 100 µL of developing solution, composed by 10 mg of o-phenylenediamine (OPD, P1526)[3] and 10 µL of hydrogen peroxide (H_2O_2) in 25 mL of citrate-phosphate buffer solution at pH 5.0.

The plates were incubated for 15 min in the dark, and the reaction was interrupted by adding 50 µL of 1N H_2SO_4 to each well. The optical density of

the wells was determined in a plate reader using a 492 nm wavelength filter. The cutoff point was established by the average of the negative controls plus twice the standard deviation thereof.

IFI

Promastigote forms of *Leishmania* (*V.*) *braziliensis* (MHOM/BR/75/M2903) were used as antigen. The slides were covered with 10 μL of the antigen and incubated at 37°C for 2 h. The sera were diluted as of 1:40 in PBS pH 7.2. After incubation in a humidified chamber at 37°C for 30 min, the slides were washed three times for five minutes in PBS and every minute in distilled water. The conjugate (Goat anti-cat IgG Fc fragment, A20-117F)[2] was diluted at 1:50 in PBS with 1:25 of Evans blue, and 10 μL of this solution was added to each well of the plate. The incubation and wash phases were repeated as previously mentioned. The slides were mounted with buffered glycerin and closed with cover slip for reading in fluorescence microscope. Titers > 1:40 were considered positive [34].

Statistical analysis

Prevalence, sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated using the Statistical Package for the Social Science (SPSS), version 16.0. The results of parasitological culture were used as reference standard. Concordance between IFI and ELISA was assessed by the Kappa (k) index, according to the following classification [40]: k = 0.00 to 0.10, virtually absent; k = 0.11 to 0.40, weak; k = 0.41 to 0.60, slight; k = 0.61 to 0.80, moderate; and k = 0.81 to 1.0, significant.

RESULTS

Sera from 34 cats from an ATL endemic area were assessed using IFI and ELISA.

Results showed positivity prevalence of 20.6% (7/34) in the IFI and 14.7% (5/34) in the ELISA, and no statistical significance was observed between these results (P = 0.75, Chi-square test, CI = 95%).

Among the 34 animals evaluated, five presented skin lesions compatible with ATL and were submitted to parasitological diagnosis, from which three individuals with positive results regarding isolation of the parasite were obtained.

Sensitivity and NPV were 100% in both the IFI and the ELISA. Specificity and PPV were 87.1% and 42.9% for the IFI and 93.5% and 60.0% for the ELISA, respectively. The samples from animals with positive isolation of *Leishmania* showed, in the IFI reaction, titers equal to or greater than 1:160, while the samples from animals with negative results for the isolation of the parasite showed titers of 1:40 and 1:80. Overall agreement between the IFI and ELISA reactions was 84.1%, presenting co-positivity and co-negativity of 14.7% and 79.7%, respectively. Although the specificity values obtained with the ELISA technique are higher than those obtained with the IFI technique, this difference is not statistically significant (P = 0.67, Chi-square test, IC = 95%).

The Kappa index value was 0.80, which classifies the concordance between IFI and ELISA as moderate [40].

Of the five felines presenting clinical suspicion for the disease, only one did not obtain diagnostic confirmation by any of the techniques applied, both serological and parasitological, so that rate of symptomatic cats was 57.1% (4/7), based on the most prevalent test. Regarding the search for antigens of FeLV and antibodies for FIV, six animals tested positive for FeLV. However, all individuals with parasitological diagnosis were negative in this test, and only one, an exclusively serum reagent cat, showed detection of such antigens.

DISCUSSION

By conducting a feline serological survey in an endemic area for ATL we seek to provide information on the involvement of this species in such epidemiological process. In this context, the participation of the cat in cycles of leishmaniases has been highlighted by several authors [4, 20,21,30,36]; infection of the phlebotomine by parasites originated from this vertebrate host can already be demonstrated by xenodiagnosis [8,23] and several cases of feline infection have been reported in endemic regions of countries such as Brazil [9,37], Spain [17,24] France [31], Portugal [4,21], Greece [10], Iran [11], Israel [30] and Italy [33].

In view of this situation, the standardization of diagnostic techniques that support the screening and monitoring of such populations becomes of utmost importance. In this study, the use of the IFI and ELISA serological techniques, showing sensitivity and NPV of 100%, confirm the use of such tools in epidemiological surveys for the detection of anti-*L.* (*V.*) *braziliensis* antibodies in domestic cats. In fact, these methodologies are frequently employed in canine surveys showing

good results [2,28,42] and the adequacy of both techniques to felines confirm the previous results [18,34].

The serological titers found in this study for *Leishmania* (*V.*) *braziliensis* are similar to those of studies carried out with cats in endemic areas of visceral leishmaniasis in Spain [17,24]. This similarity shows that the titers can be high even when L. (V.). braziliensis is the etiological agent.

Prevalence of the disease in the study area was high when compared to some works [4,41], who obtained a rate of 3.9% in a cohort of 153 cats using ELISA in the search for infection with *L. Infantum*, and rates of 2.8% and 1.9 % in a sample of 316 cats using ELISA and direct agglutination. The same occurred in others works [1,33], with 4.29% in a group of 233 animals, and with 0.9% in a sample of 110 felines, both ones carried out using IFI in studies of visceral leishmaniasis.

Our results were also higher than anothers [10,27,30]; similar to one [46]; but lower than other [24]. It is worth mentioning that these values are significant when compared with those of canine studies carried out in ATL endemic regions [12,15,39].

With respect to the clinical aspect, the presence of four animals classically symptomatic for ATL, among those with detectable immune response, oppose the high rate of asymptomatic feline leishmaniasis previous described for the visceral form [22]. Nevertheless, such result approximates that other [41], who obtained two symptomatic felines from four serum reagent cats in search for *L. infantum*. As for the non-symptomatic serum reagent animals, they evince the contact with the parasite without evolution of the clinical disease process. In fact, it is supported the hypothesis that, in infections with *L. infantum*, the immune response in cats is effective enough to control the infection and promote resistance to certain animals when there are no concomitant immunosuppressive events [43].

In this context, despite the existence of an indication that coinfection between FeLV and *Leishmania* spp. may favor the pathological process by the protozoan [17] the detection of viral antigens in only one animal serum reagent for *Leishmania* (*Viannia*) *braziliensis* indicates a dissociation between such infections in this study, as also demonstrated at the literature for the visceral form of the disease [21,24,43].

Ultimately, the sample refers to an ATL endemic region located in Brazil, one of the countries with the highest endemicity levels of cutaneous leishmaniasis in the world [45] and, therefore, an important area for the implementation and evaluation of diagnostic techniques for the generation of data on the disease and its possible hosts.

CONCLUSION

Thus the present study provides information on the occurrence of ATL by *Leishmania* (*V.*) *braziliensis* in domestic cats; demonstrates that the methodologies employed were favorable to the use of such techniques as tools in epidemiological surveys for the diagnosis of feline tegumentary leishmaniasis; and paves the way for further studies on the subject.

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***Ethical approval*.** All procedures were approved by the Ethic Committee on the Use of Animals in Research under the number P. 0221-04 (FIOCRUZ).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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