Epidemiol. Infect. (2017), **145**, 2436–2444. © Cambridge University Press 2017
doi:10.1017/S0950268817001443

Serological and molecular diagnostic tests for canine visceral leishmaniasis in Brazilian endemic area: one out of five seronegative dogs are infected

E. G. LOPES[1], A. P. SEVÁ[1], F. FERREIRA[1], C. M. NUNES[2], L. B. KEID[3], R. M. HIRAMOTO[4], H. L. FERREIRA[3], T. M. F. S. OLIVEIRA[3], M. F. D. BIGOTTO[1], F. GALVIS-OVALLOS[5], E. A. B. GALATI[5] AND R. M. SOARES[1]*

[1] *Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Sao Paulo, SP, Brazil*
[2] *Departamento de Medicina Veterinária, Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Campus Araçatuba, Araçatuba, SP, Brazil*
[3] *Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, SP, Brazil*
[4] *Centro de Parasitologia e Micologia, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil*
[5] *Departamento de Epidemiologia, Faculdade de Saúde Pública, Universidade de São Paulo, Sao Paulo, SP, Brazil*

Received 1 March 2017; Final revision 16 May 2017; Accepted 19 June 2017; first published online 20 July 2017

SUMMARY

Euthanasia of infected dogs is one of the measures adopted in Brazil to control visceral leishmaniasis (VL) in endemic areas. To detect infected dogs, animals are screened with the rapid test DPP® Visceral Canine Leishmaniasis for detection of antibodies against K26/K39 fusion antigens of amastigotes (DPP). DPP-positives are confirmed with an immunoenzymatic assay probing soluble antigens of promastigotes (ELISA), while DPP-negatives are considered free of infection. Here, 975 dogs from an endemic region were surveyed by using DPP, ELISA and real-time PCR (qPCR) for the diagnosis of VL. When DPP-negative dogs were tested by qPCR applied in blood and lymph node aspirates, 174/887 (19·6%) were positive in at least one sample. In a second sampling using 115 cases, the DPP-negative dogs were tested by qPCR in blood, lymph node and conjunctival swab samples, and 36/79 (45·6%) were positive in at least one sample. Low-to-moderate pairwise agreement was observed between all possible pair of tests. In conclusion, the official diagnosis of VL in dogs in Brazilian endemic areas failed to accuse an expressive number of infected animals and the impact of the low accuracy of serological tests in the success of euthanasia-based measure for VL control need to be assessed.

Key words: Dogs, PCR, serodiagnosis, visceral leishmaniasis.

INTRODUCTION

Visceral leishmaniasis (VL) is a global public health problem that occurs in different regions of the world such as South America, Mediterranean Europe, Africa and Asia [1, 2].

In Brazil, VL is caused by *Leishmania* (*Leishmania*) *infantum chagasi* and the most common vectors are *Lutzomyia longipalpis* and *Lutzomyia cruzi* [3, 4]. The disease has spread fast in the last decade, with new cases being recorded in urban areas. In the state of São Paulo, Brazil, VL is in rapid expansion and has already been detected in more than 100 municipalities [5].

* Author for correspondence: R. M. Soares, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo, SP, Brasil.
(Email: rosoares@usp.br)

Dogs play a key role in the epidemiological chain of VL, as they are the main urban reservoirs of the parasite [6, 7]. Therefore, the identification and culling of infected dogs is one of the legal instruments adopted to control VL by sanitary authorities in Brazil [8] making of paramount importance that the diagnostic methods of this infection have high accuracy.

Serological methods are used to identify infected dogs in official VL control programs in Brazil. However, due to the low sensitivity of serological methods, direct methods for the VL diagnosis, particularly molecular methods, have been widely studied [9, 10].

The most widespread molecular method for the diagnosis of VL in dogs is the polymerase chain reaction (PCR). This test allows selective amplification of DNA sequences of the parasite, enabling a rapid diagnostic tool with high sensitivity and specificity [11–13]. Furthermore, PCR can be used in a large variety of biological samples, such as blood, skin biopsies, lymph node puncture and bone marrow puncture [9, 10, 14].

The non-invasive methods are preferred because they allow easy sampling in field surveys, especially for mass screening, and have a better owner acceptance and collaboration. Among the non-invasive methods, we highlight conjunctival swab PCR. This method has been shown to be highly sensitive for the diagnosis of VL, both among symptomatic [14, 15] and asymptomatic dogs [16].

