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PARASITOLOGY

Canine visceral leishmaniosis in an area of fishing tourism, Bonito Municipality, Mato Grosso do Sul, Central-West Brazil

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ABSTRACT: *The study aimed to investigate Leishmania infection in a population of dogs (92 animals) in a fishing area of Bonito Municipality, and evaluate comparatively the serological methods used, immunoenzymatic assay (ELISA), indirect immunofluorescence antibody test (IFAT), and rapid Dual Path-Platform test (DPP®). Blood and aspirate of bone marrow samples were used and parasitological investigation was also performed, such as parasite isolation in Neal, Novy, Nicolle (NNN) medium culture, Woo technique, Giemsa stained smears and specific identification by polymerase chain reaction (PCR). IFAT revealed 56/92 seropositive, the ELISA 8/92 and the DPP® 41/92. Regarding the agreement between the serological tests by the Kappa index, there was a slight agreement between ELISA/IFAT and ELISA/DPP®, and fair agreement in IFAT/DPP®. The NNN culture was positive in three out of 21 dogs, and identified by PCR as Leishmania infantum chagasi. No samples were positive by the Woo technique. Our results showed low agreements between the serological tests recommended by the Brazilian Ministry of Heath, and it is necessary to associate other diagnostic techniques, such as parasitological tests and PCR, to increase the sensitivity for canine visceral leishmaniosis diagnosis, mainly regarding asymptomatic dogs in endemic areas.*
Key words: *domestic dogs, serodiagnosis, ecotourism.*

Leishmaniose visceral canina em área de turismo pesqueiro, município de Bonito, Mato Grosso do Sul, Centro-Oeste do Brasil.

RESUMO: *O estudo teve como objetivo verificar a infecção por Leishmania em uma população de cães (n=92 animais) de uma área de turismo de pesca no município de Bonito e avaliar comparativamente os métodos sorológicos utilizados, ensaio imunoenzimático (ELISA), reação de imunofluorescência indireta (RIFI) e teste rápido de plataforma dupla (DPP®). Testes parasitológicos também foram realizados, como o isolamento do parasita em meio de cultura Neal, Novy, Nicolle, técnica de Woo, esfregaços em lâminas coradas com Giemsa e identificação específica pela reação em cadeia da polimerase (PCR). A RIFI revelou sorologia positiva de 56/92, ELISA 8/92 e DPP® 41/92. Quanto à concordância entre os testes sorológicos pelo índice Kappa, houve um ligeiro acordo entre ELISA/RIFI e ELISA/DPP®, e uma concordância razoável entre RIFI/DPP®. A cultura NNN foi positiva em três cães, e identificada por PCR como Leishmania infantum chagasi. Nenhuma amostra foi positiva pela técnica de Woo. Nossos resultados mostraram baixas concordâncias entre os testes sorológicos recomendados pelo Ministério da Saúde, sendo necessário associar a outras técnicas de diagnóstico, como testes parasitológicos e PCR, para aumentar a sensibilidade ao diagnóstico de leishmaniose visceral canina, principalmente, com relação aos cães assintomáticos em áreas endêmicas.*
Palavras-chave: *cães domésticos, sorodiagnóstico, ecoturismo.*

INTRODUCTION

In Brazil, visceral leishmaniasis (VL) is a zoonosis of public health importance, and its etiological agent is *Leishmania* (*Leishmania*) *infantum chagasi*. As main vectors of this parasite, two phlebotomine sandfly species, *Lutzomyia longipalpis,* widespread in the country, and *Lutzomyia cruzi* registered in Mato Grosso do Sul and Mato Grosso States, (SANTOS et al., 1998; LAINSON & SHAW, 2005; MISSAWA et al., 2011; BRASIL, 2014) have been implicated, and the domestic dog has been considered the common reservoir, with

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canine cases normally preceding the occurrence of human cases (GONTIJO & MELO, 2004).

