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Canine visceral leishmaniasis in urban and rural areas of Northeast Brazil

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ABSTRACT

The purpose of this study was to determine the clinical and laboratory profiles of canine leishmaniasis in two distinct areas. Dogs from urban and rural areas were examined. The population studied in the metropolitan area included 54 dogs. Of these, 20 (37%) animals did not present with any signs suggestive of visceral leishmaniasis (VL). Among these, only eight were confirmed negative by ELISA (rK39 and CE) and 12 dogs, clinically negative for leishmaniasis, were seropositive by ELISA (rK39 and CE). Thinness, conjunctivitis and onychogryphosis were the most frequent clinical signs in the urban areas, followed by crusty lesions, alopecia, ulcerated lesions, hyperkeratosis and exfoliation. In the metropolitan area human VL cases occurred mainly in 1991, 1992, 1999 and 2000. In the rural areas the ELISA rK39 test detected a seroprevalence of 11.3% and ELISA CE (*Leishmania* crude extract) of 20.6%. Thirty-nine dogs were examined 6 months after the first visit. Serological exams using rK39 antigen showed seroconversion of only one dog, whereas *Leishmania* CE showed seroconversion of 13 (33.4%) dogs. In this rural environment 83.3% of the positive dogs were asymptomatic. *Lutzomyia intermedia* and *Lu. longipalpis* were the most predominant sandfly vector species. Amastigotes were identified in spleen and liver fragments of symptomatic necropsied animals. PCR amplification of DNA isolated from promastigote culture indicated that the species was *Leishmania chagasi*. This finding suggests that delayed diagnosis and euthanasia of potentially infectious animals may occur with an increased transmission risk to sandflies and subsequently to humans.

1. Introduction

Standard measures to control visceral leishmaniasis (VL) in Brazil have been based on elimination of seropositive dogs, vector control and treatment of humans with disease. However, the impact of these measures on human disease has not been fully assessed. A study (Dietze et al., 1997) in the state of Espírito Santo, Brazil, showed that the elimination of dogs alone is not sufficient to decrease human infection. Conversely, a study performed in the state of Bahia (Ashford et al., 1998) showed that the elimination of seropositive dogs led to a significant decrease in human and canine *Leishmania* seroconversion. Lainson and Rangel (2005) and Madeira et al. (2004) recently showed the presence of *Leishmania chagasi* in seropositive dogs that presented with healthy skin and no signs of leishmaniasis.

Before seroconversion, dogs infected with *L. chagasi* present with enlarged lymph nodes and dermatitis without signs of visceral leishmaniasis or change in behavior. This phase is followed by dissemination of the infection and clinical findings, including loss of appetite, fever, weight loss, alopecia, skin ulceration, onychogryphosis, keratoconjunctivitis, uveitis, bleeding, diarrhea, neuralgia, polyarthritis, interdigital ulceration and kidney insufficiency (Abranches et al., 1991; Alencar, 1959; Bettini and Gradoni, 1986; Molina et al., 1994).

This study investigated canine *Leishmania* infection in the rural areas of São Miguel, in the western region of the state of Rio Grande do Norte, and in the metropolitan area of Natal, correlating clinical signs with serological and parasitological findings. In addition, a phlebotomine sandfly survey was performed to determine the presence of sandflies capable of transmitting *L. chagasi* in these regions.

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2. Materials and methods

2.1. Human and canine visceral leishmaniasis in Rio Grande do Norte

Information on human visceral leishmaniasis in the metropolitan region of Natal and the municipality of São Miguel was obtained from hospitalized cases of the disease reported by the State Secretariat of Health and the Ministry of Health (SSP/RN, 1997). Data concerning dog serology in the metropolitan area of Natal from 1990 to 2002 was obtained from the state department of epidemiological surveillance (SINAN, 2004). Information on canine visceral leishmaniasis dating from 1999 was obtained in the municipality of São Miguel.

2.2. Study area

The study was conducted in the metropolitan area of Natal and in the rural areas of São Miguel in western Rio Grande do Norte from June 2001 to October 2002. The former has a population of 712,317 inhabitants and the latter 20,124 (IBGE, 2000). São Miguel is located in a highland region, 444 km from Natal, the state capital. Natal lies at sea level. Both areas have distinct phytogeographic characteristics.

2.3. Study population

A canine leishmaniasis incidence survey was performed in São Miguel, a municipality reporting human visceral leishmaniasis (VL) and human cutaneous leishmaniasis (CL). Ninety-seven dogs from seven areas of reported human VL or CL and from two areas with no record of human VL or CL were studied. Informed consent was obtained from the owner of each dog. All the animals were reexamined 6 months after the first visit and a second blood sample was drawn, except from the animals who had died or whose owners had moved or had refused permission.

