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Short communication

Differentiation between canine cutaneous and visceral leishmaniasis by the detection of immunoglobulin G specific for *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *chagasi* antigens using flow cytometry

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Abstract

Flow cytometry employing *Leishmania* (*L.*) *chagasi* (Lc) and *L.* (*Viannia*) *braziliensis* (Lb) antigen was used to establish the differential diagnosis between visceral (VL) and cutaneous leishmaniasis (CL) in dogs. Flow cytometry permitted the detection of *Leishmania*-specific immunoglobulin G in sera from 19 dogs: nine with CL and 10 with VL. A significant difference in the percentage of positive staining was observed in sera from dogs with CL between the homologous antigen (69% for Lb) and the heterologous antigen (42% for Lc). However, this difference was not significant in sera from dogs with VL (61% for Lb and 73% for Lc). No significant staining was observed in control sera (0.6% for Lb and 0.4% for Lc) consisting of samples from healthy dogs, or in the group with sporotrichosis (1.8% for Lb and 1.5% for Lc), a differential diagnosis of CL. The results suggest that flow cytometry might be useful for the differentiation between CL and VL in dogs, with practical applications in areas where the two infections overlap.

Keywords: Flow cytometry; *Leishmania*; Canine leishmaniasis; IgG

1. Introduction

Leishmaniases are parasitic diseases caused by protozoans of the genus *Leishmania*, which can be present in the cutaneous (cutaneous leishmaniasis, CL) or visceral (visceral leishmaniasis, VL) form. Both forms are considered to be public health problems in Brazil and in countries of the New World. The role of dogs as a reservoir in the transmission cycle of VL has been well documented. However, their importance in the transmission of CL is still a matter of discussion. In the transmission of VL, mongrel dogs are considered to be the main source of infection in urban areas, where canine cases are usually more prevalent and precede human cases (Ministério da Saúde do Brasil, 2006). These animals serve as a reservoir of parasites and develop canine VL, which is characterized by hepatosplenomegaly, lymphadenopathy, alopecia, apathy,

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and severe weight loss (Bettini and Gradoni, 1986). As a control screening protocol in Brazil, dogs with an antibody titer higher than 1:40, measured by an indirect fluorescent antibody test (IFAT, Biomanguinhos, Brazil), are considered to be positive for canine VL and are recommended to be removed from the endemic area (Ministério da Saúde do Brasil, 2006). Questionnaires regarding the elimination of dogs without definitive confirmation of their role as a source of infection in CL emphasize the importance for the availability of more sensitive and specific techniques able to distinguish VL from CL and other diseases (Falqueto et al., 1986; Reithinger et al., 2003; Ryan et al., 2003). At present, the diagnostic methods most frequently used, such as IFAT and enzyme immunoassays (ELISA), do not meet these requisites. Besides there are a lot of cross-reactions with other infectious diseases, one of them is sporotrichosis which has become epidemic in the Rio de Janeiro's State and is caused by *Sporothrix schenckii* (Schubach et al., 2006). The epidemiological, clinical and histopathological features of sporotrichosis are similar to those of canine CL and false-positive results were observed when serum from dogs with sporotrichosis was tested for CL by IFAT (Dos Santos et al., 2007).

Rocha et al. (2002) described a method for evaluation of the levels of antibodies against live promastigotes of *Leishmania* (*Viannia*) *braziliensis* by flow cytometry (FCM). These authors combined the practicality and high sensitivity (95%) of FCM with conventional immunodiagnosis. After demonstrating the possible differentiation of the humoral response between patients with and without lesions (which is not possible with IFAT), the authors proposed the use of FCM for serological assays in human CL. Recently, Carvalho Neta et al. (2006) have used FCM for the determination of anti-*L.* (*L.*) *chagasi* antibody levels in serum samples from dogs with VL. The authors showed that the technique was able to identify cases of canine VL with 96% sensitivity and 100% specificity.

The objectives of the present study were to determine the usefulness of FCM in the detection of IgG specific for *Leishmania* promastigotes in serum samples from naturally infected dogs and in the differentiation between the cutaneous and visceral form of canine leishmaniasis.

