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Short communication

Leishmania (Viannia) braziliensis: Immunoblotting analysis for the detection of IgG subclasses in the diagnosis of symptomatic and asymptomatic dogs

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ABSTRACT

During a seroepidemiological survey 2004–2006 from areas in Brazil endemic for American cutaneous leishmaniasis (ACL), serum samples from 10 dogs with ulcerated cutaneous lesions (S-ACL) and 52 asymptomatic dogs (AS-ACL) of unknown age and breed living in areas endemic for ACL were monitored for 1 year for ulcerated cutaneous lesions and immunoblotting using peroxidase-conjugated secondary anti-IgG, anti-IgG1 and anti-IgG2 dog antibodies. We reported that antibodies against *Leishmania (Viannia) braziliensis* in the sera of 22/52 dogs with asymptomatic disease showed intense reactivity to peptides larger than 66 kDa. We believe that dogs harboring subclinical amastigotes show an immunoblotting profile similar to that of symptomatic animals because a dog with self-healing presented antigens greater than 66 kDa. Such patterns can be exploited for diagnostic and epidemiological research for leishmaniasis.

1. Introduction

American cutaneous leishmaniasis (ACL) which is caused by several species of *Leishmania* protozoa from the *Leishmania* (*Viannia*) subgenera is one of the major public health problems in the world. In Brazil, ACL is one of the most relevant endemic diseases, with more than 21,000 new cases reported each year in the human population (Ministério da Saúde do Brasil, 2008). Several studies have suggested that the domestic dog (*Canis familiaris*) may have a reservoir role in the domestic transmission of human ACL caused by *Leishmania braziliensis* (Falqueto et al., 1991; Ferreira et al., 2009). However, if dogs are primary reservoir hosts of ACL, it would be feasible to eradicate domestic transmission by targeting dogs; but if dogs are secondary reservoir hosts, targeting dogs would only reduce the ACL. Any dog control strategy would be ineffective were dogs accidental hosts of ACL (Reithinger and Davies, 1999). The diagnosis of ACL is based mainly on clinical and epidemiologic features as well as on parasitological and immunological assays. Parasitological methods such as stained smears, histopathology and *in vitro* culture are the best way to confirm the diagnosis of leishmaniasis with 100% specificity. However, because of the scarcity of parasites in skin lesions, the parasitological tests display variable sensitivity (de Andrade et al., 2006). Therefore, the routine diagnosis is usually performed by combining assessment of clinical/epidemiological features with immunological methods. Serologic and molecular methods have been used for iden-

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tifying *Leishmania*-infected dogs (Day, 2007; Ribeiro et al., 2007; Oliveira et al., 2009). Several efforts have been made to establish a more reliable serological approach to ACL diagnosis using distinct antigen preparations to detect antibodies against *Leishmania* (Rocha et al., 2006). The *Leishmania* species present a genetic homology that ranges from 69% to 90%. Because of this homology, heterologous antigens have been used for immunodiagnosis and vaccine development targeting *Leishmania* infection (Gonçalves et al., 2002; Vale et al., 2009). Immunoblot analysis has shown that sera of patients with ACL have a specific and consistent pattern of reaction to the 27, 30 and 66 kDa soluble antigens as well as the 19 kDa antigen which is found in the spontaneously cured patients and may therefore play a role in protective immunity against cutaneous leishmaniasis (Brito et al., 2001). In the present study, we identified relevant antigens of *L. braziliensis* by immunoblotting, and we demonstrated the utility of this approach for serodiagnosis of ACL. In addition, we report that antibodies against *Leishmania (Viannia) braziliensis* in the sera of all dogs with asymptomatic disease showed intense reactivity to peptides larger than 66 kDa.

2. Materials and methods

2.1. Population and sera samples

The protocols employed for the collection of biological samples from study dogs followed those approved by the Ethics Committee for Animal Experimentation (CEUA-UFES) and adopted by the Brazilian College of Animal Experimentation (COBEA).

