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Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations

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Abstract

Three serological methods, indirect fluorescent immunoassay (IFI), enzyme-linked immunosorbent assay (ELISA) and direct agglutination test (DAT) that are commonly employed in the diagnosis of canine visceral leishmaniasis (CVL), have been assessed. A total of 234 domestic dogs, drawn from an area in the municipality of Belo Horizonte, Minas Gerais, Brazil, endemic for visceral leishmaniasis, were submitted to clinical and parasitological examinations and serological assay. Sera collected from confirmed non-infected dogs ($n = 20$), and from dogs with other parasitic diseases including *Trypanosoma cruzi* ($n = 7$), *Leishmania braziliensis* ($n = 5$), *Toxoplasma gondii* ($n = 5$) and *Ehrlichia canis* ($n = 3$), were also included in the study. IFI presented a lower sensitivity (72%) than ELISA (95%), although the specificities of these assays were low (52 and 64%, respectively) and both exhibited cross-reactivity with sera from dogs infected with *T. cruzi*, *L. braziliensis* and *E. canis*. In contrast, DAT exhibited a high sensitivity (93%) and a high specificity (95%) and cross-reacted with only one serum sample derived from an *E. canis*-infected dog. The reproducibilities of the ELISA and DAT assays were excellent, whilst that of IFI was considered to be acceptable. The results produced by ELISA and DAT were in complete agreement, those between ELISA and IFI were at an acceptable level of agreement, whilst the concurrence between the IFI and DAT results were either acceptable or poor depending on the clinical conditions of the group of dogs examined. Since there is no readily accessible method for the diagnosis of CVL that offers 100% specificity and sensitivity, the choice of technique employed must depend on the aim of the investigation.

Keywords: Visceral leishmaniasis; Serological assays; Dogs; Diagnosis

1. Introduction

In epidemiological terms, human visceral leishmaniasis (HVL) may be regarded as an anthroponosis when the etiological agent is *Leishmania donovani* and a zoonosis when the disease is caused by *L. infantum*

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(syn. *L. chagasi*). In the latter case, the dog is considered to be the main domestic reservoir of the agent, and canine visceral leishmaniasis (CVL) has been found to be present in 50 of the 88 countries in which HVL is endemic.

The main foci of HVL are located in Brazil, in the Mediterranean region, and in China (Alvar et al., 2004). In Brazil, HVL is considered to be a zoonosis that initially became established in rural areas of the country. Environmental degradation and especially the lack of sanitation, that has been associated with the recent migration of rural populations to urban suburbs is believed to have contributed to the urbanisation of the disease. Furthermore, the ready adaptation of the insect vectors (phlebotomine sand flies) to their modified environment has contributed to the rapid emergence of urban foci (Marzochi and Marzochi, 1997).

A number of studies have considered the role of the domestic dog in the transmission of HVL (Silva et al., 2001; Cortada et al., 2004). The most significant finding to emerge from such investigations is that dogs infected with CVL may appear to be in perfect health or, alternatively, they may present minor, moderate or severe symptoms of the disease (Mancianti and Meciani, 1988). The successful diagnosis of CVL may be difficult since the clinical signs of the disease are varied and non-specific. It is estimated that around 50% of dogs infected with CVL show no clinical signs of the disease (Abranches et al., 1991) but constitute infection sources for the insect vector (Adler and Theodor, 1932; Alvar et al., 1994; Molina et al., 1994). Since CVL-infected dogs can remain asymptomatic for long periods of time, infection prevalence in an endemic area may often be underestimated when asymptomatic animals are not included in groups to be considered for control measures.

The parasitological techniques commonly employed in the direct detection of parasites in biological samples derived from suspect animals are invasive and of relatively low sensitivity (Schallig et al., 2002). Serological assays may, on the other hand, provide realistic alternative tools in epidemiological surveys and in the diagnoses of CVL. In this context, indirect fluorescent immunoassay (IFI), enzyme-linked immunosorbent assay (ELISA) and the direct agglutination test (DAT) have been the methods most often employed in the diagnosis of CVL since they present satisfactory indices of sensitivity, although with variable specificity depending on the antigen used (Alvar et al., 2004).

