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SEROLOGICAL SURVEY FOR *Leishmania* sp. INFECTION IN WILD ANIMALS FROM THE MUNICIPALITY OF MARINGÁ, PARANÁ STATE, BRAZIL

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ABSTRACT: *Leishmania* sp. infection was investigated in wild animals from the Ingá Park, in the municipality of Maringá, Paraná state, Brazil, where American cutaneous leishmaniasis (ACL) is an endemic disease. Sixty-five mammals, comprising *Didelphis albiventris*, *Cerdocyon thous*, *Lycalopex vetulus*, *Cebus apella*, *Dasyprocta azarae*, *Dasypus novemcinctus*, *Procyon cancrivorus* and *Nasua nasua*, were captured. Blood samples were collected for parasite cultivation. Antibodies were investigated by direct agglutination test (DAT) using *Leishmania (Viannia) braziliensis* as antigen. Flagellates were observed in blood cultures of 14 (35.9%) *Didelphis albiventris*. Anti-*Leishmania* antibodies were detected in 31 (51.6%) specimens of *Cerdocyon thous, Lycalopex vetulus, Cebus apella, Dasyprocta azarae, Procyon cancrivorus* and *Nasua nasua*. These results suggest that *Cerdocyon thous* and *Lycalopex vetulus* (crab-eating fox), *Cebus apella* (capuchin monkey), *Dasyprocta azarae* (agouti), *Procyon cancrivorus* (crab-eating raccoon) and *Nasua nasua* (coati) play an important role in the ACL transmission cycle in the northwestern region of Paraná, Brazil.

KEY WORDS: *Leishmania*, wild animals, reservoir, agglutination tests.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Leishmaniasis, a primary zoonosis caused by *Leishmania* parasites that infect wild mammals, domestic animals and insect vectors, constitutes a serious health issue in numerous tropical and subtropical countries (1, 2). Parasites are transmitted by the bites of female sand flies (Diptera, Psychodidae) infecting primary and secondary reservoir hosts during blood feed (1).

There are two different clinical forms of leishmaniasis, visceral leishmaniasis or kala-azar and cutaneous leishmaniasis. The consequences of cutaneous leishmaniasis range from local lesions to serious mutilations in mucous membranes (3).

The epidemiological complexity of leishmaniasis in Brazil is related to the vast number of sand fly species involved in the disease transmission, to the numberless mammals that are natural reservoirs of the diseases and to several parasite species (4). In fact, new *Leishmania* species have recently been described (5).

About 40 mammal species hosting *Leishmania* spp., comprising rodents, xenarthrans, carnivores, primates and marsupials, have been found and other wild animal species have been pinpointed as reservoirs for different species of *Leishmania* (1, 6). *Leishmania (Viannia) guyanensis* has been isolated in common opossums (*Didelphis marsupialis*), two-toed sloths (*Choloepus didactylus*) and in collared anteaters (*Tamandua tetradactyla*) (7). Whereas *Leishmania (Leishmania) chagasi* has been isolated from crab-eating foxes (*Cerdocyon thous*), *Leishmania (Viannia) lainsoni* was found in pacas (*Agouti paca*) (8-10). *Leishmania (Viannia) shawi*, in turn, has been detected in capuchin monkeys (*Cebus apella*), bearded sakis (*Chiropotes satanas*), coatis (*Nasua nasua*) (11), three-toed sloths (*Bradypus tridactylus*) and two-toed sloths (*Choloepus didactylus*) (11). *Leishmania (L.) mexicana* has been found in big-eared climbing rats (*Ototylomys phyllotis*), black-eared rice-rats (*Oryzomys melanotis*), Yucatan deer-mice (*Peromyscus yucatanicus*) and hispid cotton rats (*Sigmodon hispidus*) (12). *Leishmania* parasites have been found in armadillos (*Dasypus novemcinctus*), foxes (*Lycalopex vetulus*) and in small rodents (*Akodon arviculoides* and *Oryzomys nigripes*) (9, 13, 14); while *Leishmania (Viannia) braziliensis* has also been isolated from black rats (*Rattus rattus)* (5, 15-18). Recently, Figueiredo *et al*. (19) observed natural infection with *Leishmania (Leishmania) chagasi* in a bush dog (*Speothos venaticus)* kept in captivity whereas Luppi *et al.* (20) reported clinical signs and positive serology for visceral leishmaniasis in a bush dog (*Spheotos venaticos*) and a hoary zorro (*Lycalopex vetulus*) as well as positive results for *Leishmania* in a crab-eating fox (*Cerdocyon*

thous), a maned wolf (*Chrysocyon brachyurus*) and a hoary zorro (*Lycalopex vetulus*), all captives. Anti-*Leishmania* antibodies, *L. braziliensis* complex DNA and *L. donovani* complex DNA were detected in an opossum (*Didelphis marsupialis*) (21).