During a seroepidemiological survey 2004–2006, serum samples from 10 dogs with ulcerated cutaneous lesions (S-ACL) and 52 asymptomatic dogs of unknown age and breed living in residences that had at least one recorded case of ACL in human or dogs were tested for ACL using immunoblotting and peroxidase-conjugated secondary anti-IgG. The positive S-ACL dogs went out from ACL endemic areas and were observed for 1 year for ulcerated cutaneous lesions and immunoblotting . The S-ACL animals had been captured in the rural areas or were donated and kept in public kennels at Zoonosis Control Centers in several states of Brazil.

The sera were divided into two groups: group I consisted of 10 serum samples from dogs with ulcerated cutaneous lesions (S-ACL), all dogs in this group had skin lesions diagnosed as *L.* (*V.*) *braziliensis* infection after staining with Giemsa, cultivation at 25 °C in liver infusion tryptose (LIT) and immunoblotting sera using peroxidase-conjugated secondary anti-IgG, anti-IgG1 and anti-IgG2 dog antibodies. Group II consisted of 52 serum samples obtained from dogs without ulcerated cutaneous lesions (AS-ACL); these dogs were clinically monitored for 1 year for ulcerated cutaneous lesions.

2.2. Parasite preparation for immunoblot

Partially soluble fractions obtained from the promastigote forms of *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903) were grown in liver infusion tryptose medium (LIT) at 25 ± 1 °C during the stationary phase of growth according to the protocol of were used as antigens. The parasites were cultured for 7 days (Laemmli, 1970), washed three times by centrifugation at 2000 rpm in phosphate-buffered saline (PBS, pH 7.2) for 10 min, maintained in an ice bath and submitted to three ultrasound treatments (40 W) of 1 min each using a Sonifier Cell Disruptor® (Branson Sonic Power, Danbury, CT, USA). The sonicated material was centrifuged at 18,500 rpm for 1.5 h at 4 °C, and the supernatant was transferred to dialysis tubes and dialyzed against PBS for 36 h with changes of PBS every 6 h. The dialyzed material was filtered through disposable sterile filters (0.22 μm) under aseptic conditions, and an aliquot was assayed for protein concentration using the method of Lowry. The remaining sterile filtrate was diluted with sterile PBS to a final protein content of 1.0 mg/ml and aliquots were stored at −70 °C before use.

2.3. Immunoblotting

Ten micrograms of soluble antigens of *L. braziliensis* per lane and SDS-PAGE Molecular Weight Standards (Biorad Laboratories; catalog number: 161-0304) were separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (Gonçalves et al., 2002) using a mini gel apparatus. Polypeptides from the gels were electroblotted onto 0.45-μm nitrocellulose membranes with a semidry blotter at 200 mA for 90 min (Biorad, USA), following the supplier's instructions. For immunodetection, strips were cut from previously blotted membranes and blocked for 90 min with 5% skimmed milk in PBS. Strips were then washed with PBS–0.05% Tween 20 (PBS-T) (three times, 10 min each), followed by incubation with sera diluted 1:100 in PBS-T for 12 h at 4 °C. After incubation with the primary antibodies, the strips were washed as described four times and incubated for 60 min with peroxidase-conjugated secondary anti-IgG, anti-IgG1 and anti-IgG2 canine immunoglobulins (Bethyl Laboratories, Inc., Montgomery, TX, USA) diluted in PBS-T at concentrations of 1:1000, 1:500 and 1:1000, respectively, for 1 h at room temperature. After the strips were washed three times with PBS-T, the color was developed by adding 0.6 mg/ml 4-chloro-1-naphthol (Sigma) in 0.15 M PBS, pH 7.3, with 0.02% H_2O_2. The final reaction was stopped by washing the strips with distilled H_2O.