In this work, dogs from an endemic region were surveyed by using serological tests used by the official surveillance system in Brazil for the diagnosis of VL [8]. Dogs were also tested by real-time PCR associated with the detection of the amplified product by hydrolysis probe (qPCR) to detect *Leishmania* spp. in blood samples, lymph node puncture and conjunctival swab.

METHODS

Sampling and ethics

The dogs were sampled from the urban perimeter of the municipality of Panorama, an endemic area for VL located in the extreme west of the state of São Paulo, Brazil (S21°21′00″ and O51°51′36″). In the year of 2012, owned dogs were surveyed by the official health system in the municipality of Panorama, by serological tests. Then, VL seropositive dogs were registered and 100 m circles in radio were mapped, taking as center of each circle the residences that contained at least one seropositive dog. The MapSource® software (Garmin®), version 6.16.3 and the Quantum GIS® software (QGIS), version 1.8.0 Lisbon, were used to plot points on a map of the municipality, thus making it possible to map the circles and dogs. Seronegative dogs resident within these circles were randomly chosen, visited again and they had biological samples collected, until completing 379 animals. Among these dogs, 321 were revisited after 6 months, and of these 321, 274 were revisited after 1 year, totaling 974 cases. This sampling was part of the methods of a study aimed at the evaluation of control measures for Canine VL, which were approved by the Ethical Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (process no. 2370/2011). The dogs were sampled from 2012 to 2013.

Biological samples

Aspirates of popliteal lymph node, peripheral blood and serum from all the 974 cases (sampling 1) were collected. Conjunctival swab specimens from the right eye were collected from the 274 dogs at the third visit. Then, 115 dogs were randomly selected from those 274 animals (sampling 2). Biological samples were taken at the owner's residence. No clinical data were collected from the animals.

The blood collection was done aseptically in two aliquots, one of them with addition of anticoagulant (sodium citrate) and another without sodium citrate. Serum was obtained from the aliquot of blood without anticoagulant. Aspiration of the popliteal lymph node was done aseptically, after disinfection with 70% alcohol iodinated at 2·5%. The lymph node aspiration was made with the aid of syringe and needle and the punctured product was suspended in 500 µl of 0·9% NaCl solution (physiological saline) in 1·5 ml microtubes. Sterile swabs without culture medium or preservatives were used for scraping the conjunctival mucosa. The end of the swabs were cut and stored in 1·5 ml microtubes without addition of any solution. All biological samples were stored at −20 °C until DNA extraction.

Serodiagnosis

Serum samples were submitted to the serological tests used by the public health service in the state of São Paulo, Brazil: (i) rapid DPP® Canine VL, Biomanguinhos, FIOCRUZ for the detection of antibodies against K26/K39 of amastigotes (DPP); and (ii) immunoenzymatic assay Canine VL, Biomanguinhos, FIOCRUZ (ELISA), for detection of antibodies against soluble antigens of promastigotes.

https://doi.org/10.1017/S0950268817001443 Published online by Cambridge University Press

DNA extraction

Conjunctival mucosal swabs were thawed, submerged for 12 h in 500 µl of physiological saline and discarded. The suspension obtained was used for DNA extraction. Blood samples, suspensions of lymph node punctures and suspensions of conjunctival swab were submitted to DNA extraction with DNeasy Blood & Tissue Kit (Qiagen®, Hilden, Germany), using in all cases the recommended protocol for DNA extraction of blood, according to the manufacturer's protocol.

Molecular tests

Nucleic acids from blood samples, lymph node puncture and conjunctival swab were submitted to qPCR using oligonucleotides and hydrolysis probes exactly as described elsewhere [17]. Positive and negative controls were used throughout all the experiment. In each qPCR assay, at least six negative controls and one positive control were included along with the test samples. Positive controls were extracted DNA from promastigotes of *L.* (*Leishmania*) *infantum chagasi* (MHOM/BR/2002/LPC-RPV) in the Schneider medium. The Leishmaniasis Research Laboratory provided this isolate (Collection of Leishmania of the Instituto Oswaldo Cruz). Negative controls were ultrapure water.

