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Major Article

Serodiagnosis of visceral and cutaneous leishmaniasis in human and canine populations living in Indigenous Reserves in the Brazilian Amazon Region

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Abstract

Introduction: Leishmaniasis is endemic to the Northern, Northeastern, Central-Western, and Southeastern regions of Brazil. We aimed to assess the epidemiological situation of leishmaniasis in humans and dogs in indigenous villages located in the States of Mato Grosso and Tocantins using a serological survey conducted in May 2011. **Methods:** Serum samples were collected from 470 humans and 327 dogs living in villages of the Urubu Branco and Tapirapé Karajá indigenous reserves. The samples were analyzed for the presence of *Leishmania* spp. antibodies using the indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) with a crude antigen (CA) and soluble antigen (SA), and Dual Path Platform (DPP®) immunoassay for canine visceral leishmaniasis. **Results:** Of 470 human samples tested, two (0.4%) were positive using IFAT. Among 327 dog samples tested, 28 (8.6%) were positive using ELISA CA, five (1.5%) using ELISA SA, two (0.6%) using IFAT, and none using DPP® immunoassay with *Leishmania infantum chagasi* antigen. When *Leishmania amazonensis* antigen was used, 20 (6.1%) samples were positive using ELISA CA and four (1.2%) using IFAT. **Conclusions:** There was a low prevalence of infection in the region, and significant differences among the main serological methods used for the diagnosis of leishmaniasis. These findings indicated that the detection of *Leishmania* spp. requires further study and improvement.

Keywords: Trypanosomatid. Zoonosis. Reservoirs. Diagnosis. Indigenous.

INTRODUCTION

Leishmaniasis is a chronic anthropozoonosis with a global distribution, and it is estimated that 0.2-0.4 million new visceral leishmaniasis (VL) cases and 0.7-1.2 million new cutaneous leishmaniasis (CL) cases occur each year worldwide[1]. In Brazil, the cutaneous form of the disease is caused mainly by *Leishmania braziliensis*, *Leishmania guyanensis,* and *Leishmania amazonensis*, while *Leishmania infantum chagasi* is responsible for the visceral form[2,3]. These diseases are endemic in the Northern, Northeastern, Central-Western, and Southeastern regions of Brazil, making it necessary to conduct serological investigations with the aim of understanding the epidemiological situation of the infection in areas with active transmission or transmission potential[4,5].

Dogs are very susceptible to VL infection and, because of their close relationship with humans, are considered to be the most important reservoirs for disease transmission to humans[6]. In the case of CL, for which domestic animals are considered to be accidental hosts, some species of rodents, marsupials, edentates, and wild canids have been recorded as hosts and possible natural reservoirs for these species of *Leishmania*[3].

Although there have been many reports of leishmaniasis in dogs in different regions of Brazil, records of infections in dogs in Brazilian indigenous villages are scarce. In Brazil, both forms of leishmaniasis affect the indigenous population: an average of 366 cases of CL and 20 cases of VL are added to the Information System for Notifiable Diseases yearly[7]. Knowledge of the epidemiological situation of this disease is of great importance so that prevention and control measures can be put in place by healthcare authorities.

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With the above in mind, we performed a serological survey among humans and dogs living in indigenous villages in the Amazon region of Brazil to identify endemic areas for *Leishmania* spp. infection.

METHODS

Ethical considerations

This study was approved by the Research Ethics Committee of the National Indian Foundation [*Fundação Nacional do Índio* (FUNAI) - protocol number 08620.002433/2007-99]. The use of animals was followed the Ethical Principles for Animal Research adopted by the Ethics Committee in the Use of Animals of Faculty of Veterinary Medicine, University of São Paulo (protocol number 1745/2009).

