Rural area of the Brazilian Pantanal wetlands associated with the occurrence of anti-*Leishmania* spp. antibodies in dogs

*Área rural do Pantanal brasileiro associada à ocorrência de anticorpos anti-*Leishmania *spp. em cães*

Andréia Lima Tomé MELO[1]; Andréa Pereira da COSTA[2]; Selma Samiko MIYAZAKI[3]; Matias Bassinello STOCCO[4]; Thiago Borges Fernandes SEMEDO[5]; Thábata dos Anjos PACHECO[4]; Rute WITTER[4]; Richard de Campos PACHECO[4]; Marcelo Bahia LABRUNA[2]; Arlei MARCILI[2,6]; Daniel Moura de AGUIAR[4]

[1] Universidade de Cuiabá, Curso de Medicina Veterinária, Cuiabá – MT, Brazil
[2] Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, São Paulo – SP, Brazil
[3] Instituto Chico Mendes de Conservação da Biodiversidade, Estação Ecológica de Taiamã, Cáceres – MT, Brazil
[4] Universidade Federal de Mato Grosso, Faculdade de Medicina Veterinária, Cuiabá – MT, Brazil
[5] Universidade Federal de Mato Grosso, Instituto de Biociências, Cuiabá – MT, Brazil
[6] Universidade de Santo Amaro, Mestrado em Medicina Veterinária e Bem-Estar Animal, São Paulo – SP, Brazil

Abstract

An evaluation was made of the presence of anti-*Leishmania infantum chagasi* antibodies in domestic dogs from the urban and rural areas of Brazil's Pantanal wetland region using serological techniques. A total of 429 dogs were sampled in three areas of the Pantanal biome, including the municipalities of Poconé, Santo Antônio de Leverger, and Barão de Melgaço, in the state of Mato Grosso, and in the municipality of Corumbá, in the state of Mato Grosso do Sul. The immunofluorescence assay (IFA) was used to detect antibodies (cut-off point 40) using *Leishmania infantum chagasi* antigen. Because of the possibility of cross-reactivity between species of the genus *Leishmania*, samples that were positive in the IFA against *L. infantum chagasi* were also tested by IFA in the same conditions, using *L. amazonensis* and *L. braziliensis* antigens. IFA-positive samples to *L. infantum chagasi* were also evaluated using Enzyme-Linked Immunosorbent Assay (ELISA). The results showed the presence of antibodies against *L. infantum chagasi* in 23 (5.36%; 95% CI: 3.50%-8.05%) dogs and at least one seroreactive dog was found in each of the municipalities evaluated in this study. Antibody titers ranged from 40 to 5,120, and all IFA positive samples were positive in the ELISA. Among the 23 positive dogs, nine were also were seroreactive for *L. amazonensis* and *L. braziliensis* antigens. The occurrence of anti- *L. infantum chagasi* antibodies in dogs was higher in rural areas (7.06%) than in urban areas (2.50%) (P < 0.05). Based on this study, we concluded that dogs from rural areas of the Pantanal wetlands were in contact with *Leishmania* species, which is relevant information given their importance to public health.
Keywords: Dog. *Leishmania* spp. Antibodies. IFA. ELISA. Pantanal.

Resumo

Neste trabalho foi realizada uma avaliação sobre a presença de anticorpos anti-*Leishmania infantum chagasi* em cães domésticos das áreas urbanas e rurais da região do Pantanal brasileiro usando técnicas sorológicas. Um total de 429 cães foram amostrados em três áreas do bioma do Pantanal, incluindo os municípios de Poconé, Santo Antônio de Leverger e Barão de Melgaço, em Mato Grosso, e o município de Corumbá, em Mato Grosso do Sul. A reação de imunofluorescência indireta (RIFI) foi utilizada para detectar anticorpos (ponto de corte de 40) de *Leishmania infantum chagasi* como antígeno. Devido à possibilidade de reação cruzada entre as espécies do gênero *Leishmania*, as amostras positivas na RIFI para *L. infantum chagasi* foram também avaliadas na RIFI utilizando *L. amazonensis* e *L. braziliensis* como antígenos. As amostras positivas na RIFI para *L. infantum chagasi* foram avaliadas utilizando o ensaio de imunoadsorção ligado à enzima (ELISA). Os resultados mostraram a presença de anticorpos contra *L. infantum chagasi* em 23 (5,36%; IC 95%: 3,50% -8,05%) cães e pelo menos um cão soro-reativo foi encontrado em todos os municípios avaliados neste estudo. Os títulos de anticorpos variaram de 40 a 5.120 e todas as amostras positivas na RIFI foram positivas no ELISA. Entre os 23 cães positivos, nove também reagiram para *L. amazonensis* e *L. braziliensis*. A ocorrência de anticorpos anti-*L. infantum chagasi* em cães foi maior nas áreas rurais (7,06%) do que nas áreas urbanas (2,50%) (P < 0,05). Com base neste estudo, concluímos que cães de áreas rurais do Pantanal tiveram contato com espécies de *Leishmania*, o que é uma informação relevante, dada a sua importância para a saúde pública.
Palavras-chave: Cão. *Leishmania* spp. Anticorpos. RIFI. ELISA. Pantanal.