The aim of the present study was to apply the serological assays IFI, ELISA and DAT to the diagnosis of dogs, from CVL-endemic areas, exhibiting different clinical manifestations of the disease, and to compare the results obtained with respect to sensitivity, specificity, reproducibility and inter-assay agreement.

2. Materials and methods

Details of the project were submitted to and approved by the Ethics Committee of the Oswaldo Cruz Foundation (FIOCRUZ). The protocols employed for the collection of biological samples from study animals followed those approved by the Ethics Committee for Animal Experimentation CEUA-FIOCRUZ (P 0119-02) and adopted by the Brazilian College of Animal Experimentation (COBEA). When appropriate, written consent was obtained from the owner of each animal prior to its inclusion in the study group.

2.1. Study animals

The study group consisted of 234 domestic dogs (*Canis familiaris*), comprising males and females of different breeds and ages, drawn from an area in the municipality of Belo Horizonte, Minas Gerais, Brazil, that is endemic for visceral leishmaniasis. Serum samples presenting negative results in parasitological assays were obtained from 20 healthy animals that had been bred and maintained under defined conditions in the experimental kennels of the Universidade Federal de Minas Gerais (Belo Horizonte, Minas Gerais, Brazil) in order to form a negative control group. For cross-reaction tests, further serum samples were obtained from animals that had been experimentally infected with the pathogens *Trypanosoma cruzi* (7 dogs), *L. braziliensis* (5 dogs), *Toxoplasma gondii* (5 dogs) and *Ehrlichia canis* (3 dogs).

2.2. Clinical and parasitological examinations

Each dog was examined carefully for the characteristic clinical signs of *Leishmania* infection, and clinical epidemiological forms containing general information about the animal (i.e., breed, gender, age, origin, possible trips to other locations, etc.) were completed.

In order to obtain samples for parasitological examinations, bone marrow punctures and ear skin biopsies were carried out on each animal, and aliquots of bone marrow cells were cultured in Novy-MacNeal-Nicolle-liver infusion tryptose (NNN-LIT) medium. Slides of Giemsa-stained bone marrow smears and cells, and imprints of skin fragments were examined for the presence of amastigote forms of *Leishmania* sp.

2.3. Serological assays

Samples of total peripheral blood were collected from 234 dogs and subsequently coded in a random manner such that the serological assays could be carried out blind. In order to evaluate the reproducibility of the assays under investigation, *ca*. 10% of the samples were processed in duplicate, each under a different and randomly selected code.

Canine IFI-Leishmaniasis Kits (Bio-Manguinhos/FIOCRUZ), with antigen prepared from *L. infantum* (syn. *L. chagasi*), were used according to the manufacture's instructions in the IFI assays. Serum samples were diluted from 1:40 (the cut-off value) to 1:640 in phosphate buffered saline (PBS). ELISA assays employed Canine Leishmaniasis EIA Kits (Bio-Manguinhos/FIOCRUZ) containing antigen prepared from parasites of the *L. donovani* complex. Serum samples were processed according to the manufacturer's instructions, and the cut-off value of absorbance was taken as >0.05 OD.

The protocol employed in the DAT assay was that described by Oskam et al. (1996). The Dog-DAT® antigen employed was derived from promastigotes of *L. donovani* and was kindly provided by KIT Biomedical Research, Amsterdam, The Netherlands, in a stable, lyophilised form. Serum samples were serially diluted from 1:100 to 1:102,400 with physiological saline solution (0.9% sodium chloride) containing 1.56% of β-mercaptoethanol, and 50 μl portions pipetted into wells of a micro-titre plate. An aliquot (50 μl) of antigen solution (concentration equivalent to 5×10^7 promas-promastigotes/ml) was added to each well, the plate was covered, gently shaken and incubated at room temperature for 18 h. The antibody titre was determined and the cut-off value taken as >1:400.