Studies have revealed a double epidemiology profile of American cutaneous leishmaniasis (ACL). Although ACL had already been considered a disease proper to wild animals, which only occasionally infects humans in contact with forest environments, it is actually expanding and may occur in deforested land and in periurban and urban areas (22).

It is endemic in Brazil and may be found in all states (23). In fact, 24,291 new cases were reported in Brazil in 2005, 473 in the southern region and 480 in the Paraná state alone (24). One of the disease transmission cycles occurs in the northwestern area of the Paraná state (25). *L (V.) braziliensis*, isolated from dogs, humans and phlebotomines, has been the main parasite species involved in cases of leishmaniasis in Paraná (26-28).

Maringá, situated in northwestern Paraná, is one the Brazilian cities with a great number of trees in its streets and avenues, featuring remnants of the Atlantic Forest too. The forest fragments within the city environment are important for the maintenance of wild fauna and, consequently, constitute a possible natural focus of ACL transmission (29). Several cases have been reported in humans and dogs residing close to the Conservation Unit Borba Gato Park or in laborers who work or live within this reserve (30). In 1996 and 2003 two cases occurred in nightwatchmen in the Ingá Park reserve. In studies on phlebotomine fauna carried out in the Ingá Park, *Nyssomyia whitmani* was predominant (29). ACL transmission in workers and inhabitants who live close to these conservation units indicate that wild animals in the neighborhood are *Leishmania* reservoirs that maintain the parasite cycle and, consequently, human infection (29). The present study investigates the infection by *Leishmania* sp. in wild animals in the Ingá Park located within the urban area of the municipality of Maringá, Paraná state, Brazil.

MATERIALS AND METHODS

Study Area

The Ingá Park is located in the central region of Maringá (23^{o} 25' S; 51^{o} 25' W), a city in the state of Paraná, Brazil. Maringá presents a subtropical temperate climate with an average yearly temperature of 21.9°C – minimum and maximum temperatures

are, respectively, 10.3 and 33.6°C – 66% relative humidity and average annual rainfall of 1,500 mm. Although predominant vegetation consists of semi-deciduous seasonal forest with trees proper to this typology, it is also characterized as a modified primary forest owing to the introduction of non-native tree species in the region.

Experimental Design

Wild animals were captured by simple and Tomahawk traps between March and September 2005 in the Ingá Park. Traps were laid at nightfall and checked at least twice during the day. Baits consisted of several types of food, such as sunflower seeds, groundnuts, corn, fruits, meat and eggs.

Captured animals were anesthetized using drugs according to each species. Subsequently, clinical exams were undertaken, biological materials were collected and subcutaneous microchips were implanted to avoid double collection. Tested animals were placed in a silent heated area until the anesthesia wore out and, then, released. Research was undertaken according to ethic standards of the Brazilian College of Animal Experimentation (COBEA) and authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), according to warrant n. 057/05.

Biological Samples

A 5-mL sample was obtained from each animal. Two drops were cultivated in blood base agar (BBA) (31). Serum was stored at –20°C until use. Skin samples were collected from animals with lesions and employed for parasite search.

Parasite Search

Lesion smears, made on glass slides for parasite direct search (PD), were stained with Giemsa and analyzed to detect the presence of *Leishmania* spp. amastigote forms.

Blood samples, cultured on BAB, were incubated at 25°C and analyzed once a week for flagellates under optical microscopy. Positive cultures were replicated weekly. The supernatants of negative ones were transferred after 30 days to a new culture medium, examined weekly and maintained for another 30-day period.