Correspondence to:
Andréia Lima Tomé Melo
Universidade de Cuiabá, Curso de Medicina Veterinária
Av. Manoel José de Arruda, 3100 – Jardim Europa
CEP 78065-900, Cuiabá, MT, Brazil
e-mail: andreialtm@gmail.com

Introduction

Leishmaniasis is a zoonotic disease caused by an obligate intramacrophage protozoan of the genus *Leishmania* (BAÑULS et al., 2007; DAWIT et al., 2013). This parasite is of medical and veterinary public health importance because it infects numerous mammal species, including humans, and is transmitted by the bite of female phlebotomine sand flies of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world. The leishmaniases may have different clinical manifestations such as American visceral leishmaniasis (AVL) and American cutaneous leishmaniasis (ACL) (ASHFORD, 2000; CHAPPUIS et al., 2007; HEUSSER JÚNIOR et al., 2010; BRASIL, 2016). In Brazil the cutaneous form of the disease is usually caused by *Leishmania braziliensis* and *Leishmania amazonensis* (BRASIL, 2016).

Leishmania infantum chagasi is the etiological agent of AVL, a chronic disease of which infected dogs are considered reservoirs able to transmit the agent to the vector (LAINSON; RANGEL, 2005; ROQUE; JANSEN, 2014). The principal vectors are *Lutzomyia longipalpis* and *Lutzomyia cruzi*, and the latter is implicated in the transmission of AVL in Corumbá and Ladário, two cities situated in the Pantanal wetland biome in the state of Mato Grosso do Sul (SANTOS et al., 1998; LAINSON; RANGEL, 2005; BRASIL, 2016).

The genus *Lutzomyia* reproduces in solid decomposing organic matter, and the lack of environmental sanitation for the elimination of this matter is associated with the presence of the vector. Therefore, the presence of agriculture and the proximity of dogs to forests, wastelands, and locations that have undergone recent environmental changes also contribute to the vector´s development. The urbanization of AVL results from anthropogenic environmental alterations and the rapid and intense migration of rural populations to urban peripheries lacking proper infrastructure, including the concurrent interaction of sylvatic reservoirs and domestic dogs (DIAS et al., 2003; SARAIVA et al., 2017).

The occurrence of *L. longipalpis* and *L. cruzi* has been observed in wetland areas. Both species have been reported in the municipality of Poconé, state of Mato Grosso, while only *L. cruzi* has been described in Santo Antônio de Leverger and Barão de Melgaço, two other municipalities located in the Pantanal wetlands in the state of Mato Grosso (MISSAWA; LIMA, 2006).

Considering the significance of the Pantanal biome as an important region of livestock production activities, fishing and safari tourism, and the presence of dogs in close contact with humans, which are also relevant in the epidemiology of leishmaniasis, the purpose of this study was to determine the occurrence of anti-*L. infantum chagasi* antibodies in domestic dogs from the urban and rural areas of the Pantanal wetlands in the Brazilian states of Mato Grosso and Mato Grosso do Sul.