Study area

The Urubu Branco indigenous reserve is located in the municipality of Confresa (S 10°38'57.0"/ W 51°34'12.6"), Mato Grosso State, in the Central-Western region of Brazil. It includes seven villages: Urubu Branco (S 10°40'16.1"/W 51°21'13.8") (the main village), Sapeva (S 10°48'12.2"/W 51°17'12.8"), Córrego da Onça (S 10°42'27.5"/W 51°18'49.0"), Buriti II (S 10°39'33.6"/W 51°17'41.2"), Codebra (S 10°52'09.6"/W 51°15'37.8"), Xexéu (S 10°51'20.6"/W 51°18'19.8"), and Santa Laura (S 10°36'23.9"/W 51°10'47.9"). In the Tapirapé Karajá indigenous reserve, the villages covered were Majtyri (S 10°39'15.36"/W 50°36'10.39"), Itxala (S 10°37'30.97"/W 50°37'30.73"), and Hawalora (S 10°38'26"/W 50°37'20"). We also included the municipality of Santa Terezinha (S 17°32'00.00"/ W 56°53'00.00") in Mato Grosso State and Macaúba village (S 10°28'18"/ W 50°28'59"), located on Ilha do Bananal (S 10° 32′ 45″/ W 50° 14′ 36″), Tocantins State, Northern region of Brazil (**Figure 1**). These indigenous groups were selected because they have had prior contact with researchers, making it easier to perform research.

Serodiagnosis in the human population

The studied indigenous population comprised 655 individuals[8] inhabiting the municipalities of Confresa and Santa Terezinha in Mato Grosso. Serum samples were collected from 470 individuals (71% of total population). All people aged > 3 years who were living in the Urubu Branco indigenous reserve and were present in the villages during the campaign