The first cases of canine and human visceral leishmaniasis in Mato Grosso do Sul date back to the 80s at the Corumbá municipality (NUNES et al., 1988; YAMAMOTO et al., 1988). By the 90s, the visceral leishmaniasis spread widely across the state being diagnosed in 56 of its 78 municipalities, affecting rural and urban populations of different age groups and the vectors have being reported in almost all the municipalities (CORTADA et al., 2004; OLIVEIRA et al., 2006; NUNES et al., 2008; LIMA-JÚNIOR et al., 2009; ANDRADE et al., 2009; MATO GROSSO DO SUL, 2014).

Researches undertaken in Bonito District have reported natural infection by *Leishmania* in sandflies and dogs in both rural and urban areas (NUNES et al., 2001; 2008; GALATI et al., 2006; SAVANI et al., 2009, ANDRADE et al., 2009; BRILHANTE et al., 2015b), and a moderate incidence of the disease in both human and canine populations (ANDRADE et al., 2009; MATO GROSSO DO SUL, 2014).

According to the Brazilian Ministry of Health, serological methods are used to diagnose and control programs of canine visceral leishmaniasis (CVL) in the country (BRASIL, 2014). However, the complexity of the diagnosis of CVL should be considered due to the low sensitivity of the tests, variance of clinical signs, presence of asymptomatic dogs and social factors caused by the culling of dogs (DE SANTIS et al., 2013).

In Águas do Miranda District, one of the major tourist resorts for ecotourism and sporting fishing in midwestern Brazil, the notification of CVL led us to investigate the infection by *Leishmania* in the canine population, and compare the performance of serological methods used, whose results are reported in this study.

MATERIALS AND METHODS

Study area

Bonito Municipality, one of the Mato Grosso do Sul municipalities, is situated in the southwest of Mato Grosso do Sul in the geomorphological unit known as Bodoquena Plateau, and consists of two districts: Bonito, containing the municipal offices, and Águas do Miranda, both considered important regional, national and international tourist resorts (IBGE 2016; BONITO, 2016).

The District of Águas do Miranda (20°45'44.4" S, 56°05'42.8" W) is 75 km from the Municipality of Bonito District containing the municipal offices (21°07'16" S, 56°28'55" W), and 180 km from Campo Grande, the capital of the Mato Grosso do Sul State (IBGE, 2016) (Figure 1). The human population consists of 450 inhabitants, which may rise to as many as 10,000 in the fishing season, from March to October. The local economy is based mainly on fishing and ecotourism (BONITO, 2009).

Sampling

With information about the occurrence of CVL cases and the help of the Zoonosis Control Center of Bonito Municipality, domiciliary visits were made in the Águas do Miranda District from June to December 2009. By consent of the owners, the 92 adult dogs, which constituted the local canine population, were submitted to clinical evaluation for the identification of manifestations suggestive of leishmaniosis. About 5 ml of venous blood was collected by jugular or cephalic vein puncture and stored in plastic tubes without and with anticoagulant (ethylenediaminetetraacetic acid - EDTA). Serum samples were stored at −20°C until the performance of the serological techniques.

For the 41 positive dogs in the Dual Path-Platform test (DPP®), a second visit was made to their owner's domicile to collect bone marrow blood samples. However, only for 21 of the dogs it was possible to obtain samples.

Serological tests

Serum samples were used for serological tests which were carried out in the laboratory of Imunodulação e Protozoologia of the Oswaldo Cruz Institute/FIOCRUZ in Rio de Janeiro. The techniques used for the diagnosis of CVL were indirect fluorescent antibody test (IFAT) (kit IFI Canine Visceral Leishmaniosis Bio-Manguinhos/FIOCRUZ); enzyme-linked immunosorbent assay (kit ELISA Canine Visceral Leishmaniosis Biomaguinhos/FIOCRUZ), and immunochromatographic rapid test (kit TR DPP® Canine Visceral Leishmaniosis-Bio-Manguinhos/FIOCRUZ). The latter is a lateral flow test for the detection of antibodies specific for *Leishmania*, using recombinant proteins K26 and K39 specific for *Leishmania infantum chagasi*. Serological techniques and immunochromatographic assay were performed according to the manufacturer's instructions. In order, to classify the positive results, the following criteria were considered: IFAT titres equal to or higher than 1:40, ELISA readings over the cut-off, and for DPP®, the emergence of two lines 15 minutes after buffer placement.