Materials and Methods

This study evaluated dogs (n = 429) from urban (n = 160) and rural (n = 269) areas sampled from 2009 to 2012 in four municipalities located in the Pantanal wetlands, namely, i) Poconé (56º37'22"W; 16º15'24"S); ii) Santo Antônio de Leverger (56º04'47"W; 15º51'47"S); iii) Barão de Melgaço (55º58'03"W; 16º11'49"S); and iv) Corumbá (57º39'44"W; 19º00'33"S) (Figure 1). The samples from the Poconé municipality were obtained from an earlier study in which the sample was determined using EpiInfo hardware version 6.0 where the dogs were sampled in the urban and rural areas (MELO et al., 2011). The remainder samples from the others municipalities were obtained through convenience sampling, where they always sought to collect blood from all the animals in the residences.

Blood samples were collected by jugular venipuncture to obtain serum. Anti-*Leishmania* spp. antibodies were evaluated by immunofluorescence assay (IFA), as described by Oliveira et al. (2008), using the promastigote forms of isolate CBT 96 (Coleção Brasileira de Tripanossomatídeos) of *L. infantum chagasi* (COSTA et al., 2015) with a cut-off point at the initial dilution of 1:40 (BRASIL, 2006). Each slide contained sera previously known to be non-reactive (negative control) and reactive (positive control). Based on the finding of cross-reactivity between species of the genus *Leishmania*, samples that were positive in the IFA against *L. infantum chagasi* were also tested by IFA in the same conditions, using *L. amazonensis* and *L. braziliensis* antigens provided by the Oswaldo Cruz Institute (OLIVEIRA et al., 2008).
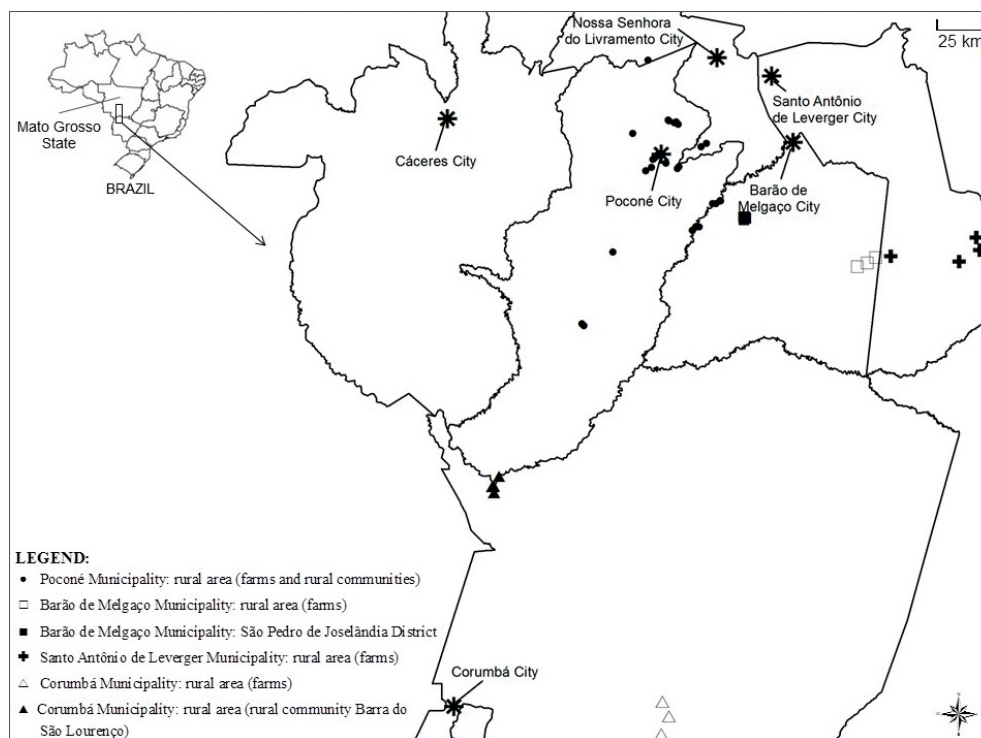
Figure 1 – Localities from the Pantanal wetland region of the States of Mato Grosso and Mato Grosso do Sul where dogs were sampled and evaluated in the presents study

IFA-positive samples were evaluated using a commercial ELISA kit based on a recombinant peptide of *L. infantum chagasi*, following the manufacturer's instructions (ELISA/S76, Biogene Laboratory®). The reaction was evaluated in an ELISA reader at an optical density (OD) of 450 nanometers. The cut-off was calculated from the arithmetic mean of the optical densities of nonreactive sera added to factor R = 0.142. A gray area (indeterminate range) was established for all the plates by subtracting the cutoff of 0.03, and serum samples with OD higher than the cut-off point were considered positive.