The qPCR was performed using Light Cycler 480® Probes Master, version 9 kit (Roche Diagnostics Ltd, Switzerland) in a final volume of 20 µl. The following reagents were used: 900 nM each primer, 200 nM hydrolysis probe, 10 µl LightCycler480 Probes Master, 2× (Roche Diagnostics Ltd, Switzerland), 3·0 µl molecular ultrapure water, 5 µl of DNA. The amplifications were conducted on the Light Cycler 480II® Thermal Cycler (Roche Diagnostic) on 96-well white LightCycler 480® multiwell plates (Roche Diagnostics Ltd, Switzerland). The following cycles of temperature variations were employed: preincubation at 95 °C for 10 min, amplification with 50 cycles of: 95 °C for 10 s 50 °C for 60 s, 72 °C for 1 s. The samples were tested in duplicate. The amplification curves were analyzed using the LightCycler 480 SW 1·5·1 software (Roche Diagnostics Ltd, Switzerland), from calculating the maximum second derivative, in accordance with the software manual.

The qPCR applied in samples of whole blood, punctured lymph nodes and conjunctival swab were, respectively, BL, LN and CS. Serological tests were DPP and ELISA.

Statistical analyzes

The agreement between two tests were calculated by Cohen's kappa coefficient (κ) [18] and McNemar test was used to verify the difference between paired proportions obtained with the diagnostic tests [19]. The interpretation of κ was performed according to Altman [20]. Cohen's κ coefficient is sensitive to the distribution of the marginal totals in 2×2 contingencies tables in case of bias between two raters (bias effect) or in case of unequal distribution of data across the two categories (prevalence effect). In order to estimate agreement between two tests for these cases, Byrt *et al.* [21] derived the formula for prevalence and bias adjusted κ (PABAK) that expresses κ in terms of bias index (BI), prevalence index (PI) and observed agreement (p_o). Thus, in addition to Cohen's κ coefficient, PABAK, 95% confidence interval of PABAK [22], BI, PI and p_o were also calculated. BI is an index of the bias between tests, and PI, an index of the differences between the overall proportion of positive and negative outputs. The higher the absolute value of either PI or HI, the higher the influence of bias and prevalence on the κ value is.

The diagnostic tests were analyzed individually or in associations. The associations of tests were performed in parallel (test 1 or 2) or in series (tests 1 and 2). The associations are explained below:

Parallel associations: cases were dogs positive by test 1 or 2 or both, while non-cases were those negative by both tests. In some cases, more than two tests were associated in parallel. In this situation, cases were animals positive by any of the tests and non-cases were those negative by all the tests.

Serial associations: cases were dogs positive by tests 1 and 2, while for any other result, the animals were considered non-cases. This is the detection criterion adopted by the official health service in the municipality of Panorama, São Paulo, SP, Brazil, to classify infected dogs using the DPP and ELISA serological tests.

For comparisons between proportions of independent samples, either Chi-squared (χ^2) statistic or Fisher's exact test was used. The 95% confidence interval for rates was calculated as described elsewhere [23].

RESULTS

Among the 974 cases of sampling 1, the highest number of positives was obtained with LN, followed by ELISA, DPP and BL, with the following values: 229 (95% CI 200·3–260·7), 213 (95% CI 185·3–243·6),

https://doi.org/10.1017/S0950268817001443 Published online by Cambridge University Press

87 (95% CI 69·7–107·3) and 37 (95% CI 26·1–51·0), respectively. With sampling 2 (115 dogs), the highest positivity was obtained with CS and the lowest with BL. In sampling 2, the numbers of positive dogs were: 54 by CS (95% CI 40·6–70·5), 42 by ELISA and LN (95% CI 30·3–56·8), 36 by DPP (95% CI 25·2–49·8), 22 by cCS (95% CI 13·8–33·3) and 11 by BL (95% CI 5·5–19·7). The statistics calculated for each pair of tests and the combination of tests are found in Table 1.

In the majority of the pairwise comparisons, agreement was at most moderate and the differences between positivities were statistically significant. Exceptions: in sampling 1, the association [LN or BL] showed a positivity of 234/974 against 213/974 of ELISA, a statistically non-significant difference. The difference in positivity between LN and ELISA was not significant, also. In sampling 2, the positivity of CS was not significantly different from ELISA, from LN, or from the associations [LN or BL or DPP] and [LN or BL or DPP or ELISA].

In sampling 1, the association [LN or BL] confirmed as positive 109 of the 213 samples positive by ELISA (51·2%). The association [LN or BL] confirmed as positive 60 of the 87 samples positive by DPP (69·0%) and 47 of the 55 samples positive by [ELISA and DPP] (85·5%). All the above proportions were significantly different from each other ($P < 0·05$).