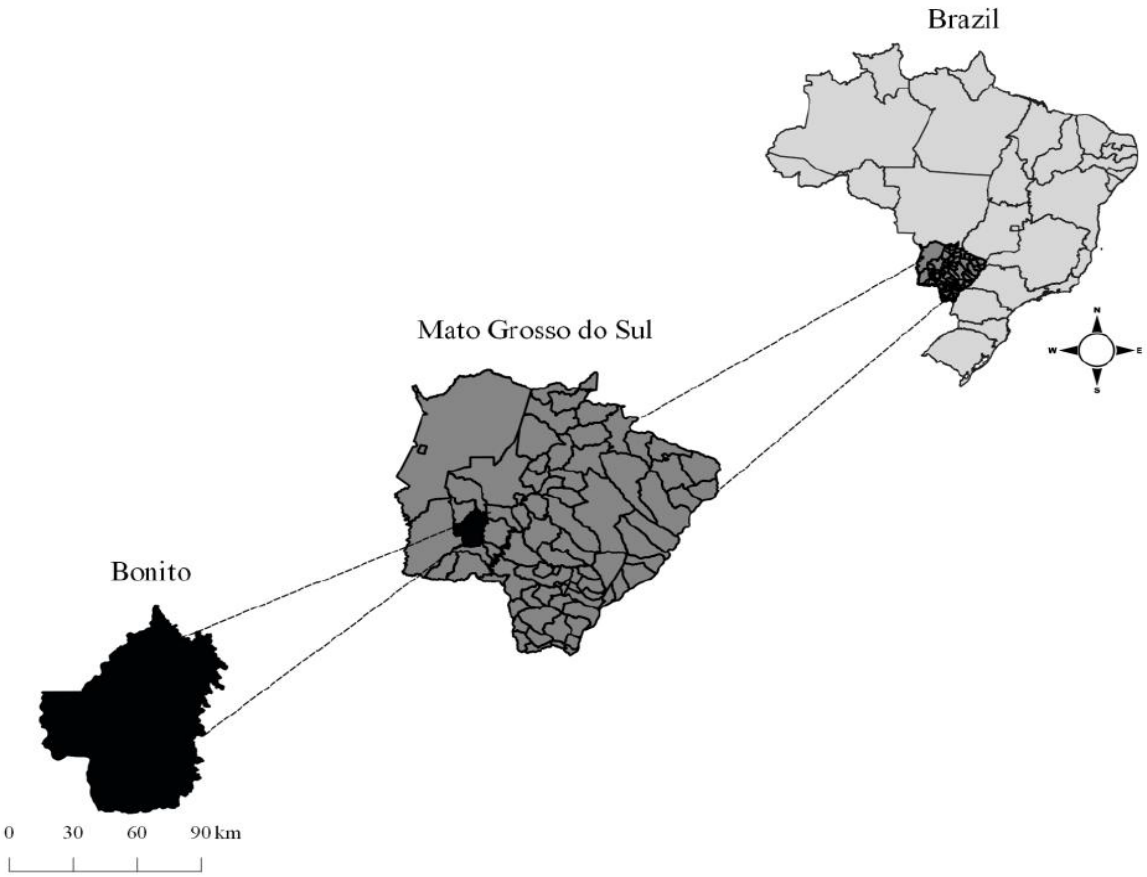

Figure 1 - Political map of Brazil emphasizing Mato Grosso do Sul State and Bonito Municipality.

Parasitological diagnosis

The Woo technique was applied to identify trypanosomatid flagellates from total blood samples in accordance with WOO (1969). The method consisted of filling two capillary tubes with approximately 0.06 ml of peripheral blood, which were flamed sealed at one end and centrifuged at 12, 000 rpm (Make of centrifuge) for 4 minutes. These tubes were then placed in a capillary tube holder and examined under a microscope using a 10 X objective to investigate trypanosomes at the junction of the plasma and buffy layer in the centrifuged blood (WOO, 1969).