2. Materials and methods

2.1. Dogs and sera

Serum samples obtained from 19 naturally infected dogs from different localities in the State of Rio de Janeiro were aliquoted at a proportion of 1:1 in glycerine (Merck, RJ, Brazil) and stored at −20 °C. In all animals, the diagnosis of CL ($n = 9$) or VL ($n = 10$) was established by isolation of the parasite in culture and subsequent characterization by determination of the isoenzyme profile of *L.* (*V.*) *braziliensis* or *L.* (*L.*) *chagasi*, respectively. In addition, all animals with VL and CL were negative for *S. schenckii*. Dogs with sporotrichosis ($n = 10$) were diagnosed by isolation of *S. schenckii* in culture of secretion or a skin lesion fragment and their parasitological diagnosis was negative for *Leishmania*. Three serum samples obtained from animals with mixed infection (isolation of *L.* (*V.*) *braziliensis* and *L.* (*L.*) *chagasi*) were also evaluated. In order to discriminate the response of recent or past infections, it were used animals where the parasitological examination had been conducted simultaneously with or without the withdrawal of serum. Confirming thus the presence of the parasite at the time of collection and discarding the possibility of past infection. So all animals used were infected and had the diagnosis of CL and VL confirmed through characterization of the species of *Leishmania*. The control group consisted of six serum samples from healthy dogs (culture, IFAT and ELISA negative for *Leishmania*). Both dogs with sporotrichosis and healthy dogs were from leishmaniasis-free areas.

2.2. Preparation of the parasite suspension

L. (*L.*) *chagasi* (MHOM/BR/74/PP75) and *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903) parasites were cultured in brain heart infusion medium (BHI) (VETEC, RJ, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab S/A, Brazil), 1% human urine, 200 U/mL penicillin (Sigma Chemical Co., St. Louis, MO) and 200 μg/mL streptomycin (FURP, Ministério da Saúde, Brazil) in a BOD incubator at 26 °C until the stationary growth phase (4 days for *L.* (*V.*) *braziliensis* and 5 days for *L.* (*L.*) *chagasi*). After refrigerated centrifugation at 7000 rpm for 10 min, the supernatant was discarded and the sediment containing the parasites was washed three times in PBS, pH 7.2, by refrigerated centrifugation (7000 rpm for 10 min). The parasite suspension was adjusted to 5×10^6 parasites/mL, 2% formalin was added (Merck), and the suspension was stored at 2–8 °C until the time of use.

2.3. Flow cytometry

The protocol for the detection of anti-*Leishmania* IgG in serum samples from infected dogs was based on the study of Rocha et al. (2002), with some modifica-

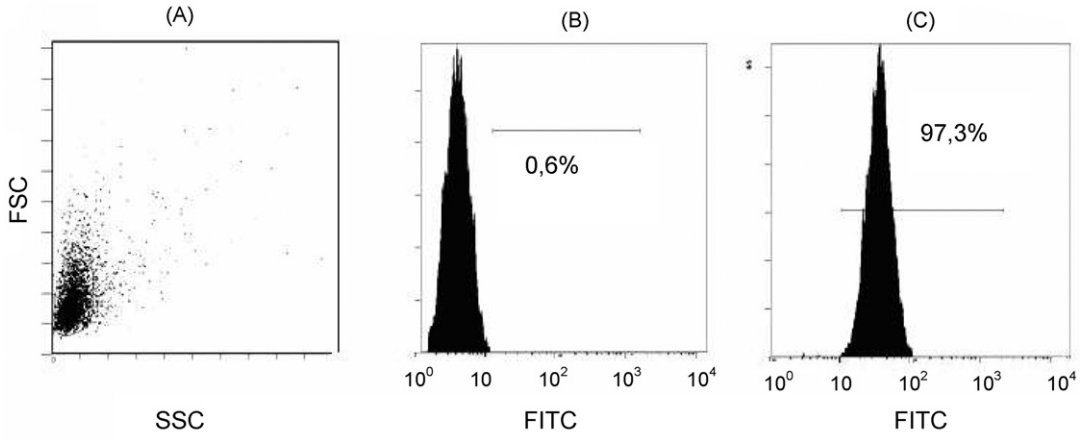

Fig. 1. Flow cytometry representation. (A) Size (FSC) and granularity profile (SSC). (B) IgG staining in serum from a healthy animal. (C) IgG staining in serum from a dog with leishmaniasis. The results are showed as the percentage of anti-IgG-FITC-stained parasites. *X*-axis in histograms B and C shows the fluorescence intensity of FITC.

tions. Fifty microliters of the serum sample diluted 1:1024 in PBS containing 10% FBS (PBS-FBS) and 50 μL of the parasite suspension (2×10^5 per well) were added to 96-well U-bottom polystyrene plates (Corning, NY). After incubation at 37 °C for 30 min, the mixture was washed twice in PBS-FBS by centrifugation at 2000 rpm for 5 min at 4 °C and the supernatant was discarded. Next, 50 μL fluorescein isothiocyanate-conjugated dog anti-IgG (intact molecule) (anti-IgG-FITC; Sigma Chemical Co.) diluted 1:200, 1:400 and 1:800 was added. After incubation for 30 min at 37 °C, the mixture was washed twice in PBS-FBS by centrifugation at 2000 rpm for 5 min at 4 °C and the sediment was resuspended in 200 μL paraformaldehyde (Sigma Chemical Co.) for fixation.

