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Short communication

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

D.A. Silva[a,d,*], M.F. Madeira[b], A.C. Teixeira[c], C.M. de Souza[c], F.B. Figueiredo[d]

[a] *Pós-graduação Stricto-sensu, Instituto de Pesquisa Clinica Evandro Chagas (IPEC), Fundação Oswaldo Cruz (FIOCRUZ), Brazil*
[b] *Laboratório de Vigilância em Leishmanioses, IPEC-FIOCRUZ, Brazil*
[c] *Laboratório de Tecnologia Diagnóstica, BIOMANGUINHOS-FIOCRUZ, Brazil*
[d] *Laboratório de Pesquisa Clínica em Dermatozoonoses em Animais Domésticos, IPEC-FIOCRUZ, Brazil*

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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

* Corresponding author at: Laboratório de Pesquisa Clínica em Dermatozoonoses em Animais Domésticos, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, Manguinhos 21040-360, RJ, Brazil. Tel.: +55 21 3865 9536; fax: +55 21 3865 9553.
E-mail address: denise.silva@ipec.fiocruz.br (D.A. Silva).

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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

molecular sequencing in another study (data to be published).

Promastigotes isolated from spleen fragments of two oligosymptomatic dogs were lost and could not be identified.

In the serological reassessment of 144 serum samples, 28% was positive for IIF while 22% was positive for EIA. Kappa value was 0.41 showing discrete concordance between the two serological tests.

Skin samples of 150 dogs were analyzed by PCR. Of those, 17 animals were positive, which three dogs had negative results to skin and spleen samples in the culture. Skin samples of seven dogs infected by *T. caninum* were negative for *Leishmania*-specific PCR. Considering the 155 animals studied, 91 (59%) were simultaneously negative in the four techniques (parasitological culture, IIF, EIA and PCR) performed. The results of laboratory tests can be found in Table 1 and Fig. 1.

4. Discussion

In the present work, following one of the VL control measures recommended by the Ministry of Health, 155 seroreactive dogs were euthanized and tested by serological, parasitological and molecular tests with objective to describe the laboratory test findings performed in these dogs.

Although it has been showed that domestic dogs are involved in the transmission cycle of VL, researches have been carried out to justify the culling of seroreactive dogs as control measure. The unreliability of current methods for identifying dogs for euthanasia is the great concern and in this context, there are still divergences between the diagnostic value of serology (Silva et al., 2005).

The municipality of Rio de Janeiro is classified as a sporadic transmission area of visceral leishmaniasis (Ministério da Saúde, 2006); however, there is a worrisome persistence of canine seroprevalence (Marzochi et al., 2009).

All the 155 dogs evaluated in this study were seroreactive for leishmaniasis by IIF assay employing eluate, however, when the same group was reevaluated by the IIF kit, using serum sample, 72% (103/144) of the animals were negative. This results can be partially explained by the type of sample tested (serum and eluate), as previously reported by other studies (Figueiredo et al., 2010; Palatnik-de-Sousa et al., 2004). However, some studies have shown similar results in the use of eluate and serum in the IIF and EIA tests for VL diagnosis (Coutinho et al., 1985; Gomes et al., 2001).

In this study, 59% of animals identified by IIF using eluate showed negative results for *L. chagasi* infection, however, the report of Figueiredo et al. (2010) showed that dogs infected by *Leishmania* could not be identified by the IIF in dried blood on filter paper, possibly maintaining the transmission cycle in endemic areas. These observations demonstrate that the results obtained, after canine surveys, by IIF assay using eluate to identify infected dogs in the VL areas should be better investigated and analyzed. The culture, employed in this study, was used for isolation and further etiologic identification. Although, this method be considered reference standard for leishmaniasis diagnosis, it is not performed in the routine of leishmaniasis control mainly due to the great volume work and specialized laboratory structure that this procedure demands. However, in areas where the circulation of other trypanosomatids occurs, parasitological confirmation of seroreactive dogs is of great important in several aspects (Madeira et al., 2009). Many studies have stated that cutaneous lesions are an important sign of canine visceral leishmaniasis. In the present study, cutaneous lesions were found in 28 (18%) dogs studied, of which five were positive for *L. braziliensis*, demonstrating that the presence of cutaneous lesions is not always associated with *L. chagasi* infection, mainly in areas with overlapping transmission of TL and VL (Madeira et al., 2006). Even as Madeira et al. (2005), the isolation of *L. braziliensis* was found only in cutaneous lesions, once again showing that this species tends to restrict to this site in naturally infected dogs. This information indicates need for parasitological investigation in suspected dogs and it corroborates discussions on the importance of the domestic dog in the transmission cycle of TL (Reithinger and Davies, 1999). In overlapping areas, both clinical and serological data should be carefully analyzed. It is important to mention that two out of five animals infected by *L. braziliensis* were positive PCR for generic targets of *Leishmania* in intact skin fragments. Primers especific for the *L. braziliensis* complex should be used to elucidate this results in the samples of these animals. Moreover, finding an animal co-infected by *L. braziliensis* and *L.* chagasi confirms the overlapping of both diseases, whose control measures are different as previously reported by Madeira et al. (2006).

L. chagasi was isolated in 14 dogs, of those only three were symptomatic for leishmaniasis. This is an important observation once asymptomatic dogs participate in the transmission cycle, but do not attract attention of owners and veterinarians, making this group of dogs silent reservoirs and then causing great damage to VL control.