For observation of amastigote forms, part of the bone marrow blood sample was used to prepare Giemsa stained smears (n=21). The remainder of each sample was inoculated intraperitoneally into two hamsters (*Mesocricetus auratus*) (n=42). Nine months after inoculation, the hamsters were necropsied and fragments of their liver and spleen were seeded in NNN culture medium supplemented with 20% fetal bovine serum. Samples were seeded in duplicate, with a total of 84 cultures.

DNA extraction and molecular test

Positive samples of the NNN culture had their DNA extracted using a DNAzol® kit in accordance with the manufacturer's instructions, and then analyzed by polymerase chain reaction (PCR) for the *Leishmania infantum chagasi* using the initiators RV1- CTTTTCTGGTCCCGCGGGTAGG e RV2- CACCTGGCCTATTTTACACCA. The PCR conditions were undertaken according to LIMA-JÚNIOR et al. (2009). The DNA reference strain was *L. infantum chagasi* (MHOM/BR/74/PP/75), supplied by the laboratory of Leishmaniasis of the René Rachou Research Center (Belo Horizonte, Brazil).

Statistical analysis

To evaluate the agreement between serological techniques (ELISA, IFAT and DPP®), the Kappa coefficient (κ) was calculated with confidence interval of 95%. The test was performed by *software* Bioestat version 5.3 (AYRES et al., 2007). Values of κ were considered according to SOLANO-GALLEGO et al. (2014) as: no agreement (κ<0), slight agreement (0< κ < 0.2), fair agreement (0.2< κ <0.4), moderate agreement (0.4< κ < 0.6), substantial agreement (0.6< κ <0.8) and almost perfect agreement (κ > 0.8).

RESULTS

The total number and percentages of positive animals in serological tests and parasitological examination according to clinical symptomatic or asymptomatic status are shown in table 1. The IFAT revealed 56/92 seropositive, the ELISA 8/92, and the DPP® 41/92. Regarding the agreement between the serological tests by the Kappa index, there was a slight agreement between ELISA/IFAT and ELISA/DPP, and fair agreement in IFAT/DPP (Table 2).

Amastigote forms were observed in the Giemsa stained smears of the bone marrow from three dogs (3/21). Promastigote forms were also observed in three cultures seeded with the spleen and/or liver fragments of the inoculated hamsters. Flagellates from the positive cultures samples were identified by PCR as *Leishmania infantum chagasi*. It is noteworthy that three dogs (3/92) were positive in all tests performed. No flagellates were observed in the peripheral blood by Woo's technique. The photograph of imprint smear from bone marrow of a positive dog demonstrating the amastigote forms and the electrophoresis in agarose gel are available in this research as supplementary material (Figure 2; Figure 3).

In the canine survey, five (5.4%) dogs showed clinical signs suggestive of CVL, such as weight loss, alopecia and onychogryphosis, and were positive in all serological tests, while 87 (94.6%) were asymptomatic. In other words, they did not present any of the symptoms described above. Of the 87 asymptomatic dogs investigated, 36 (41.4%)were positive in at least one of the diagnostic tests performed.

DISCUSSION

In Mato Grosso do Sul, a large number of seropositive asymptomatic dogs have been reported, both in rural settlements and urban areas of Bonito District (NUNES et al., 2001; ANDRADE et al., 2009) in Anastácio, a municipality adjacent to Bonito (CORTADA et al., 2004) and Campo Grande (MATO GROSSO DO SUL, 2014). Similar results were reported in Mato Grosso State (MATO GROSSO DO SUL, 2014; DE SANTIS et al., 2013) and other areas in the Brazilian south-eastern and north-eastern regions (DANTAS-TORRES et al., 2006; COURA-VITAL et al., 2011). Some studies suggested that animals without apparent clinical signs of CVL could be the source of *Leishmania* for phlebotomine sandflies (MORENO & ALVAR, 2002; LAURENTI et al., 2013); although, VERÇOSA et al. (2008) have demonstrated that asymptomatic dogs are not efficient sources of *Leishmania* for vectors.

