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Short Communication

Comparison of serological and molecular tests to investigate *Leishmania* spp. infections in stray dogs from an area of intense visceral leishmaniasis transmission in Brazil

Comparação de testes sorológicos e moleculares para investigar infecções por *Leishmania* spp. em cães errantes de uma área de intensa transmissão de leishmaniose visceral no Brasil

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Abstract

The aim of this study was to investigate the level of exposure to *Leishmania* infection in stray dogs in an area of intense visceral leishmaniasis transmission in the state of Pernambuco, Brazil. Blood samples from 178 dogs were analyzed using serological and molecular assays: rapid immunochromatographic test (ICT), enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), and conventional and quantitative polymerase chain reaction (cPCR and qPCR). Positivity values obtained with serological tests were 71.4% (127/178), 70.2% (125/178), and 50.6% (90/178) using ICT, ELISA, and IFAT, respectively, with 38.8% (69/178) of the dogs were simultaneously positive for all three tests. The positivity values obtained with cPCR and qPCR were 20.2% (36/178) and 38.8% (69/178), respectively, with 11.8% (21/178) testing positive in both molecular assays. Overall, 87.1% (155/178) were positive for anti-*Leishmania* spp. antibodies and/or *Leishmania* spp. DNA. Positivity to one or more tests was statistically associated with lymphadenomegaly, skin lesions, lymphocytosis, anemia and hyperproteinemia. The results of this study revealed a high level of exposure to *Leishmania* in stray dogs in an area of intense human visceral leishmaniasis transmission, suggesting that dogs play a role as reservoirs in the transmission cycle of this zoonosis.

Keywords: Leishmaniasis, *Canis familiaris*, zoonoosis, diagnosis, epidemiology.

Resumo

O objetivo deste estudo foi investigar o nível de exposição à infecção por *Leishmania* em cães errantes de uma área de transmissão intensa de leishmaniose visceral no estado de Pernambuco. Amostras de sangue de 178 cães foram avaliadas por testes sorológicos e moleculares: teste imunocromatográfico (IC), ensaio imunoenzimático (ELISA), teste de imunofluorescência de anticorpos (RIFI), reação em cadeia da polimerase convencional e quantitativa (cPCR e qPCR). Os valores de positividade obtidos com os testes sorológicos foram 71,4% (127/178), 70,2% (125/178) e 50,6% (90/178) com IC, ELISA e RIFI, respectivamente. E com 38,8% (69/178) dos cães mostraram-se simultaneamente positivos aos três testes. Os valores de positividade obtidos por cPCR e qPCR foram 20,2% (36/178) e 38,8% (69/178), respectivamente. E com 11,8% (21/178) dos cães foram

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positivos em ambos os testes moleculares. No geral, 87,1% (155/178) foram positivos para anticorpos anti-*Leishmania* spp. e/ou material genético de *Leishmania* spp. A positividade a um ou mais testes foi estatisticamente associada à linfonodomegalia, lesões cutâneas, linfocitose, anemia e hiperproteinemia. Os resultados do estudo revelam um alto nível de exposição à *Leishmania* spp. em cães errantes em uma área de transmissão intensa de leishmaniose visceral humana, sugerindo que eles podem desempenhar um papel como reservatórios no ciclo de transmissão dessa zoonose.

Palavras-chave: Leishmaniose, *Canis familiaris*, zoonoose, diagnóstico, epidemiologia.

Zoonotic visceral leishmaniasis (VL) is a neglected disease caused by *Leishmania infantum* in the Mediterranean Basin, the Middle East, Central Asia, South America, and Central America (Alvar et al., 2012). In Brazil, the largest focus of the disease in the Americas, VL is widespread and occurs in both rural and urban areas. This may be partly explained by the wide distribution range of *Lutzomyia longipalpis*, the primary vector of *L. infantum* in Brazil, and the frequency with which it is found in the presence of infected dogs (Dantas-Torres & Brandão-Filho, 2006).

While VL is endemic to all Brazilian regions, most human cases come from northeastern Brazil, where the disease is strongly linked to poverty (Machado et al., 2020). In this region, dogs are also highly exposed to *Leishmania* spp. The level of exposure may range widely and may be higher than 50% in highly endemic foci (Fraga et al., 2012; Dantas-Torres et al., 2020).