Two controls were used in each experiment: an internal control in which the parasites were not incubated with serum and a negative control consisting of a *Leishmania*-negative canine serum sample. Paired experiments were carried out, with each serum sample being incubated with *L.* (*V.*) *braziliensis* (Lb) and *L.* (*L.*) *chagasi* (Lc) antigens.

FCM acquisitions were performed using an EPICS ALTRA flow cytometer (Beckman Coulter, Hialeah, FL), equipped with a 488-nm argon laser and 525-nm filter for the detection of FITC emission. For each sample, a gate of the parasite population was acquired according to size (FSC) and granularity (SSC), thus removing all debris. A total of 10,000 events in the parasite gate were acquired and the result is reported as the percentage of anti-IgG-FITC-stained parasites (Fig. 1), using the Expo32 software (Beckman Coulter).

2.4. Statistical analysis

Statistical analysis was performed using the MedCalc 8.0 software, Mariakerke, Belgium. The *t*-test was used for comparison of means, with 95% confidence interval. Results with $p < 0.05$ considered that the two means were significantly different.

3. Results

3.1. Evaluation of the conjugate

After dilution assays of the anti-IgG-FITC conjugate, the titre of 1:400 was chosen because it presented the lowest staining percentage in the control groups. Mean staining was 2.7% for the negative control using *L.* (*V.*) *braziliensis* and 1.5% using *L.* (*L.*) *chagasi* and 0.3% for the internal control with each antigen.

3.2. Analysis of sera by flow cytometry

In all sera from dogs with CL, the percentage of positive staining for *Leishmania*-specific IgG was higher with the homologous antigen (*L.* (*V.*) *braziliensis*) than with the heterologous antigen (*L.* (*L.*) *chagasi*) (Fig. 2). However, only six of 10 sera from dogs with VL presented a higher percentage of positive staining for the *L.* (*L.*) *chagasi* antigen (data not shown).

Fig. 3 shows the mean percentage of staining found in each group, i.e., dogs with CL, VL, mixed infection and sporotrichosis and healthy dogs, for each antigen. In the CL group, the mean staining percentage was higher for the *L.* (*V.*) *braziliensis* antigen (69%) compared to

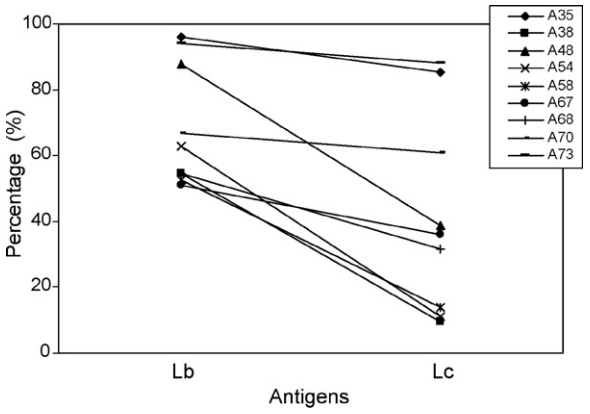

Fig. 2. Percentage of anti-*Leishmania* IgG staining in serum from nine dogs with cutaneous leishmaniasis (CL) using *L.* (*V.*) *braziliensis* (Lb) and L. (L.) chagasi (Lc) antigen. The legend insert correspond to an individual dog.

the *L.* (*L.*) *chagasi* antigen (42%) ($p = 0.03$). However, no significant difference in the percentage of positive staining between the heterologous *L.* (*V.*) *braziliensis* (61%) antigen and the *L.* (*L.*) *chagasi* antigen (73%) was observed in the VL group ($p = 0.21$). There was no expressive staining for the two antigens studied in the control group consisting of serum samples from healthy dogs (0.6% for Lb and 0.4% for Lc) or in dogs with sporotrichosis (1.8% for Lb and 1.5% for Lc). In the group of dogs with mixed infection ($n = 3$), the mean percentage of positive staining was 75% for the *L.* (*V.*) *braziliensis* antigen and 77% for the *L.* (*L.*) *chagasi* antigen, with no difference in the staining profile between the two antigens ($p = 0.90$).