In sampling 2, the confirmation of seropositives by [LN or BL or CS] occurred with the following frequencies: 37/42 (88·1%), 34/36 (94·4%) and 25/26 (96·1%), respectively for ELISA, DPP and [ELISA and DPP]. The difference between those proportions were not statistically significant ($P < 0·05$).

For the samples classified as negative by serological tests at sampling 1, [LN or BL] revealed as positive 187/919 (20·3%), 174/887 (19·6%), 125/761 (16·4%), for [ELISA and DPP], DPP and ELISA, respectively. Excluding the differences between 20·3% and 16·4%, the differences between the other proportions were not statistically significant ($P < 0·05$).

In the sampling 2, the association [LN or BL or CS] classified as positive 45/89 (50·6%), 36/79 (45·6%), 33/73 (45·2%), among the negatives by [ELISA and DPP], DPP and ELISA, respectively. In this case, no difference between proportions was statistically significant ($P < 0·05$).

Eight out of 55 cases (14·5%) classified as infected by [ELISA and DPP] were [LN or BL]-negative. When CS was added to the list of molecular tests used to confirm positive results by [ELISA and DPP], the apparent error of the associated serological tests in mistakenly classifying uninfected as positive animals dropped to 1 in 26 (3·8%).

Considering the serological status of the dogs from the samplings 1 and 2 investigated with DPP and ELISA, a significant increase was observed in the number of qPCR-positive dogs in the categories (DPP+ and ELISA−), (DPP− and ELISA+) and (DPP− and ELISA−) when CS was added to the list of molecular tests (Table 2).

DISCUSSION

In this study, specificity, sensitivity and predictive values of results of the tests were not calculated because the criteria for gold-standard definition could not be precisely established. The direct comparison between two techniques or between a technique and a set of techniques is not enough to define a gold standard situation and is not recommended by international protocols for evaluation of diagnostic techniques [24, 25].

Under the conditions of this investigation, the serially associated serological tests were found to have low capacity for detection of *Leishmania* infection when compared with molecular tests, but on the other hand, they correctly classified infected animals with high frequency, that is, they appear to have high specificity. Adding CS to the association [LN or BL] increased the ability of the molecular methods to detect infected dogs among the [ELISA and DPP]-positive animals, that is, the molecular tests effectively confirmed the positives detected by serodiagnosis, indicating that [ELISA and DPP] is trustable in identifying infected animals.

On the other hand, a non-negligible number of seronegative dogs were classified as infected by molecular tests. The combination of three molecular tests in parallel significantly increased, relative to the use of only two, the number of animals classified as infected, but erroneously indicated by the serological tests as uninfected. In Brazil, one of the official measures to control VL in endemic areas is the culling of seropositive dogs [8]. However, for this extremely controversial and unpopular measure to be effective, it is necessary to maximize the accuracy of the diagnostic tests used to discriminate infected from uninfected dogs. The use of low sensitive tests may cause infected dogs to remain in the population making the program ineffective. Tests of inadequate specificity, on the other hand, may determine that false

https://doi.org/10.1017/S0950268817001443 Published online by Cambridge University Press

Table 1. *Comparisons between tests or associations of tests for the detection of VL in dogs*