Differences in terms of exposure levels may be influenced factors, including features of the studied canine population and diagnostic tests used in the study. For instance, in a recent study carried out with owned dogs in the semi-arid region of Pernambuco, Evaristo et al. (2020) combined two serological tests and reported an overall positivity of 42.8%, ranging from 29.8% to 55.8%, depending on the municipality. Intriguingly, these values were much higher than data (i.e., 11.2%) previously reported in owned dogs in Petrolina (Araujo et al., 2016), which is also located in the semi-arid region of Pernambuco. Furthermore, Petrolina is an area of intense human VL transmission (i.e., annual average of ≥ 4.4 human VL cases in the past five years) according to the regional epidemiological risk, based on a Brazilian Ministry of Health algorithm (Brasil, 2014). Evaristo et al. (2020) suggested that the higher positivity in comparison with that reported by Araujo et al. (2016) could be related to the diagnostic tests used in both studies. To investigate this hypothesis, a cross-sectional study was conducted to investigate the level of exposure to *Leishmania* spp. infection in stray dogs in Petrolina by combining various serological and molecular assays. Additionally, clinical signs and blood count abnormalities in positive dogs were also investigated.

Located in the semi-arid region of Pernambuco, Petrolina (9°23'55" S, 40°30'03" W) occupies an area of 4,561.872 km^2 and is home to 354.317 inhabitants. This municipality is situated within the Caatinga biome, but most of its residents live in the urban area of the municipality. From 2009 to 2019, 189 human VL cases were notified in this municipality (SINAN, 2021) and *Lutzomya longipalpis* is the main vector occurring in the region (Araujo et al., 2016).

From August 2014 to July 2015, blood samples were collected from 178 adult stray dogs captured in Petrolina. Due to absence of data from local zoonosis control center, it was not possible determine with precision if the dogs were captured in the urban, rural or periurban area. There were no inclusion/exclusion criteria with regard to sex and breed.

Approximately 4 mL of blood was withdrawn from each dog and divided into two tubes (one with and another without EDTA). Whole blood was used for blood count and DNA extraction. EDTA-free blood samples were centrifuged at 1,800 *g* for 15 min, and the obtained sera were stored at –20 °C until serological testing. All procedures used in this study were approved by the Committee on Ethics of the Federal University of Vale do São Francisco (0032/260911).

A complete clinical examination was performed in all dogs enrolled by a veterinarian. Clinical signs suggestive of canine leishmaniasis (CanL) were recorded, such as weight loss, epistaxis, pale mucous membranes, lymphadenomegaly, hepatosplenomegaly, skin lesions (alopecia, dermatitis, and ulcers), onychogryphosis, and conjunctivitis (Solano-Gallego et al., 2011). In addition, data such as sex (female or male), breed (purebred or mongrel), and size (small, medium, or large) were recorded.

Blood count abnormalities were investigated in all dogs. Erythrocytes and leukocytes were counted with a semi-automatic counter and a Neubauer counting chamber. The hemoglobin concentration was verified using a spectrophotometer. The mean corpuscular volume (MCV), corpuscular hemoglobin (MCH), and corpuscular

hemoglobin concentration (MCHC) were determined by standard calculation (Anthony & Sirois, 2007). Packed cell volume (PCV) was obtained by the microhematocrit method, which uses capillary centrifugation (2,257 *g* for 10 min) followed by a holding the volume in a proper reading ruler for scale. Total plasma protein (TPP) was determined using a refractometer. Differential leukocyte count was performed on blood smears stained with a conventional hematological staining kit (Renylab®, Barbacena, Minas Gerais, Brazil), in which 100 leukocytes were counted under light microscopy followed by a calculation of absolute values.

Serum samples were initially screened using a rapid immunochromatographic test (ICT) (DPP®; Bio-Manguinhos, Rio de Janeiro, Brazil; sensitivity = 100%; specificity = 87.5–91.7%) and then by a commercial enzyme-linked immunosorbent assay (ELISA) (Imunoteste®; Imunodot, Jaboticabal, São Paulo, Brazil; sensitivity and specificity not reported by the manufacturer), which were performed according to the manufacturers' instructions. For the ELISA, the absorbance was read on an automatic EL 800G ELISA microplate reader (Bio Tek Instruments, Winooski, VT, USA) at 405 nm. Serum samples were tested using an immunofluorescence antibody test (IFAT; sensitivity and specificity unknown), as previously described by Oliveira et al. (2008). Positive and negative canine sera were used as controls.