The owner of each dog selected for blood collection was asked to fill out a comprehensive questionnaire, which included information on the animal's age, sex, and habitat (flooded area and non-flooded area).

The present study was approved by the Bioethical Committee in Animal Research of the Federal University of Mato Grosso, under protocol no. 23108.019742/09-9.

Results

All the dogs aged 1 to 72 months and belonged to different breeds. Anti-*L. infantum chagasi* antibodies were detected in 23 (5.36%; 95% CI: 3.50% - 8.05%) dogs. At least one seroreactive dog was found in each of the municipalities evaluated in this study, and the endpoint titers ranged from 40 to 5,120. All the seropositive dogs were positive in the ELISA. According to the total number of positives, 4 (2.50%) dogs were from the urban area of the municipality of Poconé and 19 (7.06%) were from the rural areas of the municipalities of Poconé, Santo Antônio de Leverger, Barão de Melgaço, and Corumbá (Table 1).

Table 1 – Distribution of antibody titers against three antigens of *Leishmania* spp. used in IFA (≥ 40) in dogs collected in the Pantanal wetland in the states of Mato Grosso and Mato Grosso do Sul – Brazil – 2009 to 2013

Municipalities	*L. infantum chagasi*		*L. amazonensis*		*L. braziliensis*	
	Positive/ tested (%)	Endpoint titers (No. of dogs)	Positive/ tested (%)	Endpoint titers (No. of dogs)	Positive/ tested (%)	Endpoint titers (No. of dogs)
Poconé	19/319 (5.95)	40 (4); 80 (2); 160 (5); 320 (3); 640 (2); 1280 (1) 2560 (2)	3/19	40 (1); 80 (2)	3/19	80 (2); 160 (1)
Santo Antônio de Leverger	1/16 (6.25)	5120 (1)	1/1	320 (1)	0/1	-
Barão de Melgaço	1/45 (2.22)	5120 (1)	0/1	-	1/1	40 (1)
Corumbá	2/49 (4.08)	40 (1); 160 (1)	1/2	80 (1)	0/2	-

Among the 23 positive dogs, nine were also positive for *L. amazonensis* and *L. braziliensis* antigens. Three dogs from Poconé were positive for *L. amazonensis* (titers ranging from 40 to 80), and three others were positive for *L. braziliensis* (titers ranging from 80 to 160). One dog from the municipality of Santo Antônio de Leverger and one dog from Corumbá were seroreactive to *L. amazonensis* with titers of 320 and 80, respectively, and one dog from Barão de Melgaço was positive for *L. braziliensis* (titer of 40) (Table 2).

In terms of the statistical analysis, only the variable habitat was associated with the occurrence of anti-*Leishmania* spp. antibodies in dogs, and a higher frequency was observed in the rural areas than in urban area (P < 0.05). The other variables were not associated with positive serology of dogs (Table 3).

Table 2 – Endpoint titer of *Leishmania infantum chagasi*, *L. amazonensis* and *L. brasiliensis* found in dogs from the Pantanal wetland – Brazil – 2009 to 2013

Municipality	Dog	*L. infantum chagasi*	*L. amazonensis*	*L. braziliensis*
		Titer	Titer	Titer
Poconé	No. 1	160	80	Negative
	No. 2	2560	40	Negative
	No. 3	2560	80	Negative
	No. 4	640	Negative	80
	No. 5	320	Negative	80
	No. 6	160	Negative	160
Santo Antônio do Leverger	No. 7	5120	320	Negative
Barão de Melgaço	No. 8	5120	Negative	40
Corumbá	No. 9	160	80	Negative

Table 3 – Analysis of the variables and results of IFA (≥ 40) in dogs from the Pantanal wetland – Brazil – 2009 to 2013