Compared tests (TEST 1/TEST 2)	+/+ (a)	+/− (b)	−/+ (c)	−/− (d)	total (*n*)	p_o	BI	PI	PABAK	95% CI PABAK	κ	95% CI κ	Δ (%)	95% CI Δ (%)
Sampling 1 (974 cases)														
ELISA/DPP	55	158	32	729	974	0805	0129	−0692	0610	0560–0660	0275	0203–0346	12·9	10·6–14·9
LN/BL	32	197	5	740	974	0793	0197	−0727	0585	0534–0636	0187	0127–0248	19·71	18·4–20·4
DPP/BL	25	62	12	875	974	0924	0051	−0873	0848	0815–0881	0·37	0259–0480	5·13	3·55–6·28
LN/ELISA	106	123	107	638	974	0764	0016	−0546	0528	0474–0581	0327	0258–0396	NS	
LN/DPP	60	169	27	718	974	0799	0146	−0676	0598	05 470−648	0288	0218–0357	14·6	12·3–16·4
ELISA/BL	28	185	9	752	974	0801	0181	−0743	0602	0551–0652	0·17	0108–0232	18·1	16·5–19·1
[LN or BL]/[ELISA and DPP]	47	187	8	732	974	0800	0184	−0703	0600	0549–0650	0257	0192–0322	18·4	16·8–19·3
[LN or BL]/ELISA	109	125	104	636	974	0765	0022	−0541	0530	0477–0583	0336	0267–0404	NS	
[LN or BL]/DPP	60	174	27	713	974	0794	0151	−0670	0587	0536–0638	0·28	0212–0348	15·1	12·8–16·9
Sampling 2 (115 cases)														
CS/LN	26	28	16	45	115	0617	0104	−0165	0235	0057–0412	0222	00469–0397	NS	
CS/BL	11	43	0	61	115	0626	0374	−0435	0252	0075–0429	0213	00986–0328	37·4	31·2–37·4
CS/DPP	28	26	8	53	115	0704	0157	−0217	0409	0242–0576	0395	0234–0556	15·6	5·2–23·2
CS/ELISA	30	24	12	49	115	0687	0104	−0165	0374	0204–0543	0363	0196–0531	NS	
CS/[LN or BL or DPP]	38	16	18	43	115	0704	−0017	−0043	0409	0242–0576	0408	0241–0575	NS	
CS/[LN or BL or DPP or ELISA]	42	12	22	39	115	0704	−0087	0026	0409	0242–0576	0413	0249–0576	NS	
[LN or BL or CS]/[ELISA and DPP]	25	45	1	44	115	0600	0383	−0165	0200	0021–0379	0285	0167–0403	38·3	30·8–40·0
[LN or BL or CS]/ELISA	37	33	5	40	115	0670	0243	−0026	0339	0167–0511	0376	0230–0521	24·3	14·5–30·1
[LN or BL or CS]/DPP	34	36	2	43	115	0670	0296	−0078	0339	0167–0511	0389	0255–0523	29·6	21·3–32·6

NS, non-significant; (+), positive result; (−), negative result; CI, confidence interval; p_o, observed agreement (p_o = (a+d)/*n*); BI, bias index (BI = (b–c)/*n*); PI, prevalence index (PI = (a–d)/*n*); PABAK, prevalence-adjusted bias adjusted κ; Δ, difference in paired proportions, calculated by using McNemar test.

Table 2. *Positivity of qPCR according to the serological status of the dogs for VL*

	Serological status (denominator)			
qPCR status (numerator)	DPP+ and ELISA+	DPP+ and ELISA−	DPP− and ELISA+	DPP− and ELISA−
Sampling 1 (974 cases)				
[LN or BL]+	47/55 (85·4%)	13/32 (40·6%)	62/158 (39·2%)	112/729 (15·4%)
Sampling 2 (115 cases)				
[LN or BL]+	21/26 (80·8%)	3/10 (30·0%)	8/16 (50·0%)	12/63 (19·0%)
[CS or LN or BL]+	25/26 (96·1%)	9/10 (90·0%)	12/16 (75·0%)	24/63 (38·1%)

positive dogs be unnecessary eliminated. In this survey, the control program is not targeting a significant part of the dog population, even though these animals are potential sources of infection. At the sampling 1, about two in ten DPP-negative animals were infected. In the second sampling, this proportion increases to five in ten, that is, half of the seronegative animals would be from infected dogs.

It is important to identify and better understand the role of dogs as sources of infection in an endemic area according to their diagnostic status. For example, dogs identified as infected only by molecular methods appear to have a lower chance of developing the disease than serologically positive dogs, as the latter develop clinical signs more rapidly and may be more efficient sources of infection [26, 27]. Therefore, the apparent negative impact caused by the error of the serological tests on leaving infected animals in the population needs to be better assessed, since these would not necessarily be efficient sources of infection.

