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Short communication

Use of crude, FML and rK39 antigens in ELISA to detect anti-*Leishmania* spp. antibodies in *Felis catus*

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ABSTRACT

Visceral leishmaniasis is a disease caused by *Leishmania (Leishmania) chagasi* and represents a serious public health problem. The dog is the main urban reservoir of the disease; however, investigations regarding the occurrence and epidemiological importance of leishmaniasis in cats have recently been initiated. This study aimed to detect cats seropositive for *Leishmania* spp. using different antigens. Additional studies were performed using sera from cats with *Toxoplasma gondii* ($n = 15$) to evaluate cross-reactivity. Serum samples ($n = 113$) from cats living in the town of Araçatuba, State of São Paulo, Brazil, an endemic area for human and canine visceral leishmaniasis, were tested by indirect ELISA using different antigens: crude (CAG-ELISA), fucose–mannose ligand (FML-ELISA) and K39 (rK39-ELISA). Anti-*Leishmania* spp. antibodies were detected in 23.0% of samples evaluated by CAG-ELISA, 13.3% by FML-ELISA and 15.9% by RK39-ELISA. Only reactive sera in all three tests were considered truly positive. No disagreement occurred among the tests ($p < 0.05$). Serum samples seropositive for toxoplasmosis tested by CAG-ELISA were negative, but one sample (6.7%) was positive for FML-ELISA and rK39-ELISA suggesting a cross-reaction between these antigens and anti-*T. gondii* antibodies. These findings indicate the occurrence of feline leishmaniasis in Araçatuba. Further studies are required to clarify the role of cats in the epidemiological cycle of leishmaniasis.

1. Introduction

Leishmaniasis is caused by protozoa of the genus *Leishmania* and represents a serious public health problem. This disease affects 12 million people in 88 countries with 350 million people at risk. Each year, 2 million new human cases are registered, 500,000 of which are visceral leishmaniasis (VL) and 1–1.5 million are cutaneous leishmaniasis (CL). A total of 90% of these cases occur in India, Sudan, Bangladesh and Brazil (Desjeux, 2004).

Although the dog is the main urban reservoir of VL (Alencar et al., 1991), cats infected by this pathogen have also been identified in several countries recently (Simões-Mattos et al., 2004). *Leishmania amazonensis*, *Leishmania braziliensis* and *Leishmania chagasi* have already been iden-

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tified in cats from Brazil (De Souza et al., 2005; Schubach et al., 2004), including an area not endemic for leishmaniasis in humans and dogs (Savani et al., 2004). Moreover, additional evidence suggests the existence of a reservoir of cats naturally infected by *Leishmania infantum*, as demonstrated by xenodiagnostic tests (Maroli et al., 2007).

Due to the difficulty of using direct diagnostic methods, such as parasitology, isolation and inoculation in hamsters, serology is widely used to diagnose leishmaniasis (Dye et al., 1993; Quinnell et al., 1997). Antigens can influence the accuracy of serological assays (Mohammed et al., 1985). Crude, soluble, recombinant and purified antigens have been described to diagnose leishmaniasis in dogs and humans by ELISA (Borja-Cabrera et al., 1999; Carvalho et al., 2003; Kumar et al., 2001; Lima et al., 2005; Palatnik-De-Sousa et al., 1995; Talmi-Frank et al., 2006); however, the performance of purified and recombinants antigens in ELISA have not been tested for the diagnosis of leishmaniasis in cats.

The purpose of this work was to investigate the occurrence of feline leishmaniasis in the municipality of Araçatuba, State of São Paulo, Brazil, an endemic area for canine and human leishmaniasis, using crude (Lima et al., 2005), fucose–mannose ligand (Borja-Cabrera et al., 1999) and recombinant K39 (Burns Junior et al., 1993) antigens in indirect ELISA. Cross-reactivity between feline anti-*Toxoplasma gondii* antibodies and these antigens was also evaluated. This is the first report concerning the use of FML and rK39 to diagnose leishmaniasis in cats by indirect ELISA.