Low agreement between the serological tests in our study can also be observed in researches carried out in Monte Negro – Rondônia (RO) State (AGUIAR et al., 2010), between ELISA and IFAT test, in Panorama - São Paulo (SP) State (LOPES et al., 2017), between ELISA and DPP tests. However, also high and moderate agreements have already been observed between the ELISA and IFAT tests in other localities of Brazil, as in Campo do Goycatazes - Rio de Janeiro State (TÁVORA et al., 2007) and Ilha Solteira (SP) (ASSIS et al., 2010; QUEIROZ et al., 2010). These observations demonstrated the need to better assess the diagnostic techniques of CVL, aiming at standardization, considering the evolution of the disease in the dog and its immune response.

Table 1 - Number and percentage of positive dogs in serological techniques for *Leishmania* by clinical status, Águas do Miranda District, Bonito, Mato Grosso do Sul, Brazil, 2009.

Clinical status	ELISA		IFAT		DPP®		ELISA, IFAT and DPP®	
	n	%	n	%	n	%	n	%
Asymptomatic (n=87)	3	37.5	51	91.0	36	87.8	2	28.6
Symptomatic (n=5)	5	62.5	5	9.0	5	12.2	5	71.4
Total (n=92)	8	8.7	56	60.8	41	44.5	7	7.6

ELISA: enzyme-linked immunosorbent assay; IFAT: indirect immunofluorescence antibody test; DPP®: Dual Path-Platform test.

Table 2 - Agreement analysis by Kappa index between serological techniques (ELISA/IFAT, ELISA/DPP and IFAT/DPP) for diagnosis of canine visceral leishmaniosis (CVL) in domestic dogs, Águas do Miranda, Bonito, Mato Grosso do Sul, Brazil.

Comparison between tests	+/+	+/-	-/+	-/-	Total	κ	CI	p	Agreement
ELISA/IFAT	8	0	48	36	92	0.115*	-0.214-0.445	0.008	Slight
ELISA/DPP	7	1	34	50	92	0.164*	-0.135-0.464	0.005	Slight
IFAT/DPP®	34	22	7	29	92	0.384*	0.190-0.578	<0.001	Fair

+: Positive; -: Negative; * Statistically significant (P ≤ 0.05), confidence interval 95%. ELISA: enzyme-linked immunosorbent assay; IFAT: indirect immunofluorescence antibody test; DPP®: Dual Path-Platform test.

In this research, the use of molecular tools was only possible in positive samples of NNN cultures. Previous studies have shown that the combination of serological and molecular techniques increase the detection of the parasite in the canine population, indicating failures in the official protocol of diagnosis of CVL in Brazil (QUEIROZ et al., 2010; LOPES et al., 2017). However, molecular tests are not available in all laboratories in the public system, and there is still no gold standard for the diagnosis of CVL, since for this technique there is the application of different protocols.

Until the year 2011, the ELISA and IFAT are methods recommended by the Brazilian Health Ministry (BHM) for CVL diagnosis. The ELISA was employed for screening large populations, and