2.4. Statistical analysis

Statistical analyses of the data were performed using the STATA statistical package (version 6.0). Values of the sensitivity, specificity and accuracy of the assays were determined on the basis of the cross distribution of positive and negative results in a 2×2 contingence table. Frequency differences of sensitivity and specificity in each test or in each clinical group were analysed using a χ^2-test: differences were considered statistically significant for p values <0.05. The reproducibility of duplicate assays was assessed from the Youlden index (at the 95% confidence interval), whilst concurrence between tests was assessed from the Kappa index of agreement (at the 95% confidence interval).

3. Results

A total of 234 dogs underwent detailed parasitological examinations and 112 were shown to be positive according to at least one of the tests: these animals were considered to represent the true positive (TP) group for the purposes of this study. Of the 234 serum samples submitted to serological assay, 148 were considered positive according to ELISA, 138 according to DAT, and 121 according to IFI. With regard to the TP group, the ELISA assay gave positive results for 108 (96%) of the 112 animals, DAT showed positive results for 104 (93%), and IFI exhibited positive results for only 81 (72%). Animals from endemic areas that presented negative parasitological examinations and negative serological assays ($n = 35$) were considered to be non-infected dogs. Healthy dogs ($n = 20$) that had been bred and maintained in an experimental kennel under defined conditions were considered as the true negative (TN) group: all of the assays produced negative results for these animals. Based on the serological results performed on TP and TN dogs, the sensitivity, specificity and accuracy of each method could be calculated, and the results are presented in Table 1. With respect to agreement between blind duplicate assays, ELISA and DAT showed very high reproducibility, whilst a much lower index of agreement was attained for duplicate samples assayed by IFI (Table 1).

In order to assess the cross-reaction of the serological assays, serum samples from 20 dogs that had been infected with other pathogens were tested. IFI showed cross-reaction in 11 samples, ELISA in eight samples and DAT in 1 sample. After the inclusion of these data, the specificities determined for ELISA and IFI showed significant reductions to 60 and 45%, respectively, whilst that of DAT remained statistically unaltered (Table 2).

On the basis of clinical examinations, taken together with the above findings, the 234 animals derived from CVL-endemic areas could be divided into four distinct groups: (i) asymptomatic dogs (AD; $n = 40$; 17%) with no clinical signs of the disease but presenting at least one positive serological and or parasitological test; (ii) oligosymptomatic dogs (OD; $n = 134$; 57%) presenting one to three clinical signs characteristic of the disease including opaque bristles and/or localized alopecia, and/or moderate loss of weight; (iii) symptomatic dogs (SD; $n = 25$; 11%) presenting more than three clinical signs characteristic of the disease; and (iv) non-infected dogs (NI; $n = 35$; 15%) with no clinical signs of the disease and negative serological and parasitological tests (Table 3).

Table 1
Sensitivity, specificity, accuracy and reproducibility of ELISA, DAT and IFI assays performed on sera of dogs from a CVL-endemic area

	ELISA		DAT		IFI	
	Positive (108[a]; 0[b])	Negative (4[a]; 20[b])	Positive (104[a]; 0[b])	Negative (8[a]; 20[b])	Positive (81[a]; 0[b])	Negative (31[a]; 20[b])
Specificity (CI 95%)	100% (100–100)		100% (100–100)		100% (100–100)	
Sensitivity (CI 95%)	96% (92–99)		93% (89–97)		72% (65–80)	
Accuracy (CI 95%)	97% (93–100)		95% (90–98)		79% (69–84)	
Reproducibility Youlden index (CI 95%)	0.95 (0.92–0.99)		0.92 (0.89–0.97)		0.72 (0.65–0.80)	

[a] TP ($n = 112$); TP, animals true positive for CVL.
[b] TN ($n = 20$); TN, animals true negative for CVL.