Analyzed variables	Nº of dogs		
		Positive to *L. infantum chagasi*	
	Analyzed	Number (%)	*P*-value
Habitat			
Urban	160	4 (2.50)	0.04
Rural	269	19 (7.06)	
Area			
No flood area	256	16 (6.25)	0.43
Flood area	173	7 (4.05)	
Gender			
Male	258	11 (4.26)	0.30
Female	171	12 (7.02)	
Age*			
0-12 months	124	3 (2.42)	0.13
12-24 months	100	8 (8.0)	0.27
24-48 months	94	9 (9.57)	0.07
48-72 months	50	1 (2.0)	0.49
>72 months	48	2 (4.17)	1.00

* A total of 13 dogs had not age determined; and none of these animals had antibodies to *Leishmania* spp.

Discussion

This study found a prevalence of anti-*Leishmania* spp. antibodies in dogs from the northern region of the Pantanal wetlands in three municipalities of the state of Mato Grosso and one in the state of Mato Grosso do Sul. The occurrence of canine visceral leishmaniasis has already been reported in the Pantanal region, and *L. cruzi* has been incriminated as a vector for this infection (SANTOS et al., 1998; BRASIL, 2016).

Antibodies against *L. infantum chagasi* antigen were detected in ~5% of dogs, with endpoint titers ranging from 40 to 5,120, suggesting that dogs were stimulated against *L. infantum chagasi*. Anti-*Leishmania* spp. antibodies in the states of Mato Grosso and Mato Grosso do Sul have been reported by other researchers, with frequencies higher than that observed in this work and according to the particular characteristics of each region under study (NUNES et al., 2001; CORTADA et al., 2004; ANDRADE et al., 2009; ALMEIDA et al., 2012; SOUSA et al., 2013; BRITO et al., 2014). This may be related to the opportunist feeding behavior of the vector. In some areas in midwestern Brazil dogs are not the preferred sources of blood of *L. longipalpis,* as it was found that it feeds preferentially on birds and rodents, in addition to humans, opossums, oxen, horses, and least of all dogs (MISSAWA et al., 2008).

Positive IFA dogs were positive by ELISA using recombinant peptides of *L. infantum chagasi* and this is relevant because the ELISA is a specific test with a medium high sensitivity (GRAMICCIA, 2011), and is currently used for confirmation of seroreagent or undetermined immunochromatographic test dogs, which is recommended for screening by the Ministry of Health of Brazil (BRASIL, 2016).

On the other hand among the 23 positive dogs to IFA using *L. infantum chagasi* antigen, nine were also seroreactive to IFA performed with *L. amazonensis* and *L. braziliensis* antigens. Considering the observation of cross-reactivity in IFA among different species of *Leishmania* spp., *Trypanosoma cruzi* (SOLANO-GALLEGO et al., 2009), and *Trypanosoma caninum* (ALVES et al., 2012), the presence of seroreagent dogs for more than one antigen suggests the possibility of a co-infection. Thus, although this test is no longer officially used by the Ministry of Health of Brazil (BRASIL, 2016), it is an important tool for the identification of *L. infantum chagasi* infection in the dogs from Pantanal region, where there are few studies about leishmaniasis

In this study most of the positive dogs clearly came from rural areas. Habitat is considered an important environmental factor in the epidemiology of leishmaniasis, mainly due to the presences of organic debris or forests close to human habitations, which can facilitate the maintenance of the vector and the occurrence of the disease (BRANDÃO-FILHO et al., 1994; MORENO et al., 2005; BIGELI et al., 2012; LOPES et al., 2014). In addition, there were more samples from rural areas than from urban areas, which may have influenced the findings of our serosurvey.

Besides degraded environments due to disordered occupation, it is known that leishmaniasis also occurs commonly in rural environments where epidemiological characteristics may facilitate the circulation of the agent (MARZOCHI et al., 2009). Furthermore, the presence of livestock and agriculture may represent a risk of disease because it can stimulate the establishment of sand flies in these environments, especially regarding that in the Pantanal area livestock activities and tourism related to fishing and safari are among the most important economic activities (MOREIRA JUNIOR et al., 2003; ALMEIDA et al., 2012). Based on these facts, the study of leishmaniasis in Brazil's Pantanal wetland is of great significance to human and animal health and it is possible to conclude that future research on this disease is essential in this region.

Acknowledgments

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