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Serological detection of antibodies against *Paracoccidioides brasiliensis* in dogs with leishmaniasis

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Abstract

The aim of this study was to detect antibodies against *Paracoccidioides brasiliensis* in dogs seropositive and seronegative for leishmaniasis. Sera from 836 dogs (449 positive and 387 negative to leishmaniasis) were analysed by ELISA and the immunodiffusion test using gp43 and exoantigen, respectively. The analysis of the 836 serum samples by ELISA and the immunodiffusion test showed a positivity of 67.8 % and 7.3%, respectively, for *P. brasiliensis* infection. The dogs positive to leishmaniasis showed a higher reactivity to gp43 (79.9%) and exoantigen (12.7%) than the negative ones (54.0% and 1.0%, respectively). The higher reactivity to *P. brasiliensis* antigens may be due to cross-reactivity or a co-infection of dogs by *Leishmania* and *P. brasiliensis*. The lower correlation (0.187) observed between reactivity to gp43 and *Leishmania* antigen reinforces the latter hypothesis.

Key words: dog, ELISA, Leishmania, *Paracoccidioides brasiliensis*

Introduction

Paracoccidioidomycosis is a systemic mycosis endemic in Latin American countries. The etiologic agent *Paracoccidioides brasiliensis* is a thermodimorphic fungus that grows as yeast in the host either at 37 °C and as mycelia at 25 °C.

The individuals that develop PCM are mainly male agricultural workers. The granulomatous lesions are frequently observed in lungs, lymphonodes, spleen, liver, skin and mucosa. The infection probably occurs by fungus propagule inhalation [1].

The ecoepidemiological aspects of PCM remains poorly understood. Despite several attempts to find the *P. brasiliensis* habitat, until now it is unknown although it is believed that the fungus lives in soil [2]. The role of other animal species in the fungus ecology also remains unclear. *P. brasiliensis* was isolated from frugivorous bats [3], penguin [4] and armadillos [5–8].

Epidemiological studies suggest that other species such as cows [9], horses [10] sheep [11], monkeys [12] and dogs [13, 14] may be infected by *P. brasiliensis*. High levels of *P. brasiliensis* infection were observed in dogs from Southern and South-eastern regions of Brazil [14, 15]. The habits of sniffing and digging the soil could increase the chance of dogs being infected. The first case of natural paracoccidioidomycosis in dogs was reported recently [16].

Taking into account that endemic areas for paracoccidiodomycosis can be endemic for other diseases that affect dogs such as leishmaniasis, the aim of this study was to evaluate the infection by *P. brasiliensis* in dogs seropositive and seronegative for leishmaniasis.

Materials and methods

Area of study

The municipality of Campo Grande (latitude 20 26′34″S, longitude 54 38′47″W, altitude 542 m) is located in Mato Grosso do Sul State, Midwestern Brazil (Figure 1). The climate is tropical-humid with mean annual temperatures of 26 °C and relative humidity of 73%. The rainfall is around 1500 mm per year, the rainy season is from September to March (annual mean). The predominant soils are medium to heavy clay.

Animals

Approximately 20,000 serum specimens were collected from dogs in the suburbs of Campo Grande. The dogs were sampled from May 2003 to May 2004 for leishmaniasis diagnosis and for this study 836 serum samples were randomly selected as follows: 449 samples seropositive and 387 samples seronegative for leishmaniasis.

Figure 1. Map showing the location of the municipality of Campo Grande, Mato Grosso do Sul State.

***Paracoccidioides brasiliensis* antigens**

Exoantigen

The exoantigen was obtained as described by Camargo et al. [17], using the *P. brasiliensis* isolate B-339.

Antigen gp43

The gp43 was purified from the *P. brasiliensis* exoantigen by affinity chromatography as previously described by Puccia and Travassos [18]. The protein concentration was determined by the Bradford method using BSA as standard [19].