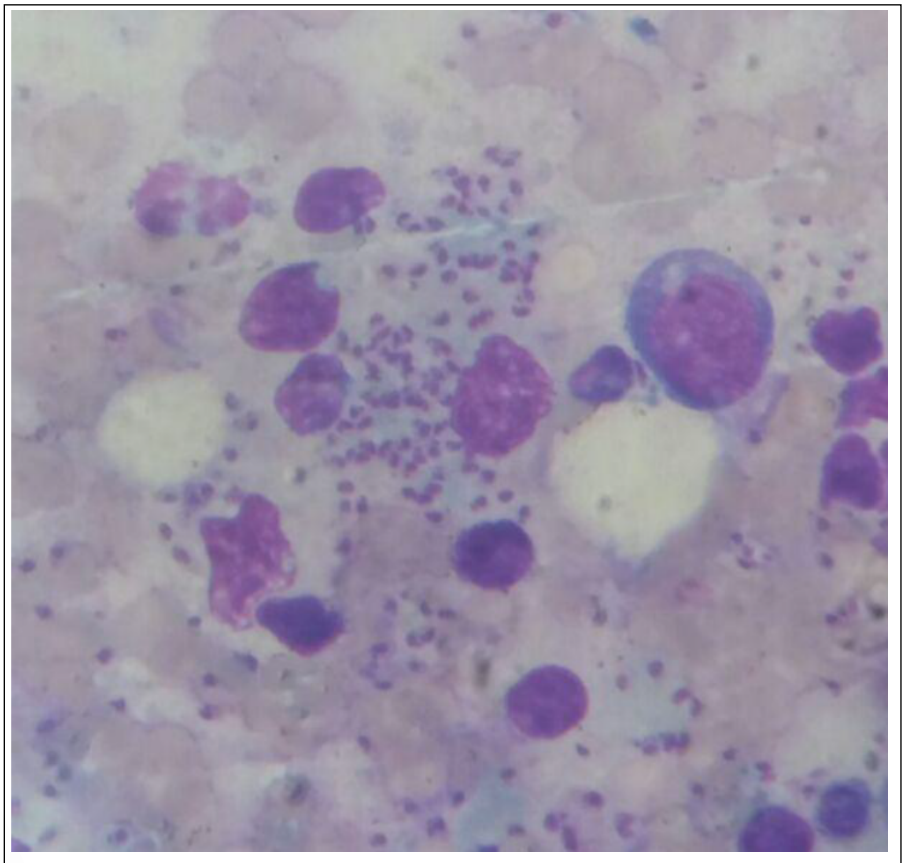

Figure 2 - Imprint smear of the bone marrow with amastigotes forms (Giemsa 100×).