3. Results

The sera of 10 dogs with S-ACL showed a variety of soluble protein profiles on SDS-PAGE; 15 bands immunoreactive to *L.* (*V.*) *braziliensis* with relative MWr values ranging from 15 to 97 kDa (17, 19, 21, 24, 27, 30, 32, 40, 45, 50, 54, 66, 70, 80 and 97 kDa) were observed after incubation with secondary anti-IgG2 canine immunoglobulins (Table 1). The *L.* (*V.*) *braziliensis* polypeptides strongly stained with Coomassie brilliant blue in SDS-PAGE showed immunoblotting reactivity in one serum sample from S-ACL in immunoblot. For the AS-ACL (Table 1), the results were provided by IgG2, which permitted discrimination from S-ACL and recognized a clearer band than 66 kDa in the sera of 22/52 dogs with asymptomatic disease. Oth-

Table 1
Positive results obtained from immunoblotting with antigenic protein fraction (*L. braziliensis*) for serum samples of dogs from groups I and II.

Protein fraction	Group I: S-ACL ($n = 10$)			Group II: AS-ACL ($n = 22$)		
	IgG	IgG1	IgG2	IgG	IgG1	IgG2
97	10	0	9	16	0	20
80	10	0	8	15	0	20
70	9	0	10	22	0	22
66	10	6	10	16	4	22
54	10	0	10	6	0	8
50	10	0	8	3	0	3
45	10	0	10	3	0	3
40	6	0	5	3	0	3
32	10	4	10	3	0	3
30	10	7	10	5	4	5
27	10	7	10	3	7	5
24	8	0	8	3	0	3
21	10	0	10	3	0	3
19	8	0	7	3	0	3
17	7	0	7	3	0	3

ers inconsistent bands with weak intensity were observed for antigens lesser than 66 kDa. A difference was observed when comparing the bands detected with IgG and IgG2 for S-ACL and AS-ACL, IgG recognized as fewer polypeptides than secondary anti-IgG2 canine immunoglobulins for AS-ACL. The secondary anti-IgG canine immunoglobulins reacted to sera from all symptomatic and 80% (16/22) of asymptomatic dogs, identifying mainly proteins of 27, 30, 54 and 66 kDa (Table 1). IgG1 showed a strong intensity of reaction only with antigens of 27, 30 and 66 kDa. The assay with anti-IgG1 presented low sensitivity of 70% (7/10 dogs) for symptomatic and 31.8% (7/22 dogs) for asymptomatic dogs (Table 1).

4. Discussion

The immunoblotting profile for soluble antigens of *L. (V.) braziliensis* obtained in this study with antibodies against symptomatic individuals has previously been reported by other authors for dogs (Vale et al., 2009), humans (Gonçalves et al., 2002) and even for human cases of subclinical cutaneous leishmaniasis (Arraes et al., 2008) or spontaneous cure (Brito et al., 2001). However, there have been no reports of identification of asymptomatic dogs with ACL by immunoblotting. PCR (de Andrade et al., 2006; Velásquez et al., 2006), ELISA (Arraes et al., 2008) and IFAT (Antunes Uchoa et al., 2001) have been used to identify ACL in humans, dogs and even primates (Souza-Lemos et al., 2008) with asymptomatic serological profiles or self-healing. We believe that dogs harboring subclinical amastigotes show an immunoblotting profile similar to that of symptomatic animals (Table 1; three dogs) because a dog with self-healing presented antigens greater than 66 kDa. Such patterns can be exploited for diagnostic and epidemiological purposes.

Although numerous studies have questioned whether infected dogs develop expression of skewed IgG subclasses, the results have been conflicting, suggesting bias toward IgG1, IgG2 or neither subclass in different investigations. This confusion might be related to the specificity of commercially available polyclonal antisera used to detect canine IgG1 and IgG2 subclasses (Day, 2007). In conclusion, our results showed a high seroprevalence in asymptomatic seropositive dogs from areas in Brazil endemic for American cutaneous leishmaniasis (ACL) and suggest a particular pattern of antibodies against *Leishmania*. These findings indicate that subclinical infections and self-healing in dogs must be better studied.

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