ELISA for leishmaniasis diagnosis

Leishmaniasis was diagnosed by a commercial ELISA kit (Bio-Manguinhos, Rio de Janeiro, RJ, Brazil). The test was carried out according to the manufacturer's instructions.

ELISA for anti-gp43 antibodies detection

The sera were analysed for detection of anti-gp43 antibodies as previously described by Eisele et al. [20]. In brief, polystyrene flat-bottom microtiter plates (Corning Costar Corporation, Corning, NY, USA) were coated with gp43 in 0.1 M carbonate buffer, pH 9.6 (250 ng well^{-1}). The plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and blocked with PBS-T 5% skim milk (PBS-T-M). After washing PBS-T, the serum samples were diluted 1:100 in PBS 1% skim milk (PBS-M) and incubated at 37 °C for 1 h. The plates were washed and incubated at 37 °C for 1 h with anti-dog IgG-peroxidase conjugate (Sigma, St Louis, MO, USA). After washing with PBS-T the solution of substrate/chromogen (H_2O_2/tetramethylbenzidine) was added to each well, and the reaction was stopped with 4 N H_2SO_4. Absorbance was measured with an ELISA reader at 450 nm. The positive and

negative controls were a serum sample from a dog immunized with *P. brasiliensis* and a pool of sera from urban dogs, respectively. Sera with two-fold or more the absorbance of the negative control were considered positive.

Immunodiffusion test

The test was performed as previously described by Eisele et al. [20] using *P. brasiliensis* exoantigen as reagent. The serum from a dog immunized with *P. brasiliensis* was used as a positive control.

Clinical exam of dogs positive in immunodiffusion test

Four animals positive in the immunodiffusion test were examined for PCM clinical signs (fever, lymph node enlargement, cough and other respiratory signs). These animals were also submitted to a chest X-ray.

Statistical analysis

The statistical analysis was performed with the program EpiInfo® 6.0. The difference was considered significant when P was less than 0.05.

Results and discussion

The analysis of the 836 dog serum samples by ELISA and the immunodiffusion test showed a positivity of 67.8 and 7.3%, respectively for *P. brasiliensis* infection (Table 1). The higher positivity observed in ELISA may be due to the greater sensitivity of this test. A seroepidemiological study carried out to determine the prevalence of *P. brasiliensis* antibodies in Brazilian blood donors showed positivity of 21% by ELISA with g43 antigen, but no reactivity was detected by immunodiffusion test [21].

Table 1. Reactivity of dogs' sera against *P. brasiliensis* antigens gp43 and exoantigen evaluated by ELISA and immunodiffusion

Sex	gp43 (ELISA) %	Exoantigen (ID) %
Male	67.9	8.6
Female	67.7	5.8
Total	67.8	7.3

ID = Immunodiffusion.

The differences in positivity were not statistically significant in relation to sex by either test (Table 1) suggesting that male and female dogs are equally exposed to the *P. brasiliensis* infection.

Similar results were observed by our group in a seroepidemiological study with dogs from rural and urban areas although no reactivity was observed in the immunodiffusion test [14]. The infection of dogs by *P. brasiliensis* was also investigated by Mós and Fava Netto [13]. The authors observed a positivity of 75% in dogs from the State of São Paulo, Brazil, using the complement fixation test with the *P. brasiliensis* polysaccharide antigen.

The dogs that showed reactivity in the immunodiffusion test probably have developed paracoccidioidomycosis because in human paracoccidiodomycosis, only patients with clinical symptoms are reactants in this test [17]. Four out of 61 dogs positive in the immunodiffusion test were examined in search of clinical signs of paracoccidioidomycosis. Discrete radiological alterations were observed in two dogs (data not shown). Probably these animals were in an initial phase of paracoccidiodomycosis with very discreet signs of illness. Recently, our group has reported an experimental infection of dogs with *P. brasiliensis* and all animals had shown humoral immune response although no significant alteration had been observed in thoracic X-ray [20].

Unfortunately the following of a greater number of animals was not possible because the serologic tests to evaluate infection by *P. brasiliensis* were carried out after the euthanasia of dogs positive for leishmaniasis.