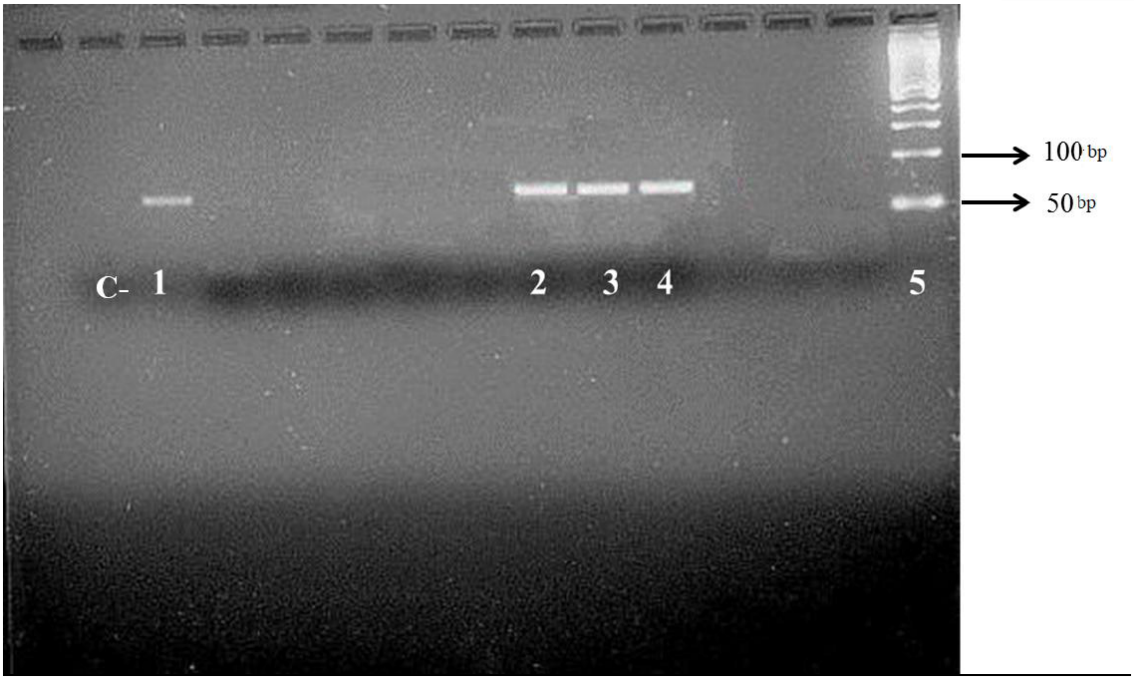

Figure 3 - PCR amplification products with primers RV1/RV2. C-: Negative control, MilliQ water; 1 Positive control, reference strain *Leishmania* (*Leishmania*) *chagasi* (MHOM/BR/74/PP/75); 2, 3 e 4: positive dog samples; 5: molecular marker 50bp.

the IFAT for the confirmatory diagnosis. After some studies, and through a BHM technical note (Nota Técnica nº 01/2011 – CGDT/CGLAB/DEVIT/SVS/MS), this context has been modified to DPP® for screening and ELISA for confirmatory, allowing this screening to be performed in all Brazilian municipalities (BRASIL, 2014).

However, if in our study we had adopted the strategy recommended by the BMH, a low number of infected dogs, as confirmed by ELISA (8/92), would have escaped detection. Regarding the DPP®, it is considered easy to apply in field research. In the studies undertaken by GRIMALDI JÚNIOR et al. (2012), this test showed high sensitivity in symptomatic dogs. This result is corroborated in our study since all symptomatic animals were positive in this diagnostic test. The differences reported in the present study between the reactivity of the serum samples may result from false positive reactions due to cross-reactivity with agents other than *Leishmania* and the sensitivity thresholds of other tests (LIRA et al., 2006; SANTOS et al., 2010). However, some studies have reported no cross-reactivity between *Leishmania* and other pathogens (OLIVEIRA et al., 2008; GUIMARÃES et al., 2009). Another possibility is that the levels of anti-*Leishmania* antibodies were below the cut-off point used as positive, as shown by SILVA et al. (2009), which IFAT assay using a 1:20 dilution showed a better contingency coefficient.

The use of the Woo technique is justified, given the occurrence of *Trypanosoma evansi* in domestic animals at the Bodoquena Plateau, in the Pantanal of the Mato Grosso do Sul and in Bonito Municipality (STEVENS et al., 1989; NUNES et al., 1994; SAVANI et al., 2005), also present in a dog from the rural area of the São Paulo State (COELHO et al., 2013).

The isolation of the parasite by NNN culture has its importance for obtaining parasite "in-mass" for use of techniques such as monoclonal antibodies and biochemical characterization, as observed in studies by DE SANTIS et al. (2011).

In Águas do Miranda, the evidence of dogs carrying *L. infantum chagasi* and the presence of *Lutzomyia longipalpis* circulating in the peridomicile environment (BRILHANTE et al., 2015a) together with insufficient knowledge of the population about the disease, its severity and the forms of transmission, points to the possibility of introducing visceral leishmaniasis to the residents of that district, as well as for tourists, like other areas of canine cases preceding human cases of parasitosis (GONTIJO & MELO, 2004).

CONCLUSION

Our results showed low agreement between the serological tests recommended by the BHM, and it is necessary to associate other diagnostic techniques, such as parasitological tests and PCR, to increase the sensitivity for CVL diagnosis, mainly regarding the asymptomatic dogs in endemic areas. Although, these methods are expensive and require high professional qualification, the proposal should be discussed among Brazilian health authorities, since euthanasia of positive dogs is one of the main measures of control of VL in Brazil.

In addition, our findings may help the surveillance agencies in Bonito, because the high prevalence of seropositive dogs in this endemic region may contribute to an increase in the prevalence of infection in humans, since the municipality of Bonito is considered a moderate transmission area of leishmaniasis.

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BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was approved by the Ethics Committee of Animal Use of the Universidade Anhanguera/Uniderp, Unidade Agrárias, Campo Grande, MS (authorization: 76-011/09-MS). Results of this research were sent to the Center of Zoonosis Control of Bonito Municipality, so that control measures could be taken.

DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflicts of interest.

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