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Short communication

Laboratory tests performed on *Leishmania* seroreactive dogs euthanized by the leishmaniasis control program

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ABSTRACT

In 2008, in the west zone of Rio de Janeiro municipality—Brazil, the leishmaniasis control program identified 155 dogs with titers ≥40 by Indirect ImmunoFluorescence (IIF) on blood collected onto filter paper. The objective of this study was to describe the laboratory test findings performed in dogs euthanized by the leishmaniasis program control of Rio de Janeiro municipality. Dogs were examined, subjected to euthanasia and collection of clinical specimens. Parasite isolation was obtained in 29 animals: *Leishmania chagasi* was isolated in 14 dogs; *Leishmania braziliensis* was isolated in five dogs; *Trypanosoma caninum* was obtained in seven animals and one dog had mixed infection (*L. braziliensis* and *L. chagasi*). By Polymerase Chain Reaction, seventeen animals were positive in intact skin fragments. In the serological reassessment of serum samples, 28% and 22% were positive for IIF and enzyme immunoassay, respectively. Ninety-one (59%) dogs were negative for all tests performed in this study. The findings indicate that the visceral leishmaniasis control program needs to be adjusted in order to avoid non-infected dogs from being removed or permit that dogs infected with *L. chagasi* to remain undetected in endemic areas.

1. Introduction

Both Visceral Leishmaniasis (VL) and Tegumentary Leishmaniasis (TL) are complex diseases in different aspects. In Brazil, VL is caused by *Leishmania (Leishmania) chagasi*, whose main vector is *Lutzomyia longipalpis* and the reservoir in domestic and peridomestic environment is the domestic dog (*Canis familiaris*). The Brazilian Ministry of Health recommends euthanasia of seroreactive dogs, especially in endemic areas (Ministério da Saúde, 2006). Thus, Indirect ImmunoFluorescence (IIF) assay in blood samples collected on filter paper is employed. This technique, however, is limited in areas where VL and TL occur in overlapping (Madeira et al., 2006), or even in areas where other trypanosomatids such a *Trypanosoma caninum* circulate (Madeira et al., 2009) due to cross-reaction that can occur in serological tests. Appropriate control measures must be applied to the epidemiological context of each endemic area to be effective (Ministério da Saúde, 2006, 2010). To control VL, dogs with titers ≥40 by IIF are subjected to euthanasia (Ministério da Saúde, 2006). The same is not indicated in the case of TL (Ministério da Saúde, 2010) although dogs with TL may be seroreactive for *Leishmania* parasites (Madeira et al., 2005). Thus, in areas with overlapping transmission of VL and TL, differentiated control measures for each disease should be based on parasitological diagnostic methods together with the identification of the *Leishmania* species involved.

The objective of this study was to describe the laboratory test findings performed in dogs euthanized by the

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leishmaniasis program control of Rio de Janeiro municipality.

2. Materials and methods

This study was approved by the Ethics Committee on the Use of Animals of FIOCRUZ (CEUA/FIOCRUZ), license number L-023/06.

2.1. Animals and samples

In 2008, the leishmaniasis control program identified 155 dogs with titers ≥40 by IIF on blood collected onto filter paper (eluate) in the west area of Rio de Janeiro municipality. At Instituto de Pesquisa Clínica Evandro Chagas (IPEC/Fiocruz/RJ), these animals were sedated with ketamine (10 mg/kg) associated with acepromazine (0.2 mg/kg). After sedation, the dogs were examined and classified as asymptomatic (no clinical signs), oligosymptomatic (1–3 signs) and symptomatic (more than three clinical signs) based on Mancianti et al. (1988) criteria.

Blood samples were collected by venipuncture cephalic or jugular and placed in tubes without anticoagulant to serological reassessment. Euthanasia was performed with overdose of sodium thiopental 5%. After euthanasia, cutaneous lesions, intact skin of the scapular region and spleen fragments were collected.

2.2. Serological tests

Serum samples were tested for anti-*Leishmania* antibodies using IFI-Leishmaniose-Visceral-Canina-Bio-Manguinhos and EIE-Leishmaniose-Visceral-Canina-Bio-Manguinhos kits (Bio-Manguinhos, Rio de Janeiro, Brazil), according to the manufacturer's instructions.