Table 2
Results of cross-reaction tests on sera from dogs infected with different pathogens

Pathogen (*n*)	ELISA		DAT		IFI	
	Positive	Negative	Positive	Negative	Positive	Negative
Trypanosoma cruzi (7)	04	03	0	07	07	0
Leishmania braziliensis (5)	03	02	0	05	02	03
Toxoplasma gondii (5)	0	05	0	05	0	05
Ehrlichia canis (3)	01	02	01	02	02	01
Total (20)	08	12	01	19	11	09
Specificity (CI 95%)	60% (39–82)		95% (85–100)		45% (23-67)	

The levels of concurrence between the serological tests, analysed 2 × 2, was generally good with average percentage agreements taken across the AD, OD and SD groups of 94% for ELISA × DAT, 81% for ELISA × IFI, and 80% for IFI × DAT. The AD group showed the lowest concurrence between the assays, whilst the highest was observed in the SD group (Table 4).

4. Discussion

Parasitological tests for the diagnosis of CVL are invasive and offer low sensitivity compared with serological assays. Unfortunately, however, a number of these simple alternative assays suffer from problems of cross-reactivity with other diseases (Boelaert et al., 1999) giving rise to reduced specificity. In evaluating the validity of diagnostic methods, such problems need to be taken fully into account in order to obtain reliable and accurate results. In this respect, one of the major difficulties in the assessment of diagnostic methods for CVL is that there is no reliable gold standard against which alternative assays may be compared. This problem could be minimised by using a latent class analysis (LCA) model (Boelaert et al., 2004) that estimates disease prevalence, and sensitivity and

Table 3
Results of parasitological and serological tests carried out on dogs of different clinical groups

Clinical condition	Total of samples	Positive results (%)			
		ELISA (%)	DAT (%)	IFI (%)	Parasitological examination (%)
Asymptomatic	40	34 (85)	30 (75)	34 (85)	19 (48)
Oligosymptomatic	134	95 (71)	89 (66)	71 (53)	76 (57)
Symptomatic	25	19 (76)	19 (76)	16 (64)	17 (68)
Non-infected	35	0	0	0	0
Total	234	148	138	121	112

Table 4
Concurrence between ELISA, DAT and IFI assays according to the different clinical groups of the dogs examined

	ELISA × DAT	ELISA × IFI	IFI × DAT
Asymptomatic dogs ($n = 40$)			
Agreement (%)	85%	70%	65%
Kappa index	0.54	0.18	0.08
CI 95%	0.22–0.85	−0.27 to −0.08	−0.34–0.186
Oligosymptomatic dogs ($n = 134$)			
Agreement (%)	95%	79%	78%
Kappa index	0.90	0.57	0.54
CI 95%	0.79–0.97	0.44–0.70	0.40–0.68
Symptomatic dogs ($n = 25$)			
Agreement (%)	100%	88%	88%
Kappa index	1.00	0.72	0.72
CI 95%	1.00–1.00	0.43–1.00	0.43–1.00

specificity of all of the diagnostic tests. The application of the LCA model in biomedical research is, however, still somewhat limited. In the present study, we have taken positive parasitology as the criterion of positive detection of CVL in an animal since parasitological examination is the most accurate test when positive. The criteria for negative CVL animals were negative parasitology together with negative results in IFI, ELISA and DAT assays.

Among the serological techniques that are commonly employed in the diagnosis of CVL, IFI is considered to be the standard. On the other hand, ELISA and DAT have been reported to be more sensitive and practical for epidemiological surveys (Evans et al., 1990; Rami et al., 2003), and the results of the present study confirm the lower sensitivity of IFI.

All three serological assays exhibited 100% specificity when assessed using sera from dogs presenting positive or negative parasitology for leishmaniasis alone. However, in cross-reaction tests involving serum samples derived from dogs infected with different pathogens commonly encountered in Brazil, DAT showed a positive reaction against a single sample from an animal infected with *E. canis* whilst ELISA and IFI showed reactions against 8 and 11 of the samples, respectively. These data demonstrate that when the validation experiment is extended to include serum samples from dogs infected with other pathogens, only the specificity index of DAT remains at a high level (95%) whilst ELISA and IFI present, respectively, much reduced specificities of 60 and 45%, values that are not significantly different from each other.

