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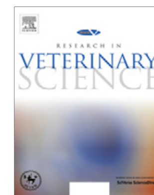

Evaluation of serological cross-reactivity between canine visceral leishmaniasis and natural infection by *Trypanosoma caninum*

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ABSTRACT

In order to evaluate if the presence of *Trypanosoma caninum* can lead to a confuse diagnosis of canine visceral leishmaniasis (CVL), we investigated the serological status of dogs infected by *T. caninum* and assessed the serological cross-reactivity with CVL. A set of 117 serum samples from dogs infected by *T. caninum*, *Leishmania chagasi* and not infected dogs ($n = 39$ in each group) was tested using commercial kits – indirect immunofluorescence (IFI-LVC), ELISA (EIE-LVC) and immunochromatographic test (DPP) – and in house tests with *T. caninum* (IIF-Tc and ELISA-Tc) and *L. chagasi* antigens (IIF-Lc and ELISA-Lc). IIF-Tc and ELISA-Tc presented sensitivity of 64.1% and 94.9% and specificity of 23.1% and 35.9%, respectively. The sensitivity of the IFI-LVC, EIE-LVC and DPP tests was 100% and the specificity was 70.5%, 68% and 97.5% respectively. The concordance between the tests was considered as satisfactory. The specificities of IFI-LVC, EIE-LVC and DPP were higher when the group Tc was excluded, with significant values for IFI-LVC ($\chi^2 = 4.36$, P-value = 0.036), thus suggesting that the infection by *T. caninum* can confuse the diagnosis of CVL.

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1. Introduction

Leishmaniasis is a globally neglected disease affecting mostly poor or vulnerable populations (WHO, 2010). In Brazil, visceral leishmaniasis (VL) is endemic with autochthonous transmission in all federal states. In the urban environment, the domestic dog acts as a reservoir of *Leishmania chagasi* (syn. *L. infantum*) the etiological agent of VL in the Americas (Ministério da Saúde, 2006). One of the control measures includes canine monitoring by serological surveys through enzyme linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF) for *Leishmania* sp. The seroreactive dogs are collected and euthanized (Ministério da Saúde, 2006). A disadvantage of the serological tests is the possibility of cross-reactions with other agents, thus in areas where other dermotropic *Leishmania* species or other trypanosomatids are also present, the serologic diagnosis must be carefully used since these agents can share antigenic determinants and lead to confusing results (Vexenat et al., 1996; Madeira et al., 2006; Silva et al., 2011).

Dual-Path Platform (DPP) is an immunochromatographic rapid test that provides an alternative for CVL diagnosis. It is being produced by Fiocruz (Bio-Manguinhos – Rio de Janeiro, Brazil) and uses rK26 and rk39 recombinant agents specific for *L. chagasi* (Grimaldi et al., 2012) which is being validated to be used in the Brazilian VL control program.

In this context, the occurrence of *Trypanosoma caninum*, a parasite detected for the first time in dogs from Rio de Janeiro municipality (Madeira et al., 2009a; Pinto et al., 2010) and, later in different Brazilian regions is a matter of concern (Almeida et al., 2011; Barros et al., 2012). So far, 53 cases of natural infection by *T. caninum* have been registered in different municipalities of five states in the Southeastern and Midwestern regions, suggesting overlapping of a natural cycle of this parasite in areas of visceral leishmaniasis. Although apparently *T. caninum* is not pathogenic for the dog, aspects related to its natural infection are still unknown.

Based on the hypothesis that dogs infected by *T. caninum* could present serological cross-reactivity in the tests used for the diagnosis of canine visceral leishmaniasis, this study aims at investigating the serological status of animals infected by *T. caninum* and assess

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the cross-reactivity with serological tests routinely used for VL control in Brazil.

2. Materials and methods

2.1. Design of the study

The present study aims at evaluating the use of serological tests as a tool for the diagnosis of infection by *T. caninum* and the cross reactivity with the commercial tests commonly used for canine visceral leishmaniasis diagnosis.

2.2. Sample selection

We studied a set of 117 serum samples from dogs. All samples were obtained during serological surveys conducted for VL control between 2008 and 2011, in different Brazilian regions and stored at −20 °C at the Laboratory for Leishmaniasis Surveillance (VigiLeish/IPEC/FIOCRUZ). The clinical specimens (serum and tissue fragments) were obtained according a protocol approved by the Ethics Committee on the Use of Animals (CEUA/FIOCRUZ), under licenses P-0276/05, L-017/06 and L-038/08.