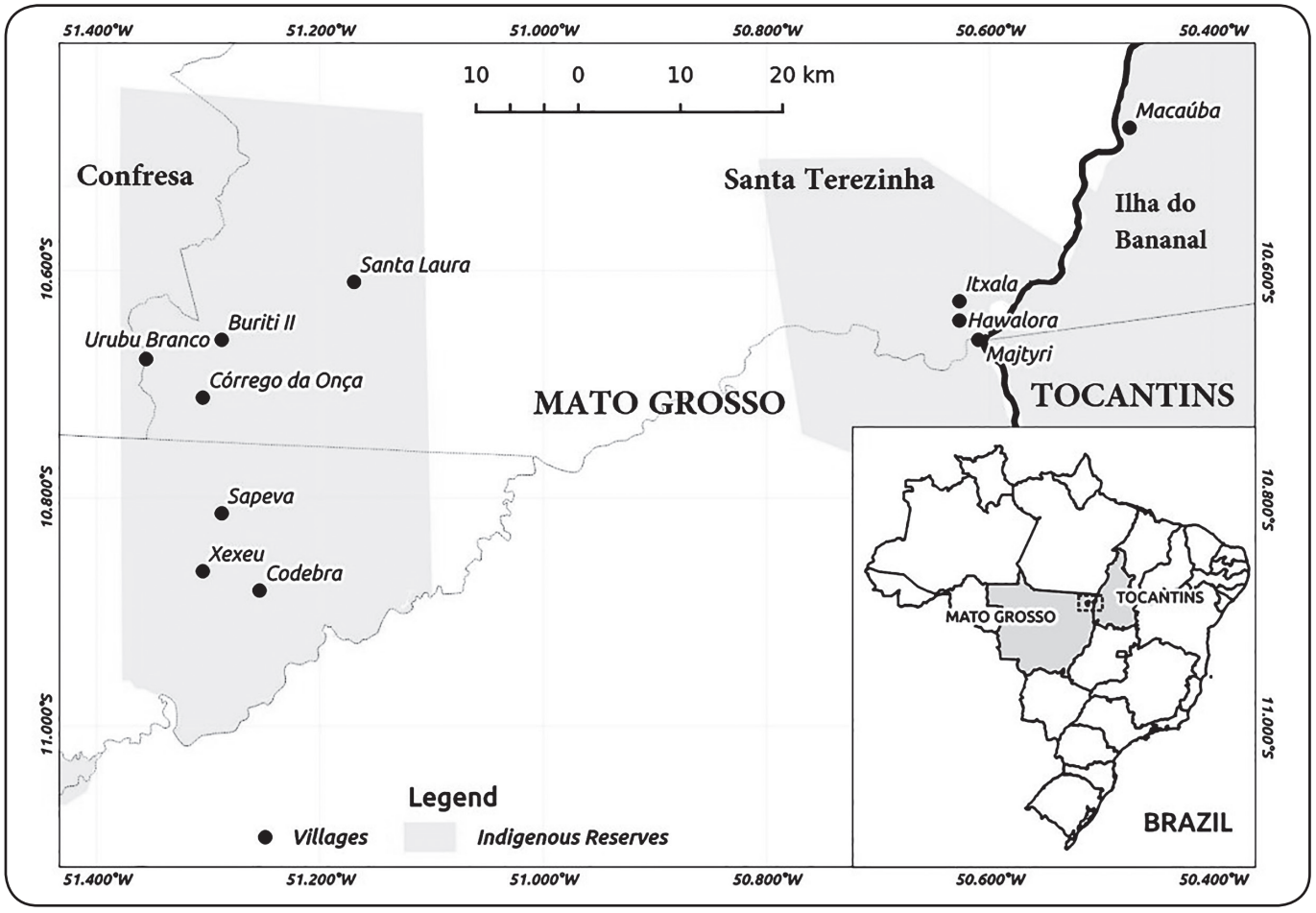

FIGURE 1. Location of the villages in the Urubu Branco (at left) and Tapirapé Karajá (at right) indigenous reserves within Mato Grosso and Tocantins, Brazil.

were eligible for participation. An indirect fluorescent antibody test (IFAT) was performed as previously described[9] for *L. infantum chagasi* and *L. amazonensis* antibody detection. In other indigenous reserves, the caciques did not authorize us to collect material from the human population.

Serodiagnosis in the canine population

Serum samples were collected from 114 dogs living in villages in the Urubu Branco indigenous reserve and 213 dogs living in villages in the Tapirapé Karajá indigenous reserve (327 animals). This number represents all dogs, both young and adult, of both genders, inhabiting and present in these villages during the campaign. Serum samples were identified and stored at -20ºC until analysis. For *L. amazonensis*, the enzyme-linked immunosorbent assay with crude antigen (ELISA CA) and IFAT techniques were performed. For *L. infantum chagasi,* ELISA CA, ELISA with soluble antigen (SA) (soluble antigen), IFAT, and Dual Path Platform (DPP®) CVL (Bio-manguinhos, Fiocruz – RJ) tests were performed. The samples that were positive according to one of the three methods were considered positive, whether from a human or dog.

Enzyme-linked immunosorbent assay with crude antigen

The *L. infantum chagasi* strain MHOM/BR/72/strain46 was isolated from a human case of VL that arose in the municipality of Mantena, and was characterized using monoclonal antibodies[10] and isoenzymes[11] at the Evandro Chagas Institute (IEC), Belém, Brazil. It was then maintained in hamsters with subcultures taken every two to three months. The *L. amazonensis* strain MHOM/BR/73/M2269 was isolated from a human case of anergic diffuse cutaneous leishmaniasis identified in the municipality of Belém and characterized at the IEC using the same methods. For antigen production, the parasites were isolated in Schneider culturing medium with 10% heat-inactivated fetal bovine serum. After two to three passages through cultures, the promastigote forms in the stationary phase of culturing were washed three times in sterile phosphate-buffered saline (PBS) solution and centrifuged at 1,620g at 4°C for 10 minutes. To prepare the antigen, the precipitate containing the promastigote forms was frozen in liquid nitrogen and defrosted at room temperature (three consecutive times) and then sonicated at power level 4 in a 1-minute cycle (Sonic Dismembrator, Fisher Scientific, USA). Protein assays were performed using the Bradford method (Bio-Rad Protein Assay, Hercules, USA).

After antigens were obtained, the ELISA technique was performed for each of the antigens as previously described[12]. Reference serum samples from the serum bank of the Laboratory of Parasitic Diseases in the Department of Preventive Veterinary Medicine and Animal Health at the College of Veterinary Medicine (FMVZ), University of São Paulo (USP), were included as negative and positive controls. Serum was considered positive if the optical density (OD) was 3 standard deviations higher than the mean OD of the negative controls.