Blood samples were subjected to DNA extraction using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA), following the manufacturer's instructions. Extracted DNA was eluted in 50 µL and subsequently tested by conventional PCR (cPCR) and quantitative PCR (qPCR). The cPCR was conducted with the primers RV1 and RV2, using reagents and thermal conditions described by Le Fichoux et al. (1999). These primers were originally designed to amplify a specific 145 bp fragment of the *L. infantum* kDNA (sensitivity = <1 parasite per reaction), but may actually amplify other *Leishmania* spp. (Lachaud et al., 2002). cPCR assays were run on a Biocycler® thermal cycler, and amplification products were analyzed by electrophoresis in a 1.5% agarose gel. The bands were stained with ethidium bromide and visualized under ultraviolet trans-illumination.

The qPCR was conducted with the primers LEISH-1 and LEISH-2 and a TaqMan-MGB probe, using reagents and thermal conditions described by Dantas-Torres et al. (2017). These primers were also originally designed to amplify a specific 120 bp fragment of the *L. infantum* kDNA (sensitivity = <1 parasite per reaction), but may actually amplify other *Leishmania* spp. (Dantas-Torres et al., 2017). The qPCR assays were run on a QuantStudio® 5 (ThermoFisher scientific/LSA28139) Real-Time PCR system (Applied Biosystems™), and the results were analyzed using QuantStudio® Design and Analysis Software v1.3.1 (Applied Biosystems™). For cPCR, DNA extracted from blood samples of infected and non-infected dogs were used as positive and negative controls, respectively. For qPCR, DNA from *L. infantum* promastigotes and DNA-free water were used as positive and no-template controls, respectively.

The minimal sample size (n = 153) was calculated using the software WinEpi (http://www.winepi.net/uk/index.htm), considering a margin of error of 5%, confidence level of 95%, an unknown population size, and an expected positivity of 11.2%, based on a previous study conducted in Petrolina (Araujo et al., 2016). The associations of various variables (sex, size, clinical signs, and blood count abnormalities) with seropositivity were assessed using Chi-square test, Fisher's exact test or G-test with a significance level of 5% (P < 0.05). Statistical analyses and calculations were performed using BioEstat version 5.3 (Mamirauá Institute, Tefé, Amazonas, Brazil) and GraphPad Prism 5.0 software (GraphPad Software Inc., CA, USA).

Of the 178 stray dogs sampled, 71.9% (128/178) were females and 28.1% (50/178) were males. All dogs were mongrel, with 84.8% (151/178), 8.4% (15/178), and 6.7% (12/178) being medium, large, and small, respectively. All the dogs were adults, but the age could not be precisely estimated and, therefore, age data was not further analyzed.

Overall, 155 dogs (positivity = 87.1%; 95% CI: 81.2%–91.6%) were positive to at least one test. Of these, only 19 dogs were positive to one test and the remaining 136 dogs were positive to two or more tests. The number of dogs positive to all tests, alone or in combination, is shown in Figure 1.

The positivity values detected with serological tests were 71.4% (127/178; 95% CI: 64.1–77.9%), 70.2% (125/178; 95% CI: 62.9–76.8%), and 50.6% (90/178; 95% CI: 43.0–58.1%) with ICT, ELISA, and IFAT, respectively. Considering the test combinations ICT + ELISA, IFAT + ELISA and ICT + IFAT, 55.6% (99/178; 95% CI: 48.0–63.1%), 44.9% (80/178; 95% CI: 37.5–52.6%) and 43.8% (78/178; 95% CI: 36.4–51.4%) of the dogs were positive, respectively. Overall, 38.8% (69/178; 95% CI: 31.6–46.3%) of the dogs were simultaneously positive to all three tests. Molecular testing showed that 20.2% (36/178; 95% CI: 14.6%–26.9%) and 38.8% (69/178; 95% CI: 31.6%–46.3%) of the dogs were positive by cPCR and qPCR, respectively, with 11.8% (21/178; 95% CI: 7.5–17.5%) of them being simultaneously positive in both methods.