The samples were classified in three different groups composed by 39 samples each:

Tc group: Naturally infected dogs by *T. caninum*, from the states of Rio de Janeiro, Minas Gerais, São Paulo, Goiás and Mato Grosso. The diagnosis of this group was confirmed by culture isolation of *T. caninum*, identified by the analysis of nucleotide sequences obtained through nested-PCRs that targeted a partial sequence of the 18S rDNA gene (Almeida et al., 2011).

Lc group: Naturally infected dogs by *L. chagasi*, from Rio de Janeiro municipality and Minas Gerais. The diagnosis of this group was confirmed by culture isolation of *Leishmania* sp. and etiologic identification through isoenzyme technique (Madeira et al., 2009b).

Co group: Healthy dogs in good physical conditions used as control group. The animals in this group were from Rio de Janeiro municipality and, at that time, presented two serological tests – IIF and ELISA negative for anti-*Leishmania* sp. antibodies; besides culture and molecular tests (nested-PCR, gene 18S rRNA) negative for *Leishmania* sp. and *Trypanosoma* sp. in intact skin fragments.

2.3. Serological tests and antigens

Anti-*Leishmania* and anti-*T. caninum* specific antibodies were assessed by IIF and ELISA using antigens produced in house and commercial kits manufactured by Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, Brazil and distributed to the public service: IFI-Leishmaniose visceral canina (IFI-LVC), EIE-Leishmaniose visceral canina (EIE-LVC) and Dual-Path Platform (DPP). Antigens of *T. caninum* (MCAN/BR/2003/R847) and *L. chagasi* (MCAN/BR/2007/R1112) the strains of which were properly characterized (Madeira et al., 2009a,b) were used in the in house tests. Both parasite samples were cultured in Schneider's medium supplemented by the addition of 10% fetal bovine serum (FBS) and antibiotics (penicillin – 200 UI/mL and streptomycin – 200 μg/mL) and processed during the stationary phase of growth.

The parasites for IIF were washed twice in physiological buffered solution (PBS, pH 7.2) through centrifugation and the resultant sediment was resuspended in 2 mL of 2% buffered formalin and stored at 4 °C until the time of usage.

For ELISA, around 2 mL of lysis buffer containing protease inhibitors (1 mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride, 1 mM phenanthroline) were added to the sediment of parasites after washing. The suspension was subjected to 40 cycles of freezing (dry ice + ethanol) and thawing (water bath at 60 °C) and then sonicated (Transsonic 310-Elma®) during 2 h until complete destruction of the parasites. After centrifugation (10.000 rpm/5 min/4 °C), the sediment was discarded and the protein content of the supernatant was determined by the Folin–Lowry method (Lowry et al., 1951), separated in aliquots ant stored at −20 °C until the time of usage.

2.4. Indirect immunofluorescence test

Three IIF test were used: (a) IFI-LVC commercial kit; (b) IIF test with *T. caninum* antigen (IIF-Tc) and (c) IIF test with *L. chagasi* antigen (IIF-Lc), the reactions of which were previously standardized. The procedure with the kit followed the manufacturer's instructions and the in house tests were performed as briefly described as follows: about 10 μL of antigen suspension was placed in slides and after drying was added of 10 μL of serum diluted 1:2 from 1:40 until 1:640 dilution. After incubation (37 °C/30 min), the slides were washed in PBS (pH 7.2) and after drying, the conjugate anti-canine IgG conjugated to fluorescein isothiocyanate previously titrated, was added. A new incubation and washing were conducted as described above. Then, the slides were mounted with buffered glycerol and examined using an epifluorescent microscope (BX40 – Olympus®). Titers equal or above 1:40 were considered positive.

2.5. Enzyme linked immunosorbent assay

As for IIF, we used three protocols of immunoenzymatic assay: (a) EIE-LVC commercial kit; (b) ELISA with *T. caninum* antigen (ELISA-Tc) and (c) ELISA with *L. chagasi* antigen (ELISA-Lc). The procedure with the kit followed the manufacturer's instructions and the in house tests were conducted as follows: 100 μL of 10 μg/mL antigenic solution, diluted in 0.06 M carbonate–bicarbonate buffer (pH 9.6), were adsorbed to the wells of polystyrene plates (Imuno Maxisorp – NUNC®) and incubated overnight at 4 °C. Then, they were washed four times (PBS + 0.05% Tween 20), and the serum samples diluted 1:40 in 1% skim milk solution (PBS 0.01 M, containing 0.05% Tween 20) were placed and the plates were incubated (37 °C/45 min). After two washings the anti-canine IgG conjugated to peroxidase previously titrated was added. The reaction was revealed using 3,3′,5,5′-tetramethyilbenzidine (TMB) and the optical density (OD) was measured in absorbance (405 nm) using a microplate ELISA spectrophotometer (Genious – Tecan®). The cut-off of the EIE-LVC test was defined following the manufacturer's instructions considering the mean of the OD of the negative controls multiplied by 2. The cut-off of the in house tests was established based on the ODs and defined by the Receiver Operating Characteristic Curve (ROC Curve). The performance of these reactions was compared through the area under the curve (AUC) with its respective confidence interval of 95% using the *MedCalc* 11.6.0.0 software. Samples with ODs above the cut-off were considered positive.