Enzyme-linked immunosorbent assay using soluble antigen

The sample of *L. infantum chagasi*[13] was isolated from bone marrow aspirate from a naturally infected dog in the municipality of Olympia, São Paulo, and was characterized using molecular methods[14,15]. The parasites were cultured in Roswell Park Memorial Institute (RPMI) liquid medium supplemented with 10% heat-inactivated fetal bovine serum and, after two to three passages, the promastigote forms in the stationary phase of culturing were centrifuged at 735*g* for 10 minutes and the supernatant was discarded. Subsequently, the parasites were subjected to two washes using incomplete RPMI medium and one wash using homogenization buffer -0.25M sucrose, 1mM ethylenediaminetetraacetic acid (EDTA), and 5mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl). They were then counted on a Neubauer chamber to reach a concentration of 2×10^6 promastigotes/mL. After counting, they were resuspended in homogenization buffer. The parasite suspension was then subjected to seven freezing cycles at -70°C, followed by defrosting at 37°C. The resulting suspension was centrifuged at 12,000*g* for 30 minutes at 4°C. The protein content of the soluble antigen was determined using the bicinchoninic acid assay (BCA) using a BCA reagent kit (Pierce Chemical Company, USA) in accordance with the manufacturer's recommendations.

After the antigen had been obtained, the ELISA test was performed as described previously[16] for *Babesia bovis*, with small modifications[17]. Reference serum samples from the serum bank of the Laboratory of Parasitic Diseases in the Department of Preventive Veterinary Medicine and Animal Health at the *Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo* (FMVZ-USP) were included as negative and positive controls. Serum was considered positive if the OD was 2.5 standard deviations higher than the mean OD of the negative controls.

Indirect fluorescent antibody test

The *Leishmania infantum chagasi* (CBT56 – Coleção Brasileira de Tripanossomatídeos FMVZ/USP) sample was isolated from a popliteal lymph node from a naturally infected dog in the city of Cuiabá, and was characterized using molecular methods[18]. The *L. amazonensis* strain IFLA/BR/1967/PH8 was obtained courtesy of the Leishmaniasis Research Laboratory, Oswaldo Cruz Foundation, and forms part of the Oswaldo Cruz Institute's *Leishmania* Collection. The parasites were cultured in a biphasic medium composed of a blood agar base with 15% defibrinated sheep blood and liquid liver infusion tryptose medium supplemented with 10% heat-inactivated fetal bovine serum. After two to three culturing passages, the promastigote forms in the stationary phase were washed three times in sterile PBS and centrifuged at 1,620g at 4°C. The precipitate at the bottom of the tube was resuspended in buffered formalin solution at 2% and kept at 4°C for 24 hours. After fixation of the promastigote forms, these were washed again in sterile PBS and counted in a Neubauer chamber. The parasite concentration was adjusted to 2×10^6 promastigotes/mL, and 20µL per well were applied to immunofluorescence slides. These slides were dried at room temperature and stored at -20°C until needed.

After the antigens had been obtained, the IFAT technique was performed on each of the antigens as described previously, with a cutoff value of 1:40[9]. Reference serum samples from the serum

bank of the Laboratory of Parasitic Diseases in the Department of Preventive Veterinary Medicine and Animal Health at the FMVZ, USP, were included as negative and positive controls.

Dual Path Platform® - canine visceral leishmaniasis

The rapid DPP® CVL assay is a chromatographic immunoassay for detecting specific antibodies for *Leishmania* in the serum, plasma, or venous whole blood of dogs. The antigen used in this test is a fusion of the recombinant proteins K26 and K39, which is specific for *L. infantum chagasi*. The marker used is a combination of protein A, extracted from *Staphylococcus aureus*, which has high affinity for immunoglobulins, conjugated with colloidal gold particles. These are both bonded to a nitrocellulose membrane[19]. The test was performed in accordance with the manufacturer's recommendations. The positive control serum was provided in the DPP® CVL kit.

Statistical analysis

To evaluate the concordance index between the ELISAs performed with different antigen preparations, the kappa test[20] was used according to previously suggested criteria[21], in which zero indicates absence of concordance and one equals total concordance.

RESULTS

Among the 470 indigenous inhabitants analyzed using IFAT, only two (0.4%) presented reactive results when tested for the *L. amazonensis* antigen at the dilution level of 1:40. Both were females, with different ages (5 and 25 years), living in different villages (Urubu Branco and Santa Laura). None of the individuals sampled yielded titers when tested for the *L. infantum chagasi* antigen.