From June 2001 to October 2002, 300 dogs were captured in VL-endemic areas by technicians from the Center for Zoonosis Control of Natal (CCZ). Fifty-four dogs from this population were randomly assessed.

The study protocol was approved by the Postgraduate Program in Biochemistry and the Health Secretariat of São Miguel. In the metropolitan region of Natal the population studied was the same population examined by the technicians of the Center for Zoonosis Control, a department of the Health Secretariat of Natal responsible for the elimination of positive dogs as part of the effort to control VL.

2.4. Clinical examinations and blood sample collection

The animals were identified on charts and submitted to physical examination for assessment of clinical signs suggestive of leishmaniasis. According to animal size, 5–10 ml of blood was drawn and aliquoted into sterilized tubes for serum acquisition and DNA extraction.

2.5. Anti-leishmania serology

The detection of anti-*Leishmania* antibodies was performed by immunoenzymatic assay (ELISA) using *L. chagasi* recombinant protein rK39 (Braz et al., 2002), provided by Steven G. Reed (Institute of Infectious Diseases Research) and *Leishmania* crude extract (CE) as previously reported (Evans et al., 1992). Data of the canine control program were obtained from the Health Secretariat of Rio Grande do Norte. A cut-off of 0.108 was established for CE and 0.339 for rK39, which corresponds to the mean absorbances of negative control serums plus three standard deviations. All the animals with positive serology at the first visit were euthanized according to the recommendations of the Brazilian Program for the Control of Leishmaniasis (MS, 1999). In Brazil immunofluorescence serological tests are used to diagnose infection by leishmanias and when ELISA is performed, raw leishmania extract is used.

2.6. Parasite identification by the molecular method

Anti-*Leishmania* seropositive dogs were euthanized by the State Health Secretariat. The animals were first anesthetized intravenously with ketamine (10–15 mg/kg) and xylazine (0.5–1.0 mg/kg) followed by a lethal injection of 10% potassium chloride. Spleen and liver fragments were removed for parasite culture and histopathological examination. The fragments were macerated and inoculated in biphasic culture (NNN) using Schneider as the liquid phase (blood agar base, Merck; TC-100 Insect Medium Life Technologies, Gibco BRL). *Leishmania* isolate was also typed by isoenzymes at Fundação Oswaldo Cruz, Rio de Janeiro, a World Health Organization-sanctioned laboratory.

Identification of leishmania was also done by polymerase chain reaction (PCR), following a previous protocol (Smyth et al., 1992). To the reaction were added 20 ng of DNA at a mixture of 4.18 μl of sterile bidistilled water, 1.25 μl of 10× PCR buffer (Tris–HCl 100 mM, KCl 500 mM, $MgCl_2$ 15 mM, pH 8.3), 1.25 μl each of oligonucleotide at a concentration of 20 nmol (forward: 5′-GGGGTTGGTGTAAAATAG-3′ and reverse: 5′-CCAGTTTCCCGCCCCG-3′), 2.5 μl of a solution containing 1.25 mM of deoxyribonucleoside triphosphate (dGTP, dATP, dCTP, dTTP, Boehring Mannheim Corporation, Indianapolis, IN, EUA) and 0.0625 μl of thermal-resistant DNA polymerase 5 U/μl (Gibco BRL Life Technologies, Grand Island, NY, USA). Samples were initially denatured at 93 °C for 3 min and then submitted to 30 denaturation cycles at 93 °C for 30 s, annealing at 60 °C for 1 min and final extension at 72 °C for 1 min. The PCR product was identified by 1% agarose gel electrophoresis in TBE buffer (Tris 0.04 M, boric acid 0.04 M, EDTA 0.02 M). Five microliters of the amplified DNA were mixed with 1 μl of ethidium bromide (1 g/ml). A negative (water) and positive (*Leishmania* DNA) control of the reaction were used in each experiment.

2.7. Sandfly capture and identification

Four captures of phlebotomine sandflies were made using six CDC light traps (Haushers Machine Works, New Jersey, USA) set in São Miguel and Natal. Two captures were carried out during the rainy season (June and July) and two during the dry months (December and January). Sandflies were identified according to Young and Duncan (1994).

2.8. Statistical analysis and climate data

The analysis and comparison of the data was performed by Statistica software 6.1. The São Miguel mean rainfall and temperature data were provided by the Empresa de Pesquisa Agropecuária do Rio Grande do Norte (EMPARN).

3. Results

3.1. Canine and human leishmaniasis in Rio Grande do Norte

The recorded cases of human VL in the metropolitan area of Natal and São Miguel are shown in Fig. 1. In the metropolitan area human VL cases occurred mainly in 1991, 1992, 1999 and 2000. Peak occurrences of the disease are coincident in both areas. Canine vis-