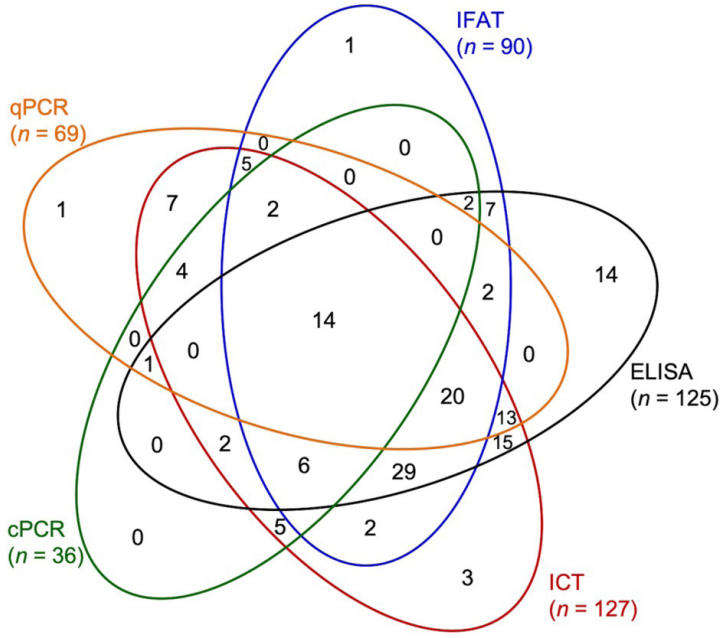

Figure 1. Venn diagram of *Leishmania* seropositivity in stray dogs from Petrolina (Pernambuco, Brazil), based on different tests alone or in combination; immunochromatographic test (ICT), enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), conventional polymerase chain reaction (cPCR) and quantitative polymerase chain reaction (qPCR). Numbers represent the number of positive dogs for one or more tests.

Regarding the 155 positive dogs, 72.3% (112/155) were females and 27.7% (43/155) were males. Most of the positive dogs were medium size (83.9%; 130/155), followed by large (9.0%; 14/155), and then small (7.1%; 11/155). No statistical difference was found between positive and negative dogs in relation to sex (Chi2 = 0.072, *df* = 1, *P* = 0.7886) or size (G-test = 0.9988, *df* = 2; *P* = 0.6069).

The most frequent clinical signs among positive dogs were lymphadenomegaly (80.7%; 125/155), onychogryphosis (65.2%; 101/155), skin lesions (52.9%; 82/155), and weight loss (49.7%; 77/155). The most frequent blood count abnormalities among positive dogs were lymphocytosis (71.0%; 110/155) and anemia (61.3%; 95/155). Positivity was significantly associated with lymphadenomegaly, skin lesions, lymphocytosis, anemia and hyperproteinemia (Table 1).

We confirmed a high level of exposure to *Leishmania* spp. among stray dogs from an area of intense human VL transmission in northeastern Brazil. The positivity varied according to the test used. In a previous study conducted with privately-owned dogs in Petrolina, seropositivity values of 14.5% and 37.3% with IFAT and ELISA, respectively (Araujo et al., 2016). By combining both methods, the authors found a seropositivity of 11.2%. In our study with stray dogs, we found seropositivity values of 50.6% and 70.3% with IFAT and ELISA, respectively. By combining these tests, 44.9% of the dogs were positive. These findings suggest that stray dogs are much more exposed to *Leishmania* infection ricks than privately-owned dogs.

Our results also support the hypothesis that differences in terms of positivity may also be related to the tests used. In fact, considering each individual test used in the present study, the level of exposure varied widely, ranging from 20.2% (cPCR) to 71.4% (ICT). This highlights that tests used in epidemiological studies should be chosen with caution and that combining more tests increase the probability of finding more positive animals. Nonetheless, differences reported in previous studies (Araujo et al., 2016; Evaristo et al., 2020) may also be due to other epidemiological factors.

Regardless of the serological test used, the use of a single test may increase the probability of false-negative results, especially when dealing with infected, but healthy dogs (Otranto et al., 2009). This is a real problem with practical implications for public health authorities, considering that false-negative dogs may remain in endemic areas, potentially serving as *Leishmania* spp. reservoirs for phlebotomine sand fly vectors. In turn, false-positive dogs may also be unnecessarily euthanized, which is ethically arguable and represents an unnecessary public spending.

Combing two tests as a diagnostic criterion may be plausible, but the tests currently used in are not entirely reliable and may increase the probability of false-negative results if these tests are used consecutively, as discussed

Table 1. Clinical signs and blood count abnormalities detected among stray dogs from Petrolina (Pernambuco, Brazil), in relation to positivity to anti-*Leishmania* antibodies and/or *Leishmania* DNA. Statistically significant results are in bold.