Among the 114 dogs living in the Urubu Branco indigenous reserve, five (4.4%) presented reactive results for *L. infantum chagasi* when tested using the ELISA CA technique, versus three (2.6%) that presented with optical density (OD) higher than the cutoff point when tested using the ELISA SA technique. IFAT showed that two dogs (1.8%) were reactive to *L. infantum chagasi* at the 1:40 dilution level. Only one sample presented reactive results for all three techniques. Excluding the positive sample in the three techniques, the others were negative in all techniques or positive in only one. None of these samples presented positive results when tested using the DPP® CVL.

When tested for *L. amazonensis* using the ELISA CA technique, four dogs (3.5%) yielded positive results. Using IFAT, two (1.8%) samples presented positive fluorescence at the 1:40 dilution level, and one of these was also reactive using ELISA CA. The five samples positive to any of these techniques when using the *L. amazonensis* antigen were also positive to at least one technique when using the *L. infantum chagasi* antigen.

In the villages of the Tapirapé Karajá indigenous reserve, 213 animals were sampled, and the ELISA CA test showed that 23 (10.8%) were reactive to *L. infantum chagasi*, while ELISA SA showed that only two (0.9%) were reactive. The two animals that were reactive to ELISA SA were also reactive to ELISA CA. However, all samples presented negative results when tested using the IFAT and DPP® CVL.

When used to test samples for *L. amazonensis*, ELISA CA showed that 16 (7.5%) samples presented OD higher than the cutoff point, and two (0.9%) also presented positive fluorescence when tested using the IFAT. Only two samples that tested positive for *L. amazonensis* using ELISA CA were negative for any of the techniques using *L. infantum chagasi*.

Table 1 presents the number of dogs positive for antibodies against *L. infantum chagasi* and *L. amazonensis* using the ELISA, IFAT, and DPP® CVL techniques.

In total, among the 28 dog samples that were positive in the ELISA CA test for *L. infantum chagasi*, three were also positive in the ELISA SA test and only one sample was reactive using IFAT. When using *L. amazonensis* antigen, among the 20 samples that were positive using ELISA CA, only three were positive when tested using IFAT.

TABLE 1

Number of dogs from the Urubu Branco and Tapirapé Karajá indigenous reserves examined and positive to antibodies to *Leishmania infantum chagasi* and *Leishmania amazonensis* by different techniques in Mato Grosso and Tocantins.

Indigenous reserves	Examined dogs (n)	Positive dogs (n)							
		Leishmania infantum chagasi					*Leishmania amazonensis*		
		ELISA CA	ELISA SA	IFAT (1:40)	ELISA CA + ELISA SA + IFAT (1:40)	DPP CVL	ELISA CA	IFAT (1:40)	ELISA CA + IFAT (1:40)
Urubu Branco	114	5	3	2	1	0	4	2	1
Tapirapé Karajá	213	23	2	0	0	0	16	2	2
Total	327	28	5	2	1	0	20	4	3

ELISA CA: enzyme-linked immunosorbent assay using crude antigen; **ELISA SA:** enzyme-linked immunosorbent assay using soluble antigen; **IFAT:** indirect fluorescent antibody test; **DPP CVL:** Dual Path Platform® for canine visceral leishmaniasis.

The concordance between tests performed with different antigen preparations to ascertain the presence of anti-*L. infantum chagasi* antibodies was evaluated by determining the kappa coefficient, which showed that there was poor concordance ($k = 0.16$) between ELISA CA and ELISA SA results.

To analyze the occurrence of CVL , the results obtained using DPP® CVL were used because this test is part of the official diagnostic protocol for this disease in Brazil[22]. To analyze the occurrence of infection due to *L. amazonensis* in dogs, reactive samples from ELISA CA and IFAT were both taken into consideration, given that both of these methods are recommended by the Ministry of Health[3].

DISCUSSION

In this study, we observed that the two humans who tested positive for *L. amazonensis* were negative when tested for *L. infantum chagasi*, demonstrating that there was likely no cross-reactivity between the two antigens. In dogs, of the 21 samples that were positive for *L. amazonensis* using any technique, only two were negative when tested for *L. infantum chagasi*. In this case, co-infection or cross-reaction may have occurred.