Direct Agglutination Test (DAT)

DAT was performed according to Garcez *et al*. (32) with modifications. Promastigote forms of *L. (V.) braziliensis* were cultivated in 199 medium supplemented with 10% fetal calf serum. Parasites were washed five times (3,000 x g, 15 minutes, 4°C) with Locke's solution (LS) (NaCl 0.15 M; KCl 5.6 mM; $CaCl_2$ 2.1 mM; $NaHCO_3$ 2.3 mM; glucose 5.5 mM). The, sediment was resuspended at 1/20 (weight/volume) in LS with 0.4% trypsin (Sigma, Brazil) at 4°C and incubated for 45 minutes at 37°C. Subsequently, parasites were washed once again and resuspended at 2 x 10^8 promastigotes/mL. An equal volume of formaldehyde 2% was added to LS and incubated overnight at 4°C. Parasites were washed three times in physiological saline (PS) (4,000 x g, 10 minutes, 4°C) and resuspended in Comassie Brilliant Blue (Sigma, Brazil) at 0.02% in PS at a concentration of 1 x 10^8 promastigotes/mL. After that, parasite suspensions were moderately shaken for 90 minutes, centrifuged and washed three times with PS.

The concentration was adjusted to 1 x 10^8 promastigotes/mL in PS with 1% formaldehyde and filtered in nylon. Reagent was stored at 4°C until use. Sera were watered down to 1/10 in diluting solution (PS with 1% fetal calf serum and 0.7% 2-mercaptoethanol) in V-shaped bottom microplates. Plates were incubated for one hour at room temperature. An equal volume (50 µL) of antigen suspension was, then, added and incubated overnight at 25°C. Diluting solution was used as agglutination control. Serum samples from ACL dogs were used as positive controls and those from healthy dogs were employed as negative controls in all reactions. All serum samples were tested in duplicates.

Data Analysis

Proportions and confidence intervals were calculated using OpenEpi version 2.2.1 software.

RESULTS

Capture of Animals

Sixty-five animals from Canidae, Cebidae, Dasyproctidae, Dasypodidae, Didelphidae and Procyonidae families, comprising eight species, were captured. The common opossum (*Didelphis albiventris*) was the most frequent captured mammalian species (39 individuals). Four specimens of crab-eating fox (*Cerdocyon thous* and *Lycalopex*

vetulus), 12 specimens of agouti (*Dasyprocta azarae*), two specimens of coati (*Nasua nasua*), two specimens of crab-eating raccoon (*Procyon cancrivorus*), two specimens of armadillo (*Dasypus novemcinctus*) and four specimens of capuchin monkey (*Cebus apella*) were captured. Some of the aforementioned animals belong to the captive fauna of the Ingá Park.

Parasite Search

Although a crab-eating fox (*Cerdocyon thous*) had lesions on its nostrils, direct test for *Leishmania* spp. and the culture of a lesion fragment revealed no positive results. Growth of trypanosomatids (epimastigotes and trypomastigotes) occurred in blood cultures of 14 (out of 39) opossums (*Didelphis albiventris*). In other animals, growth of flagellate forms in blood culture was not observed.

Search for Antibodies by DAT

DAT was applied to 60 serum samples for anti-*Leishmania* antibody detection (Table 1). Antibody titers ≥ to 10 were detected in four (100%) crab-eating foxes (*Cerdocyon thous* and *Lycalopex vetulus*), in four (100%) capuchin monkeys (*Cebus apella*), in nine (75%) agoutis (*Dasyprocta azarae*), in 12 (35%) common opossums (*Didelphis albiventris*), in one (50%) crab-eating raccoon (*Procyon cancrivorus*) and in one (50%) coati (*Nasua nasua*). No anti-*Leishmania* antibodies were found in the two armadillos (*Dasypus novemcinctus*).

Table 1. Anti-*Leishmania* antibodies by direct agglutination test (DAT) of different wild animal species captured in the Ingá Park, a reserve area in the municipality of Maringá, Paraná state, Brazil

Species	Antibody Titers									
	< 10	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**≥2560**
Cerdocyon thous (n = 2)					1* 50.00% (2.5-97.5%)**			1 50.00% (2.5-97.5%)		
Lycalopex vetulus (n = 2)					1 50.00% (2.5-97.5%)		1 50.00% (2.5-97.5%)			
Cebus apella (n = 4)				3 75.00% (24.23-97.75%)	1 25.00% (1.25-75.77%)					
Dasyprocta azarae (n = 12)	3 25.00% (6.79-54.12%)		3 25.00% (6.79-54.12%)			1 8.33% (0.42-34.75%)	3 25.00% (6.79-54.12%)		1 8.33% (0.42-34.75%)	1 8.33% (0.42-34.75%)
Dasypus novemcinctus (n = 2)	2 100% (22.36-100.00%)									
Didelphis albiventris (n = 34)	22 64.71% (47.72-79.27%)	4 11.76% (3.85-25.98%)	3 8.82% (2.29-22.16%)	2 5.88% (0.99-18.10%)	3 8.82% (2.29-22.16%)					
Procyon cancrivorus (n = 2)	1 50.00% (2.5-97.5%)						1 50.00% (2.5-97.5%)			
Nasua nasua (n = 2)	1 50.00% (2.5-97.5%)					1 50.00% (2.5-97.5%)				
Total (n = 60)	29 48.33% (35.92-60.91%)	4 6.67% (2.15-15.3%)	6 5.00% (1.29-13.00%)	5 8.33% (3.12-17.51%)	6 5.00% (1.29-13.00%)	2 3.33% (0.56-10.58%)	5 8.33% (3.12-17.51%)	1 1.67% (0.08-7.95%)	1 1.67% (0.08-7.95%)	1 1.67% (0.08-7.95%)