2.3. Parasitological culture and characterization by multi-locus enzyme electrophoresis (MLEE)

Cutaneous lesions, intact skin of the scapular region and spleen fragments were immersed in saline containing 100 μg of 5′fluorocytocine, 1000 IU of penicillin and 200 μg of streptomycin per milliliter and stored at 4 °C for 24 h. After this period, each fragment was transferred aseptically to a biphasic culture medium (NNN supplemented Schneider's medium with 10% fetal bovine serum) and stored at 26–28 °C. Fresh cultures were observed weekly for thirty days.

Multi-locus enzyme electrophoresis (MLEE) was used for characterization of isolates, according by Cupolillo et al. (1994). Five enzymatic systems were employed to analyze all the isolated samples: malic enzyme (ME, E.C.1.1.1.40), nucleosidase (NH1 and NH2, E.C.3.2.2.1), glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), glucose phosphate isomerase (GPI, E.C.5.3.1.9) and 6-phosphogluconate dehydrogenase (6PGDH, E.C.1.1.1.43). *Leishmania braziliensis* (MHOM/BR/75/M2903), *L. chagasi* (MHOM/BR/74/PP75) and *L. amazonensis* (IFLA/BR/67/PH8)) were used as reference samples.

2.4. Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from samples of intact skin using Illustra™ Tissue & Cells Genomicprep Mini spin kit (GE Healthcare, New York, USA), following the manufacturer's instructions. DNA extracted were analyzed by PCR using primers: 5′-(G/C) (G/C) (C/G) CC(A/C)CTAT(A/T)TTACACAACCCC-3′ and 5′-GGGGAGGGGCGTTCTGCGAA-3′ described by Degrave et al. (1994), which amplify a 120 base pair fragment of the conserved region of minicircle kinetoplast of *Leishmania* DNA. Each PCR run included positive control (*L. chagasi* DNA) and negative controls (all reaction components, except the DNA and negative DNA sample for *Leishmania*).

The cycles included an initial step at 94 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min. All products were analyzed by electrophoresis in agarose gels 2% stained with ethidium bromide and amplicons fragments were visualized with an image analyzer L-PIX HE (Loccus Biotechnologia®, São Paulo, Brazil).

2.5. Statistical analysis

Statistical analyses of the data were performed using the Statistical Package for Social Science (SPSS) version 16.0. In order to evaluate the concordance index between the serological tests, Kappa measurement (k) was used according to classification proposed by Shrout (1998): $k = 0.00–0.10$ virtually no reliability; $k = 0.11–0,40$ low; $k = 0.41–0.60$ discrete; $k = 0.61–0.80$ moderate and $k = 0.81–1.0$ substantial.

3. Results

Of the 155 dogs studied 40% were asymptomatic, 54% oligosymptomatic and 6% symptomatic for VL. Twenty-eight dogs (18%) had cutaneous lesions located in the ears, muzzle, face, scrotal bag, and limbs and 74% were mongrel dogs.

Parasite isolation was obtained in 29 (19%) animals. After isoenzyme analysis with isolated from different sites, it was observed that:

(a) *Fourteen (48%) animals were infected by L. chagasi*. This species was isolated from fragments of intact skin, lesion and spleen. Two animals were asymptomatic, nine oligosymptomatic and three symptomatic for VL.
(b) *Five (17%) animals were infected by L. braziliensis*. This species was isolated exclusively from fragments of cutaneous lesions of three asymptomatic animals and two oligosymptomatic animals for VL.
(c) *One animal showed co-infection by L. braziliensis and L. chagasi*. *L. braziliensis* was isolated from cutaneous lesion of muzzle and *L. chagasi* from spleen fragment. The animal was oligosymptomatic for VL.
(d) *Seven (24%) dogs were infected by T. caninum*. Of these animals, epimastigotes were isolated in intact skin fragments: three were asymptomatic, three oligosymptomatic and one symptomatic for VL. These samples were identified as *T. caninum* by PCR and