Variables	ICT	IFAT	ELISA	cPCR	qPCR	Serology and/or molecular test	Statistics (test and *P* value)
	% (positive/total)	% (positive/total)	% (positive/total)	% (positive/total)	% (positive/total)	% (positive[a] /total)	
Lymphadenomegaly							
Present	68.2 (101/148)	51.3 (76/148)	70.9 (105/148)	21.6 (32/148)	37.2 (55/148)	84.4 (125/148)	**Fisher's exact test, *P* = 0.0157**
Absent	86.7 (26/30)	46.7 (14/30)	66.7 (20/30)	13.3 (4/30)	46.7 (14/30)	100.0 (30/30)	
Onychogryphosis							
Present	72.2 (83/115)	49.6 (57/115)	73.0 (84/115)	20.9 (24/115)	35.6 (41/115)	87.2 (101/115)	Chi2, *P* = 0.6879
Absent	69.8 (44/63)	52.4 (33/63)	65.1 (41/63)	19.0 (12/63)	44.4 (28/63)	85.7 (54/63)	
Skin lesions							
Present	76.1 (67/88)	58.0 (51/88)	78.4 (69/88)	22.7 (20/88)	44.3 (39/88)	93.2 (82/88)	**Chi2, *P* = 0.0164**
Absent	66.7 (60/90)	43.3 (39/90)	62.2 (56/90)	17.8 (16/90)	33.3 (30/90)	81.1 (73/90)	
Weight loss							
Present	75.0 (63/84)	50.0 (42/84)	70.2 (59/84)	15.5 (13/84)	47.6 (40/84)	91.7 (77/84)	Chi2, *P* = 0.0845
Absent	68.1 (64/94)	51.1 (48/94)	70.2 (66/94)	24.5 (23/94)	30.8 (29/94)	83.0 (78/94)	
Pale mucous membranes							
Present	66.7 (14/21)	57.1 (12/21)	76.2 (16/21)	9.5 (2/21)	28.5 (6/21)	90.5 (19/21)	Fisher's exact test, *P* = 0.7460
Absent	72.0 (113/157)	49.7 (78/157)	69.4 (109/157)	21.6 (34/157)	40.1 (63/157)	86.6 (136/157)	
Conjunctivitis							
Present	81.8 (9/11)	27.3 (3/11)	90.9 (10/11)	18.2 (2/11)	36.4 (4/11)	100.0 (11/11)	Fisher's exact test, *P* = 0.3634
Absent	75.1 (118/157)	52.1 (87/167)	68.9 (115/167)	20.3 (34/167)	38.9 (65/167)	86.2 (144/167)	
Hepatosplenomegaly							
Present	83.3 (5/6)	66.7 (4/6)	83.3 (5/6)	33.3 (2/6)	50.0 (3/6)	100.0 (6/6)	Fisher's exact test, *P* = 0.6040
Absent	70.9 (122/172)	50.0 (86/172)	69.8 (120/172)	19.8 (34/172)	38.4 (66/172)	86.7 (149/172)	
Epistaxis							
Present	100.0 (1/1)	100.0 (1/1)	100.0 (1/1)	0.0 (0/1)	100.0 (1/1)	100.0 (1/1)	Fisher's exact test, *P* = 1.000
Absent	71.2 (126/177)	50.3 (89/177)	70.0 (124/177)	80.2 (142/177)	38.4 (68/177)	87.0 (154/177)	
Lymphocytosis							
Present	70.2 (92/131)	49.6 (65/131)	65.6 (86/131)	23.7 (31/131)	35.1 (46/131)	84.0 (110/131)	**Fisher's exact test, *P* = 0.0430**
Absent	74.5 (35/47)	53.2 (25/47)	83.0 (39/47)	10.6 (5/47)	49.0 (23/47)	95.7 (45/47)	
Anemia							
Present	73.3 (74/101)	43.6 (44/101)	63.4 (64/101)	23.8 (24/101)	43.6 (44/101)	94.0 (95/101)	**Chi2, *P* = 0.0015**
Absent	68.8 (53/77)	59.7 (46/77)	79.2 (61/77)	15.6 (12/77)	32.5 (25/77)	77.9 (60/77)	
Hyperproteinemia							
Present	87.9 (29/33)	51.5 (17/33)	72.7 (24/33)	24.2 (8/33)	51.5 (17/33)	75.7 (25/33)	**Fisher's exact test, *P* = 0.0435**
Absent	67.6 (98/145)	50.3 (73/145)	69.6 (101/145)	19.3 (28/145)	35.9 (52/145)	89.6 (130/145)	
Neutrophilia							
Present	66.7 (12/18)	50.0 (9/18)	66.7 (12/18)	11.1 (2/18)	44.4 (8/18)	83.3 (15/18)	Fisher's exact test, *P* = 0.7054
Absent	71.9 (115/160)	50.6 (81/160)	70.6 (113/160)	21.2 (34/160)	38.1 (61/160)	87.5 (140/160)	