2.6. Dual-Path Platform Immunochromatographic test

Following the manufacturer's recommendations, 5 μL of serum sample were placed in well #1 of the kit device and 2 drops of buffer (also supplied in the kit) were added. After 5 min, 4 drops of the same buffer were placed in well #2, and after 20 min, the reading was performed as recommended by the manufacturer. The results were considered positive when two lines, one in the control area and the other in the area with the sample under test were visualized. The visualization of just one line indicates a negative result.

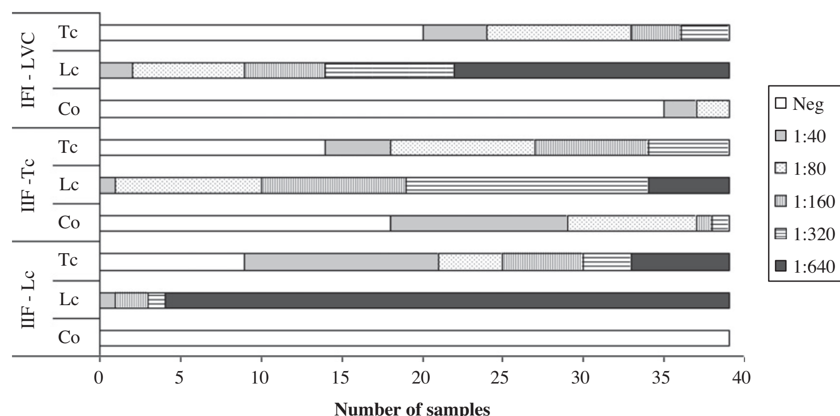

Fig. 1. Distribution of the serological titers of the 117 serum samples of dogs infected by *Trypanosoma caninum* (Tc group), *Leishmania chagasi* (Lc group) and not infected control animals (Co group), obtained in indirect immunofluorescence tests with IFI-LVC (IFI-Leishmaniose visceral canina, Bio-Manguinhos/FIOCRUZ) commercial kit and with IIF-Tc (*T. caninum* antigen) and IIF-Lc (*L. chagasi* antigen) in house tests. (Neg., negative result.)

2.7. Data analysis

The description of the IIF and DDP results was performed by the absolute and relative frequency of positives.

The validation parameters (sensitivity and specificity) of all the tests conducted in this study were supplied by the *MedCalc* 11.6.0.0 software. The parameters of the commercial kits (IFI-LVC, EIE-LVC and DPP) were compared considering the total number of samples and excluding the group of samples from dogs infected by *T. caninum*.

Chi-squared (χ^2) was used to compare specificity value of commercial tests (IFI-LVC, EIE-LVC and DPP) excluding the group of dogs infected with *T. caninum*. The differences were considered statistically significant when *P*-values <0.05.

The total concordance (positive + negative) was calculated between the commercial tests (IFI-LVC, EIE-LVC and DPP) and between in house tests for diagnosis of *T. caninum* infection (IIF-Tc × ELISA-Tc) and LVC diagnosis (IIF-Lc × ELISA-Lc).

3. Results

In the test with the IFI-LVC kit we found 48.7% (n = 19), 100% and 10.3% (n = 4) of positive samples for the groups Tc, Lc and Co respectively, with titers of 1:40–1:320 for the Tc group and of 1:40–1:640 for the Lc group. Two samples of the Co group were reactive at dilution 1:40 and two at 1:80.

In the in house tests, initially using *T. caninum* antigens (IIF-Tc) we found 64.1% (n = 25), 100% and 53.8% (n = 21) of positive samples for the groups Tc, Lc and Co respectively, with titers of 1:40–1:320 for the Tc group and of 1:40–1:640 for the Lc group. The Co group presented titers of 1:40–1:320. With *L. chagasi* antigen (IIF-Lc), the Tc group presented 77% (n = 30) of positive samples with titers of 1:40–1:640 and the Lc group presented 100% positivity with titers of 1:640 in about 90% of the samples. In this test, 100% of the samples of the Co group were negative.

The distribution of the serological titers found in each group is shown in Fig. 1.