Regarding the clinical condition of the dogs, the capacity of the symptomatic dogs to be sources of infection of VL more efficient than the asymptomatic ones is not unanimity among researchers. While some authors have shown that asymptomatic dogs are ineffective in transmitting the agent [28] or less effective in doing so than symptomatic dogs [27], other authors have shown that dogs of both categories have the same ability to transmit the parasite to the vector [7]. In our study, the clinical condition of the dogs was not investigated. Given the complexity of the survey, which was made through visits to owners, the clinical parameters were not properly collected. The determination of the clinical status of a dog for diagnosing VL should include blood cell counts, complete serum biochemical profile, urinalysis and an adequate profile of tests for differential diagnosis with other diseases [29]. Thus, the classification of clinical status of animals into healthy or diseased, based only on physical examinations, does not represent an acceptable approach [30]. The imprecise determination of signs such as lymphadenomegaly, hepatomegaly, splenomegaly, skin diseases could cause important biases in determining the attributes of laboratory tests for diagnosis of VL used in symptomatic and asymptomatic animals. Studies, in which the clinical parameters of the investigated animals were adequately verified, clearly show associations between positivity in diagnostic tests and the presence of clinical signs, which is not the case when the measurement of clinical data is not satisfactory [30].

If the clinical condition of a dog is not associated to the fact that it is an efficient source of infection [7], on the other hand, the biological sample where the parasite is detected in an infected dog seems to be determinant for this condition. Thus, the parasitism in the skin seems to have a lower correlation with the vector transmission than the parasitism in lymph nodes [7], which shows that LN-positive dogs play relevant role in VL transmission, irrespective of their clinical or serodiagnostic status.

It is noteworthy the unsatisfactory agreement of most pairwise comparisons. Cohen's κ have been used as chance-adjusted measure of agreement between two raters, but some issues (termed 'paradoxes') related to its interpretation have been pointed out [31]. Kappa is susceptible to the distribution of the marginal totals in a pairwise comparison and the agreement between two tests might be underestimated when the frequencies of the observed event are low (first paradox or prevalence effect). Kappa may also be affected if the two tests have rather different capabilities of detecting an event (second paradox or bias effect). In order to overcome these problems, Byrt *et al.* [21] proposed PABAK, a statistic that depends solely on the observed agreement (PABAK = $2p_o$–1; where p_o is the observed agreement). These authors recommend that index agreement be interpreted along with the presence of bias and/or the

https://doi.org/10.1017/S0950268817001443 Published online by Cambridge University Press

prevalence effect. From our data, the pairwise comparisons in sampling 1 seem to be under the effect of prevalence and PABAK is modally higher than κ. On the contrary, in sampling 2 PABAK did not improve the estimates of agreement. From the values of BI and PI, one can infer that the prevalence effect affected the agreement between tests in sampling 1, but not in sampling 2. Prevalence of VL in sampling 2 was higher than in sampling 1 because the former is composed by the same dogs of the sampling 1, but that had been sampled 6 months to 1 year before. In consequence, dogs from sampling 2 were obviously at higher risk of VL infection than dogs of the sampling 1.

Irrespective of using either κ or PABAK, the agreement was at most moderate and the difference between test positivities was significant in most pairwise comparisons. The exception was the pairwise DPP/BL with a high value of PABAK. Despite the pairwise agreement between DPP and BL is the most influenced by prevalence effect, both techniques have low positivity. Thus, the negative agreement, i.e., the number of negatives by both tests, highly contributed to the value of PABAK.

These results suggest that the different diagnostic tests indicate the presence of the infection in a complementary way. The absence of the parasite in lymph node samples does not predict the absence of the parasite in conjunctival mucosa scraping. Likewise, the absence of humoral response revealed by the serological tests in association does not predict that the parasite is absent in lymph nodes or cells obtained from mucosal scrapings. These results corroborated several others that evidenced that humoral response is not a sensitive marker of VL [32, 33]. Thus, criterion of identification of infected dogs for euthanasia-based control programs needs to be rethought.

The PCR applied in swab samples has proved to be a promising approach for the diagnosis of VL in dogs, which was confirmed in this study.

Under the conditions of this study in which asymptomatic and symptomatic dogs were not differentiated, whole blood samples did not appear to be adequate for the diagnosis of VL because none of the CS or LN-negative samples were positive for BL (sampling 2). Still, the positivity for BL was significantly lower than the other two tests. The controversial value of blood samples for the diagnosis of VL in dogs was also pointed elsewhere. Lombardo *et al.* [30] found positivities of 24·5% (40/163), 22·1% (36/163) and 5·5% (9/163), for qPCR directed to lymph node, conjunctival swab and whole blood, respectively. Positivity of blood samples was also lower than that of other samples such as skin, bone marrow, lymph node aspirate, oral swab and conjunctival swab [15, 33].