[a]Positive to one or more test. For more details, see Methods section of this article; Abbreviations: ICT, immunochromatographic test; IFAT, immunofluorescence antibody test; ELISA, enzyme-linked immunosorbent assay; cPCR, conventional polymerase chain reaction; qPCR, quantitative polymerase chain reaction.

elsewhere (Lira et al., 2006). Furthermore, the use of a second serological test in as confirmatory test is controversial because it does not eliminate the false-positive problem (Mendonça et al., 2017). The use of two tests in parallel has been recommended when higher sensitivity is required, reducing the number of false-negative results (Lira et al., 2006; Arruda et al., 2016). Using this criterion, dogs are considered positive when positive to any of the two tests or both (Lira et al., 2006). In this study the combination ICT + ELISA detected more positive dogs, when compared with ICT + IFAT or IFAT + ELISA, probably due to the higher sensitivity of the ICT and ELISA (Lira et al., 2006; Coura-Vital et al., 2014). As expected, when all five test results were used in parallel (ICT, IFAT, ELISA, cPCR and/or qPCR), the positivity observed was higher than any individual test, alone or in combination.

Molecular tests are powerful diagnostic tools for detecting truly infected dogs, but they are rarely used in public health, due to their higher costs and complexity as compared to serological tests. The qPCR employed in this study detected more positive dogs than cPCR (38.8% versus 20.2%), which agrees with other studies (e.g., Ramos et al., 2012). It is known that qPCR presents a higher sensitivity than cPCR, especially when using blood samples (Travi et al., 2018). Another advantage of qPCR is its quantification capability, which allows the individualization of so-called super spreaders (Duthie et al., 2018), making them useful tools not only in clinical settings but also for public health. In fact, six positive dogs resulting positive with the molecular assays used herein were negative with ICT, the official screening test used in Brazil. In particular, three of these dogs were positive by qPCR, two by cPCR and one by both molecular assays. Of these six dogs, five were positive by ELISA, indicating a higher sensitivity of this test as compared with the ICT, as demonstrated by Laurenti et al. (2014). Doing a simple theoretical count, this suggests that around 20% of the dogs screened with ICT would be positive if tested by one of the PCR assays used herein. From a national perspective, it means that thousands of seronegative dogs may actually be infected, as has been suggested before (Alves & Bevilacqua, 2004). Still, the ICT test has many advantages over molecular tests, including rapidity, easiness to perform and to interpret, as wells as low cost. Furthermore, one should also consider that the sensitivity of molecular tools in blood samples is lower as compared to more invasive samples, such as bone marrow (Dantas-Torres et al., 2017).

Regarding clinical signs and laboratory abnormalities, dogs with skin lesions or anemia were significantly more positive than those without these alterations. Nonetheless, these data should be interpreted with caution, considering that the presence of concomitant infections in the studied stray dogs cannot not be ruled out. While clinicopathological abnormalities have limited diagnostic value, they may reveal that otherwise apparently healthy dogs may have some clinicopathological anomalies. This in turn is important for clinically staging, and therefore managing the disease, in infected animals (Solano-Gallego et al., 2011). Hyperproteinemia, an abnormality that is commonly reported in CanL (Solano-Gallego et al., 2011), occurred at a low frequency in this study.

Finally, the present study indicates that stray dogs may play a more important role as *Leishmania* spp. reservoirs than privately-owned dogs, in this area of intense VL transmission. Thus, even if privately owned dogs could be systematically targeted by public health authorities, stray dogs would continue to provide a source of *Leishmania* parasites to phlebotomine sand flies. This is in line with a previous longitudinal study conducted in Jequié (Bahia state, northeastern Brazil), which revealed that serological positivity in stray dogs was consistently higher than privately owned dogs throughout the years (Fraga et al., 2012).

In conclusion, the results of this study reveal a high level of exposure to *Leishmania* spp. in stray dogs in Petrolina and highlights the importance of using different diagnostic methods to reduce the probability of false-negative results. The test combination ICT + ELISA enable the detection of more positive dogs than any other test combination. Moreover, our study indicates that stray dogs may play an important role as parasite reservoirs in this region of intense human VL transmission, emphasizing the importance of pet owner education on responsible ownership as well as castration campaigns, ultimately to reduce the population of stray dogs and their associated zoonotic risks.

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