In the State of Mato Grosso, although the phlebotomine species *Lutzomyia longipalpis* and *Lutzomyia flaviscutellata* (the ones most frequently correlated with the *Leishmania* species) are present,[3] low dissemination of the infection was found amongst the indigenous population of Tapirapé ethnicity. In the present study, IFAT performed on serum samples from humans showed that only two individuals were reactive to the *L. amazonensis* antigen. None of the samples showed reactivity for *L. infantum chagasi*. These results corroborate the data provided by the epidemiological surveillance program of the *Secretaria de Saúde* of the State of Mato Grosso during the period 2006-2008, in which only three cases of indigenous CL were recorded in the municipality of Confresa, Mato Grosso[23], where the Urubu Branco indigenous reserve is located. In the municipalities of Santa Terezinha, Mato Grosso, and Ilha do Bananal, no epidemiological data concerning VL in humans or dogs have been recorded.

Based on the results obtained by DPP® CVL in the present study, serologic VL was absent from dogs in the Urubu Branco and Tapirapé Karajá indigenous reserves. However, the other serological tests used in this study showed positive results, suggesting that the DPP® CVL test may not be the best choice for screening in this context, as has been recommended by the Ministry of Health. These results corroborate those found in a previously reported study[19], which observed low sensitivity (50%) of the DPP® CVL in the detection of asymptomatic infected dogs.

Regarding infection by *L. amazonensis* in these animals, we found a seroprevalence of 0.9% in both indigenous reserves. However, these results are of little relevance because dogs do not play an important role in the epidemiology of CL[24].

The low prevalence observed in the present study can be attributed to a variety of factors, both environmental and ecological. Little is known about the role that wild animals may serve as reservoirs for leishmaniasis in this region. Furthermore, the inhabitants of the Urubu Branco indigenous reserve do not generally raise animals such as chickens, pigs, or small ruminants for consumption. Besides being attractive to phlebotominae themselves, these animals lead to the accumulation of organic material, thereby contributing to maintaining the vector in that location.

There is some controversy regarding the efficacy of serological methods for diagnosing VL. These techniques often produce false-positive results, leading to the culling of healthy animals, or false-negative results, contributing towards maintaining the reservoirs in endemic áreas[25]. The platforms for the serodiagnosis of *Leishmania* spp. depend greatly on the standardizations and adaptations that are used in each laboratory. These methods tend to be created according to the preferences of each laboratory, and may cause discordant results between different laboratories. In addition, crude antigen preparations made from whole promastigotes or their soluble extracts limit the standardization of testing and the reproducibility of results[26-28]. When using ELISA as a serological test, we found significantly conflicting results when different antigen preparations were used.

The antigens used in the ELISA CA method in this study corresponded to the total product from the promastigote forms of *Leishmania*. In contrast, the antigen used for ELISA SA corresponded to the water-soluble fraction of the total antigenic extract of the promastigote forms of the parasite. In general, the use of total fractions of antigens compromise the specificity of serodiagnostic methods because these fractions are composed of many antigenic components of the parasite, which shares epitopes with other pathogens that may be involved in the immunological profile of the animals analyzed[29]. In addition, different strains of *L. infantum chagasi* were used for each ELISA test. The fact that different fractions and strains were used may explain the low concordance rate between the ELISA methods used.

Some cases that were seropositive according to IFAT were negative according to ELISA. This was an unexpected finding because ELISA tends to be more sensitive than IFAT, particularly in the case of leishmaniasis serodiagnosis[30]. These findings corroborate the fact that the techniques normally used for leishmaniasis serodiagnosis offer low reliability and accuracy.

In conclusion, the indigenous areas studied yielded a low occurrence rate of leishmaniasis, probably due to the low degree of modification undergone by the villages. It is also likely that the low number of production animals in the villages contributed to the low occurrence of *Leishmania* spp. infections in the Urubu Branco and Tapirapé Karajá indigenous reserves.

The serological tests commonly used for the diagnosis of leishmaniasis offer low reliability and accuracy, indicating that there is a need for standardized techniques and studies of the correlations between direct and indirect laboratory tests in order to allow safer diagnosis in the future.

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Conflict of interest

The authors declare that there is no conflict of interest.

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