These findings are in accord with previous reports in which IFI and ELISA have been shown to cross-react when serum samples from dogs infected with *T. cruzi* and *L. braziliensis* were analysed. Thus Costa et al. (1991) reported 75% cross-reaction with cutaneous leishmaniasis and 83% cross-reaction with Chagas disease following IFI assays of canine serum samples, whilst ELISA was shown to give false positive reactions with both human and canine serum in the presence of other bacterial and parasitic diseases, particularly *T. cruzi* (Roffi et al., 1980; Badaró et al., 1986; Mancianti et al., 1996). On the other hand, in assessing the application of the lyophilised antigen Dog-DAT in CVL diagnosis, Oskam et al. (1996) found no cross-reaction with serum samples from dogs infected with *Babesia* and *Leptospira*, and reported that the DAT assay attained 100% sensitivity and 98.8% specificity.

With respect to the diagnosis of HVL, comparative studies employing IFI, ELISA and DAT assays with human sera showed that DAT was as sensitive and specific as IFI when samples from humans with trypanosomiasis infection were not included in the validation (Harith et al., 1987). When such samples were included, however, the results obtained using DAT were similar to those of ELISA, and both techniques were less specific than IFI. Additionally, El Safi and Evans (1989) demonstrated that DAT was at least as good as ELISA for the diagnosis of HVL, whilst Andrade et al. (1987) reported that the results obtained with DAT and IFI were similar.

In the present study, good general concurrence was observed between the results obtained using IFI, ELISA and DAT, although the specific level of agreement varied according to the clinical condition of the group of dogs examined. Thus, the three serological assays gave results that were in perfect agreement between all comparison pairs when sera from non-infected dogs from both CVL-endemic and non-endemic areas were assayed. Moreover, excellent concurrence was observed between the results of ELISA and DAT when sera

samples from OD animals were analysed, whilst the agreements between ELISA and IFI, and between IFI and DAT, were considered satisfactory. The lowest concurrence was found for the AD group: in this case, although the level of agreement between ELISA and DAT was satisfactory, those between ELISA and IFI, and between IFI and DAT, were weak. It is of interest to note that, according to Dye et al. (1993), even if a highly efficient serological assay is employed, some 20% of cases of CVL infection may remain undetected especially in animals at the stage of disease incubation or seroconversion. During these phases, the levels of circulating antibodies can be highly variable thus giving rise to unpredictable serological results, and this may explain the poor level of agreement between the assays when asymptomatic dogs are assessed.

In a serological survey of leishmaniasis in Turkey, Ertabaklar et al. (2005) reported that the level of agreement between DAT and IFI was 94.4%, and that *ca*. one-third of the seropositive dogs were asymptomatic. The difference between these data and the results of the present study may result from the different assay protocols employed and the type of antigen used, since these two elements impact upon specificity, sensitivity and reproducibility indices (Sundar and Rai, 2002).

Little information has been published concerning the reproducibility of diagnostic assays, although Rosário et al. (2005) reported levels of reproducibility that varied between 0.74 and 1.0 for IFI and ELISA according to the antigen used. In the present study, the ELISA and DAT assays were shown to be highly reproducible, whilst the reproducibility of IFI was considered only to be acceptable indicating, perhaps, the lower reliability of this assay.

The role of the dog as the main domestic reservoir of the agent of HVL implies that the use of accurate diagnostic tools with which to detect CVL is crucial in the implementation of measures designed to control the spread of the disease. Recently, molecular methods such as the polymerase chain reaction (PCR) have been developed for the identification of CVL, and these offer high sensitivity and specificity (Lachaud et al., 2002; Cortes et al., 2004). However, such diagnostic tools are currently not practical for use in large scale screening, as required in epidemiological surveys, by reason of potential cost and applicability issues (Reed, 1996). In particular, the PCR technique needs to be improved in regard to its application in developing countries by reducing the operational costs involved and increasing the simplicity of the method (Gontijo and Melo, 2004). Currently, therefore, sero-diagnostic tools remain the most appropriate and practical choice for use in campaigns aimed at the control of HVL. Very recently, Silva et al. (2006) recommended a change from IFI to DAT for the sero-diagnosis of CVL. In support of this suggestion, the results of the present investigation indicate the clear superiority of DAT and ELISA compared with IFI (applied under the conditions described) in a number of specific aspects.

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