In the ELISA with the EIE-LVC kit, a cut-off value of 0.130 was obtained, and 48.7% (n = 19), 100% and 15.4% (n = 6) of positive samples were found for groups Tc, Lc and Co respectively. To in house test, using *T. caninum* antigen (ELISA-Tc), the cut-off obtained by the ROC Curve was 0.212, with an area under the curve of 0.590 (CI 95% = 0.496–0.680). With this cut-off, 94.9% (n = 37), 100% and 28.2% (n = 11) positive samples were found for groups Tc, Lc and Co, respectively. The cut-off established in the tests employing *L. chagasi* antigen (ELISA-Lc) was 0.282 and the area under the curve was 0.990 (0.950–0.999), which is equivalent to 12.8% (n = 5) and 100% of positive samples respectively for groups Tc and Lc, and 100% negative results for group Co. The distribution of the ODs and the cut-offs established for the different ELISA tests are shown in Fig. 2.

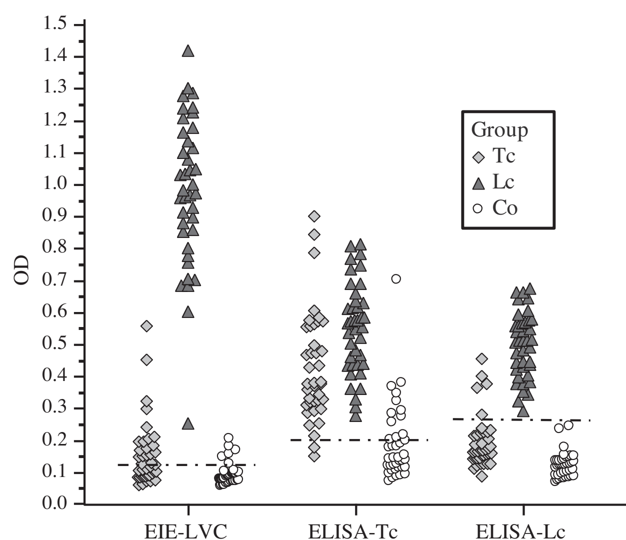

Fig. 2. Distribution of optical densities (OD) of 117 serum samples obtained from dogs infected by *Trypanosoma caninum* (Tc group), *Leishmania chagasi* (Lc group) and not infected control animals (Co group) in immunoenzymatic assays: EIE-LVC (EIE-Leishmaniose visceral canina kit, Bio-Manguinhos/FIOCRUZ); ELISA-Tc (*T. caninum* antigen); ELISA-Lc (*L. chagasi* antigen). A cut-off value (dotted line) was established as described in Section 2.

When the rapid DPP test was employed, 5.1% (n = 2) and 100% of positive samples were found for groups Tc and Lc, respectively, and the Co group presented 100% of negative results.

The validation parameters of the tests conducted in the present study are shown in Table 1.

No difference was found in the specificity EIE-LVC (χ^2 = 2.87, *P*-value = 0.089) and DPP (χ^2 = 0.05, *P*-value = 0.818) when Tc group was excluding, however with IFI-LVC this value was significant (χ^2 = 4.36, *P*-value = 0.036).

The total concordance between IFI-LVC × EIE-LVC, DPP × EIE-LVC and DPP × IFI-LVC were 74%, 80% and 82% respectively. Between the in house tests (ELISA-Tc × IIF-Tc and ELISA-Lc × IIF-Lc) the total concordance were 74% and 77% respectively.

Table 1
Sensitivity and specificity of indirect immunofluorescence tests (IFI-LVC, IIF-Lc and IIF-Tc), immunoenzymatic assays (EIE-LVC, ELISA-Lc and ELISA-Tc) and immunochromatographic test (DPP) with sera from dogs with *Trypanosoma caninum*, *Leishmania chagasi* infection and healthy controls.

Tests	Sensitivity	Specificity	Sensitivity[a]	Specificity[a]	*P*-value
IFI-LVC	100 (91–100)	70.5 (59.1–80.3)	100 (91–100)	89.7 (75.8–97.1)	0.036
IIF-Lc	100 (91–100)	61.5 (49.8–72.3)	NE	NE	NE
IIF-Tc	64.1 (47.2–79)	23.1 (14.3–34)	NE	NE	NE
EIE-LVC	100 (91–100)	68 (56.4–78,1)	100 (91–100)	84.6 (69.5–94.1)	0.089
ELISA-Lc	100 (91–100)	93.6 (87.7–98)	NE	NE	NE
ELISA-Tc	94.9 (82.7–99.4)	35.9 (25.3–47.6)	NE	NE	NE
DPP	100 (91–100)	97.5 (91–99.7)	100 (91–100)	100 (91–100)	0.818

IFI-LVC – IFI-Leishmaniose visceral canina kit, Bio-Manguinhos/FIOCRUZ; IIF-Tc – *T. caninum* antigen in house test; IIF-Lc – *L. chagasi* antigen in house test; EIE-LVC – Leishmaniose visceral canina kit, Bio-Manguinhos/FIOCRUZ; ELISA-Tc – *T. caninum* antigen in house test; ELISA-Lc – *L. chagasi* antigen in house test; DPP – immunochromatographic test; NE – not evaluated.
[a] Parameters evaluated excluding the samples of the infected dogs by *T. caninum*. IC = 95%.