* number of samples; ** 95% confidence interval.

DISCUSSION

The information that wild animals are important *Leishmania* reservoirs is in agreement with several studies (1, 14). Garcez *et al*. (32) affirm that deforestation in the New World and forest management, or other activities that involve the forest ecosystem, may trigger the emergence of human cutaneous leishmaniasis. Environmental conditions of Maringá, with its exuberant tree-lined streets and downtown forest fragments, are a favorable milieu for the maintenance of wild fauna that may constitute possible natural foci of ACL transmission (23, 29). The occurrence of ACL cases in humans and dogs from the neighborhood of the Conservation Unit Borba Gato Park and in persons that work or reside in the forest reserve, as nightwatchmen of the Ingá Park, indicate the possible existence of reservoirs at these sites (30). It is well-known that wild animals, the parasite's natural hosts, render possible the maintenance of *Leishmania* cycle and, consequently, human infections (29). Nevertheless, probable reservoirs in the northwestern region of the Paraná state are unknown.

Difficulties with wild animal capture, taxonomic identification and standardization of laboratory techniques for ACL diagnosis constitute restricting factors in the understanding of leishmaniasis epidemiology (17). Several authors, employing *Leishmania* promastigotes, had utilized DAT in epidemiological studies (32-35). In fact, the test does not require special equipment or species-specific immunoglobulin conjugate, and may be useful in cases of natural and experimental infections (34). The assay had been previously employed for serum diagnosis of canine infection by *Leishmania* in animals with asymptomatic visceral leishmaniasis (36, 37). In the current study, the authors are reporting the presence of anti-*Leishmania* antibodies in *Cerdocyon thous, Lycalopex vetulus, Cebus apella, Dasyprocta azarae, Procyon cancrivorus* and *Nasua nasua*. However, *Leishmania* infection failed to be detected in blood culture from these animals.

Since *Leishmania* infection and/or presence of anti-*Leishmania* antibodies had been already reported in *Cerdocyon thous* (8, 9, 20), *Lycalopex vetulus* (9, 20), *Cebus apella* (11) and *Nasua nasua* (11), these animals were pinpointed as *Leishmania* spp. reservoirs. The high titers of antibodies in *Dasyprocta azarae* corroborate finds by Forattini (38), who described amastigote forms in cutaneous lesions from *Dasyprocta azarae* in an endemic area of cutaneous leishmaniasis.

On the other hand, no reports are found in the literature on natural infection by *Leishmania* sp. in *Procyon cancrivorus*. Moreover, anti-*Leishmania* antibodies were

not found in *Dasypus novemcinctus* specimens. Alcantara de Castro *et al*. (39) did not observe flagellates in *Dasypus novemcinctus*, although Lainson and Shaw (40) detected parasites of the *Leishmania* genus in this species.

Anti-*Leishmania* antibodies in *Didelphis albiventris* may be due to cross reactions, since flagellates were detected in the blood culture of these animals. Polymerase chain reaction of flagellates with *Leishmania (Viannia*) primers revealed no results (data not shown). Flagellates were not identified, but these species were found to be naturally infected by *Trypanosoma cruzi* (41, 42).

Current data show serological evidence of infection by *Leishmania* sp. in the wild species *Cerdocyon thous* and *Lycalopex vetulus* (crab-eating fox), *Cebus apella* (capuchin monkey), *Dasyprocta azarae* (agouti), *Procyon cancrivorus* (crab-eating raccoon) and *Nasua nasua* (coati) and suggest that these mammals have an important role in ACL transmission in northwestern Paraná, similar to other ACL endemic regions. Studies with more specimens and more sensitive techniques have been undertaken in our laboratories to achieve a better evaluation of the importance of these species as *Leishmania* reservoirs in Paraná.

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