Regarding parasite isolation, of the 155 dogs evaluated, the presence of *T. caninum* was confirmed in seven samples, indicating that parasite circulation in the municipality of Rio de Janeiro as reported by Madeira et al. (2009) and Pinto et al. (2010).

All animals evaluated in this study were seroreactive for VL, a primary condition for euthanasia; however, considering all techniques used, 59% of dogs showed negative results, indicating that some seroreactive dogs subjected to euthanasia showed no infection for *L. chagasi* or other agent, although *Leishmania* or other protozoan infection cannot be completely ruled out. Based on these results, two significant questions arise.

1. Can the overlap of TL and VL areas and the occurrence of other agents such as *T. caninum* influence the results of serological survey?

   The overlap of endemic areas is undoubtedly a complex factor in canine diagnosis once serological tests are not able to discriminate between *L. braziliensis* and *L. chagasi* infection, which have different control measures for domestic dogs. Several studies indicate that antigens

Table 1
Laboratory test results of 155 *Leishmania* seroreactive dogs euthanized by the leishmaniasis control program of Rio de Janeiro, Brazil, 2008.

Culture/isoenzymes diagnosis, *n* = 155	PCR, *n* = 150	Serological tests, *n* = 144	
		IIF	EIA
Leishmania chagasi infected dogs, *n* = 14	11	10	13
Leishmania braziliensis infected dogs, *n* = 5	2	2	3
L. chagasi and *L. braziliensis* co-infected dogs, *n* = 1	–	1	1
Trypanosoma caninum infected dogs, *n* = 7	–	3	2
Dogs with no parasites identification, *n* = 2	1	2	2
Dogs with negative culture, *n* = 126	3	23	10

PCR, Polymerase Chain Reaction; IIF, Indirect Immunofluorescence; EIA, enzyme immunoassay.

of different species of *Leishmania* can interfere with serological tests.

In this study, the occurrence of mixed infection by *L. braziliensis* and *L. chagasi* is once more confirmed. Besides, it is important to mention the presence of *T. caninum* in dogs in municipality of Rio de Janeiro. *T. caninum* circulation among these animals may confound the results of canine surveys in leishmaniasis area.

2. Are the tools currently used in Rio de Janeiro safe for VL diagnosis?

Concerning this issue, the sample used in the serological evaluation of animals has to be taken into account. Initially, eluate was used and then serum. As previously mentioned, according to collection and storage conditions of eluate, there may be interference in antibodies detection. In the present study, positive serological results, initially obtained, could not be confirmed with serum samples.

In conclusion, our results showed that 59% of dogs were unnecessarily destroyed when the decision was based on a single serological test and the combination of different laboratorial tests could not confirm the infection. In this context, an alternative approach should be required in epidemiological surveys, however, is important, emphasizes the particularities of Rio de Janeiro municipality that should be considered in the surveys and the need for adjustment of the VL control by responsible agencies in order to avoid non-infected dogs from being removed or permit that dogs infected with *L. chagasi* to remain undetected in endemic areas.

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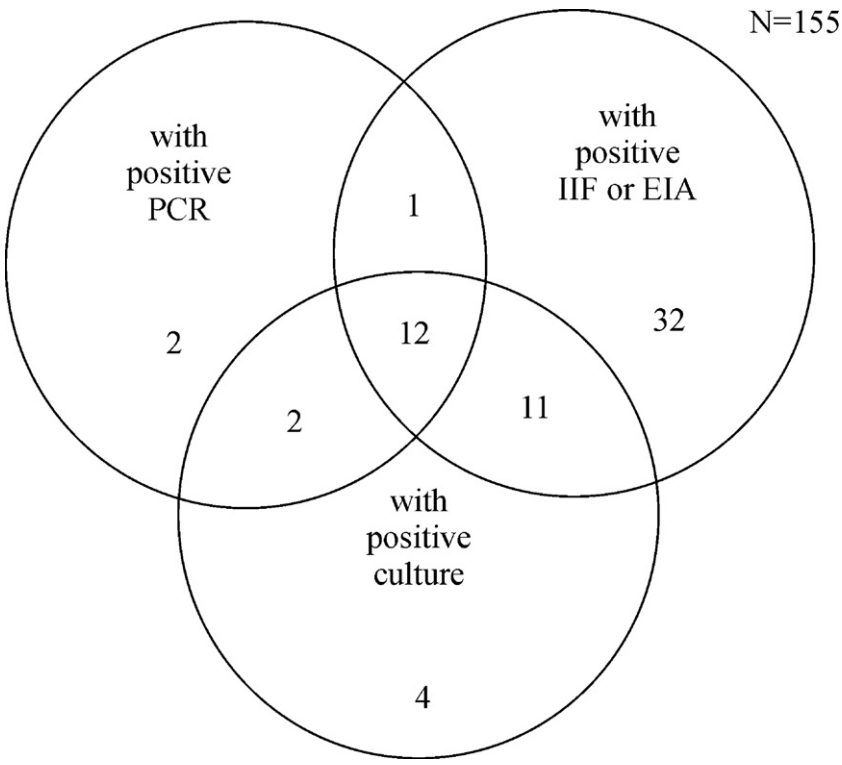

Fig. 1. Results from the combination of parasitological (culture), serology (IIF/EIA) and molecular (PCR) assays performed in 155 seroreactive dogs euthanized by the leishmaniasis control program of Rio de Janeiro, Brazil. Ninety-one dogs (59%) were negative in all tests.

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