4. Discussion

In Brazil, the diagnosis of canine visceral leishmaniasis (CVL) used in control programs is based on serological tests (Ministério da Saúde, 2006). The overlapping of areas where closely related etiological groups are present is a limiting aspect of the interpretation of the serological data due to possible serologic cross-reactions (Caballero et al., 2007). Descriptions of the presence of *T. caninum* in areas endemic for CVL led us to evaluate the possibility of serological cross reactivity between those infections. Accuracy indices of the diagnostic tests may vary as a function of the group studied and the criteria adopted to establish positive and negative gold-standards (Lucey and Weina, 2008). The infection by *L. chagasi* or *T. caninum* was proved through determination of the etiological agent in all the dogs of the Lc and Tc groups, respectively, thus supporting the results.

When we observe the group of animals infected by *T. caninum*, we verify that at least nine animals were negative to IIF and three to ELISA, regardless of the test. This result suggest that *T. caninum* may be little immunogenic for dogs, at least in the induction of humoral immune response. In the same way, the general condition of the animal is not affected during infection by *Leishmania braziliensis* (Dantas-Torres, 2007). *T. caninum* has been most frequently isolated from healthy dogs (Silva et al., 2011; Almeida et al., 2011). If this hypothesis is true, the serological test would have little applicability for the diagnosis and tracking of this parasite.

On the other hand, the results obtained with the Co group, where 53.8% and 28.2% of the samples presented reactivity to IIF-Tc and ELISA-Tc tests respectively called our attention. This result may be related to the poor knowledge on the natural canine infection by *T. caninum* and the sensitivity and specificity of the methods used to define the Co group. This group was formed with dogs from Rio de Janeiro municipality, where the presence of *T. caninum* has been reported in different occasions (Madeira et al., 2009a; Pinto et al., 2010; Silva et al., 2011), and thus, the possibility of infection by *T. caninum* cannot be disregarded. This hypothesis is supported by the results of other tests of the Co group, mainly when *L. chagasi* antigen was employed.

The use of homologous antigens is considered a positive factor in the performance of diagnostic tests (Baleeiro et al., 2006). In the present study, the IIF-Tc and ELISA-Tc presented low specificity values, which reflects a poor performance of the serological methods for *T. caninum* diagnosis. A curious fact, with possible implications for CVL control actions, is that this group presented a higher frequency of reactors in IIF-Lc than in IIF-Tc.

The animals infected by *L. chagasi* were 100% reactive in the tests that employed homologous antigens (*L. chagasi*) or heterologous (*T. caninum*), demonstrating the serological cross-reactivity between both agents. In this group, the ELISA-Lc presented higher specificity than EIE-LVC, although the same result was not obtained for IIF-Lc. This can be explained by the nature of the assays (ELISA and IIF), which react with different antigens. The IFI-LVC and EIE-LVC tests, both routinely used for VL surveillance in Brazil presented sensitivity of 100% and specificity around 70% (Ministério da Saúde, 2006). The specificity of these tests was higher when the group of animals infected by *T. caninum* was excluded from the analysis. The sensitivity and specificity indices of DPP remained high, regardless the inclusion or exclusion of the animals infected by *T. caninum*. These results support the choice of this rapid test as an alternative for CVL diagnosis (Grimaldi et al., 2012). Only two animals infected by *T. caninum* were seroreactive to DPP. Both dogs were from areas with VL, although the laboratory examinations did not identify mixed infection.

A hundred percent of the dogs infected by *L. chagasi* would be properly diagnosed by the tests currently employed by the Brazilian Health Ministry and designated for euthanasia. False negative results could represent a problem in leishmaniasis control, but they were not detected in the present study. However, considering EIE-LVC as a screening test and IFI-LVC as a confirmatory test, 23% ($n = 9$) of the animals infected by *T. caninum*, would be erroneously diagnosed as CVL.

Further studies are needed for better understanding the natural infection by *T. caninum* in domestic dogs and determining the more adequate tools for the diagnosis and tracking of this parasite.

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