The dogs positive for leishmaniasis showed a higher reactivity to gp43 (79.9) than the negative ones (54%) (Table 2). Similar results were observed in the immunodiffusion test taking into account that 12.7% of the samples positive to

Table 2. Reactivity of dogs' sera seropositive and seronegative for leishmaniasis against *P. brasiliensis* antigens gp43 and exoantigen evaluated by ELISA and immunodiffusion

	gp43 (ELISA) %	Exoantigen (ID) %
Leishmaniosis positive (n = 449)	79.9 (n = 359)	12.7 (n = 57)
Leishmaniosis negative (n = 387)	54.0 (n = 209)	1.0 (n = 4)

ID = Immunodiffusion.

leishmaniasis were positive for *P. brasiliensis* exo-antigen against only 1.0% for the leishmaniasis negative samples (Table 2).

This higher reactivity to *P. brasiliensis* antigens observed in serum samples positive to leishmaniasis may suggest a cross reactivity with *Leishmania* antigens as previously described [22]. In order to rule out this hypothesis a correlation test was performed to compare reactivity of serum samples to *P. brasiliensis* (gp43) and *Leishmania* antigens. The very low correlation coefficient observed ($r = 0.187$) suggests that reactivities against the two antigens systems are not related (Figure 2). Taking into account that the municipality of Campo Grande is endemic for paracoccidioidomycosis [23] and leishmaniasis [24], dogs at higher risk of infection by *Leishmania* could be also more exposed to *P. brasiliensis* infection.

The dogs under 1-year-old showed lower reactivity to gp43 suggesting that the age is a risk factor for *P. brasiliensis* infection (Figure 3). Older animals have a higher probability of being infected by the fungus during their lifetime. Otherwise the puppies that are at lower risk of infection are probably more susceptible to developing paracocccidioidomycosis than adults [25].

The data of this study suggest that co-infection of dogs by *P. brasiliensis* and *Leishmania* may be occurring. The association between these pathogens in dogs is very interesting because the protective immune response to paracoccidioidomy-cosis and leishmaniasis is TH1 type [26, 27]. In this way, dogs susceptible to developing leishmaniasis could be also more susceptible to developing paracoccidioidomycosis.

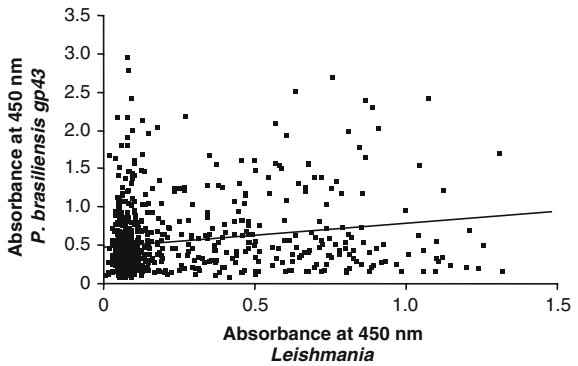

Figure 2. Correlation between absorbances of dogs' sera analysed by ELISA for reactivity to antigens from *Leishmania* and *P. brasiliensis* (gp43).

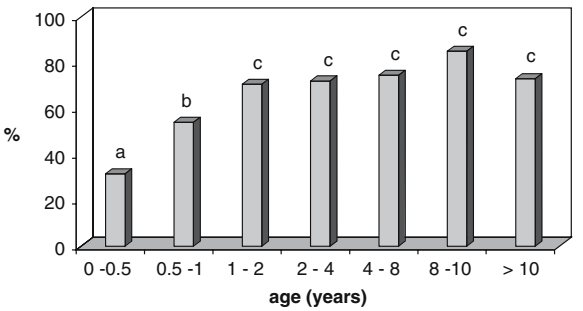

Figure 3. Relative frequency of positivity against gp43 evaluated by ELISA in dog serum samples ($n = 836$) according to age.

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