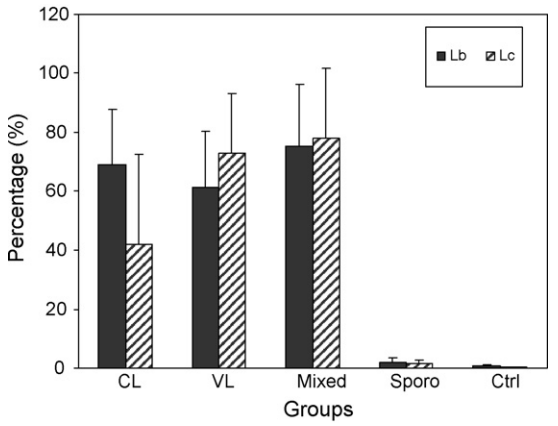

Fig. 3. Comparison of the mean percentage (±S.D.) of *Leishmania*-specific anti-IgG in sera from dogs with cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), mixed infection (mixed) and sporotrichosis (Sporo), and control animals (Ctrl) using *L.* (*V.*) *braziliensis* (Lb) and L. (L.) chagasi (Lc) antigen. Two means were significantly different ($p = 0.03$). See Section 2.

4. Discussion

The diagnosis of canine VL is a public health problem due to the similarity of VL to other infectious diseases, the presence of nonspecific histopathological alterations, the lack of a 100% specific and sensitive serological diagnostic test, and the high percentage of asymptomatic and oligosymptomatic dogs (Ministério da Saúde do Brasil, 2006). Since conventional serology is unable to discriminate between CL and VL, positive dogs are sacrificed irrespective of the *Leishmania* species involved. However, the role of dogs as a reservoir in CL has not been confirmed. As a consequence, there is no indication for euthanasia in these animals.

Furthermore, IFAT and ELISA present highly variable specificity and sensitivity, a fact that contributes to the maintenance of VL transmission in endemic areas due to the lack of identification and consequent culling of infected dogs. Berrahal et al. (1996) demonstrated 80% positivity by PCR in dogs that tested negative by both IFAT and ELISA, and suggested that most dogs had been exposed to *Leishmania*. In Greece, Leontides et al. (2002) showed that most dogs remained seronegative despite being infected (63% testing positive by PCR and only 12% by IFAT), generating a false estimate of the prevalence of infection.

Other more sensitive techniques have been used for the diagnosis of canine leishmaniases, including immunoblotting (Berrahal et al., 1996), ELISA with purified (Barbosa-de-Deus et al., 2002) or recombinant antigens (Scalone et al., 2002), PCR (Reithinger et al., 2003), and analysis of IgG subclasses (Deplazes et al., 1995; Rocha et al., 2006; De Andrade et al., 2007). Moreira et al. (2004) suggested that the failure of elimination of seropositive dogs as a control strategy might be related to the poor sensitivity and specificity of available serological methods and the consequent failure to identify infected dogs. The present results agree with those reported by other investigators (Rocha et al., 2002, Carvalho Neta et al., 2006) and suggest that FCM might be used for the diagnosis of leishmaniasis with high sensitivity and specificity.

The mean percentage of positive staining obtained for the internal and negative controls of the experiments did not exceed 0.3% and 2.7% (data not shown), respectively, suggesting that the conditions chosen for the test, i.e., fixed antigens and dilution of the sera and conjugate, did not affect the specificity or sensitivity of the assays. Cordeiro et al. (2001) discussed the possibility that the process of fixation of parasites

may induce conformational changes able to increase cross-reactions. In the present study, we did not test the use of live parasites or metacyclic forms since this type of antigen would render this method unsuitable as a diagnostic tool.

The choice of a homologous antigen might be an important tool to increase the sensitivity and specificity of serological tests in leishmaniasis. In the present study, we demonstrated the ability of FCM to differentiate between dogs naturally infected with *L.* (*V.*) *braziliensis* and *L.* (*L.*) *chagasi* using homologous and heterologous antigens. Other investigators observed that in ELISA using sera from dogs with CL the sensitivity and specificity were higher with the *L.* (*V.*) *braziliensis* antigen compared to the *L.* (*L.*) *chagasi* antigen (Ribeiro et al., 2007).

The present results suggest that the higher percentage of positive staining with the *L.* (*V.*) *braziliensis* in dogs with CL compared to the *L.* (*L.*) *chagasi* antigen might be used to distinguish between asymptomatic cases of CL and VL.

5. Conclusion

The present results indicate the possible application of FCM to the differentiation between CL and VL in naturally infected dogs in areas where the two infections overlap by detection of IgG specific for *L.* (*L.*) *chagasi* or *L.* (*V.*) *braziliensis*.

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