2. Materials and methods

2.1. Samples

One hundred and thirteen blood samples were collected by venipuncture from cats of different ages and breeds kept at the Zoonosis Control Center (ZCC) in Araçatuba, SP, Brazil, and were used for serology by indirect ELISA. Fifteen serum samples from cats positive for toxoplasmosis by IFAT (Camargo, 1974) from a nonendemic area for leishmaniasis were used to investigate cross-reactivity between the tests performed in this study. Thirty-six serum samples from cats negative for leishmaniasis by cytological examination of lymph node and bone marrow aspirates and seronegative for toxoplasmosis by IFAT (Camargo, 1974) from Araçatuba, SP, Brazil, were used to set the cutoff. Acepromazine (0.05 mg/kg, i.v.) was used for sedation. All samples were centrifuged for 15 min at 3250 × *g*. The serum was removed and stored at −20 °C until serological analysis. This study was approved by the institutional Ethics and Animal Welfare Committee (*Comissão de Ética em Experimentação Animal*, CEEA, UNESP, process number 2010-005231).

2.2. ELISA

Serial titrations were performed to determine the best dilution for the tests. Dilutions of protein A at 4 μg/ml and 2 μg/ml and cat serum samples in the proportions of 1:25, 1:50 and 1:100 were tested. The dilutions 1:50 and 2 μg/ml were chosen for serum and protein A, respectively. The ELISA cutoff points were determined by the mean plus 3 standard deviations of the readings obtained using 36 serum samples from cats with negative serology (Voller et al., 1980).

2.3. Total antigen (CAG-ELISA) or recombinant K39 (rK39-ELISA)

Plates (Greiner Bio-One Microlon® 600, Germany) were coated with 50 μl/well of 20 μg/ml of crude antigen extract from *L. chagasi* promastigote culture, MHOM/BR00/MERO2 (Lima et al., 2005) or 25 ηg/ml of rK39 in carbonate/bicarbonate buffer (0.05 M, pH 9.6). After incubation at 4 °C overnight, the plates were washed three times with PBS (pH 7.0) and 0.05% Tween® 20 (washing buffer) and blocked with 150 μl/well of PBS containing 10% fetal bovine serum for 1 h at room temperature, then washed three times again. Serum samples (50 μl) were diluted 1:50 in PBS containing 10% bovine fetal serum and 0.05% Tween 20® and added to each well, in duplicate, then incubated for 3 h at room temperature. After washing three times, 100 μl/well of protein A peroxidase conjugate (Sigma, St. Louis, USA) at 2 μg/ml were added and incubated for 1 h at room temperature. After washing three times, 100 μl/well of substrate solution were added, containing 0.4 mg/ml O-phenylenediamine (Sigma) and 1.6 μl/ml H_2O_2 in phosphate-citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μl of 16% HCl. The optical density (OD) was measured using a Spectra Count™ reader (Packard Bio Science Company, USA) at a wavelength of 490 nm. Duplicates were included in blank wells, together with positive and negative control in all plates.

2.4. Glycoprotein fucose–mannose ligand (FML-ELISA)

Plates (Greiner Bio-one Microlon® 600, Germany) were coated with 50 μl/well of purified glycoprotein FML (40 μg/ml) in carbonate/bicarbonate buffer (0.05 M, pH 9.6), incubated at 37 °C for 1 h and then at 4 °C overnight. Plates were washed with PBS containing 1% milk powder (Molico®, Nestlé) and 0.05% Tween® 20 (PBS–MT). Sera samples were added at 50 μl/well, in duplicate, diluted 1:50 in PBS–MT and incubated at 37 °C for 1 h. Next, 50 μl/well of protein A peroxidase conjugate (Sigma) were added at 2 μg/ml in PBS–MT. After 1 h at 37 °C, the plates were washed with PBS–MT and revealed with 50 μl O-phenylenediamine (Sigma) at 0.4 mg/ml and 1,6 μl/ml H_2O_2 in phosphate-citrate buffer (pH 5.0). The reaction was stopped by the addition of 10 μl/well of 16% HCl. The OD was measured by a Spectra Count™ automatic reader (Packard Bio Science Company) at a wavelength of 490 nm (Borja-Cabrera et al., 1999 modified). Duplicates were included in blank wells, together with positive and negative control in all plates.

2.5. Statistical analysis

The nonparametric McNemar test was used to evaluate disagreement between the different indirect ELISA tests performed, while the kappa coefficient (k) was used to eval-