It was not possible to demonstrate with statistical significance, that the positivity of qPCR in conjunctival swab samples was different from that of qPCR in lymph node aspirates, as has been reported in other studies [33]. For comparison between proportions, non-parametric statistical tests such as χ^2 or McNemar test indicate a significant difference when the value of significance probability is less than the significance value (usually, $P = 0{\cdot}05$), which allows one to reject the hypothesis of equivalence between positivities. However, when the value of probability of significance is higher than the value of significance, the indication is that there is no evidence to reject the hypothesis of equivalence between positivities, which is different from accepting that the proportions compared are equivalent. Therefore, by the results of the comparisons between diagnostic performance of LN and CS, it is not possible to detect difference between the positivity of both techniques. On the other hand, the κ agreement value among them is one of the lowest among all registered comparisons, which seems to indicate that LN and CS should detect the parasite at different stages of infection.

In conclusion, the protocol for official diagnosis of VL in dogs in Brazilian endemic areas failed to accuse an expressive number of infected animals. Thus, the impact of such a low accuracy over the success of euthanasia-based measure for VL control needs to be assessed. The results presented here strongly suggest that the sensitivity of CS or LN does not accredit them to be used as the only method for VL diagnosis in control programs or cross-sectional studies, but CS and LN may be associated in parallel to significantly increase the diagnostic sensitivity.

Although qPCR apparently improved the sensitivity of the VL diagnosis, one must consider the intrinsic difficulties of including molecular tests in public health services. The limitations of qPCR and other molecular techniques have to be considered. Molecular diagnosis of VL needs to be validated and consolidated by mean of kits and automation, in order to minimize variation and achieve intra and inter laboratory reproducibility. The PCR for VL diagnosis is far to be a consensus among laboratories, as a number of methods and molecular markers have been proposed by several research groups. In addition, molecular diagnosis needs for expensive facilities, including equipment,

https://doi.org/10.1017/S0950268817001443 Published online by Cambridge University Press

consumable items and highly skilled operators. These imply high costs, making such tests difficult to be included in public-financed programs of control, especially in developing countries.

ACKNOWLEDGEMENTS

This study was sponsored by FAPESP (grant number 2011/21796-2). F. Ferreira, C. M. Nunes and E. A. B. Galati are recipient of productivity fellowship from CNPq. E. G. Lopes received scholarships from FAPESP (grant number 2011/14892-5).

DECLARATION OF INTEREST

None.

REFERENCES

1. **Desjeux P.** Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology & Infectious Disease* 2004; **27**: 305–318.
2. **World Health Organization.** Sustaining the drive to overcome the global impact of neglected tropical diseases. *Second WHO Report on Neglected Tropical Diseases* 2013; **3·9**: 67–71.
3. **Santos SO, *et al.*** Incrimination of *Lutzomyia cruzi* as a vector of American visceral leishmaniasis. *Medical and Veterinarian Entomology* 1998; **12**: 315–317.
4. **Lainson R, Shaw JJ.** Biology and epidemiology: evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick R, eds. *The Leishmaniasis in Biology and Medicine*, vol. **1**. London: Academic Press, 1987, pp. 1–120.
5. **Rangel O, *et al.*** Classificação epidemiológica dos municípios segundo o programa de vigilância e controle da leishmaniose visceral americana no estado de São Paulo para 2013. *Boletim Epidemiológico Paulista* 2013; **10**: 3–14.
6. **Barrouin-Melo SM, *et al.*** Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of *Leishmania chagasi* infection in dogs. *Memórias do Instituto Oswaldo Cruz* 2004; **99**: 195–197.
7. **Laurenti MD, *et al.*** Asymptomatic dogs are highly competent to transmit *Leishmania* (*Leishmania*) *infantum chagasi* to the natural vector. *Veterinary Parasitology* 2013; **196**: 296–300.
8. **Brasil, Ministério da Saúde.** *Manual de Vigilância e controle da Leishmaniose Visceral*. Brasília: Ministério da Saúde do Brasil, 2014, p. 122.
9. **Martínez V, *et al.*** Canine leishmaniasis: the key points for qPCR result interpretation. *Parasites & Vectors* 2011; **4**: 57.
10. **Almeida ABPF, *et al.*** Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. *Diagnostic Microbiology and Infectious Disease* 2013; **76**: 321–324.
11. **Degrave W, *et al.*** Use of molecular probes and PCR for detection and typing of *Leishmania*- a mini review. *Memórias do Instituto Oswaldo Cruz* 1994; **89**: 463–469.
12. **Lachaud L, *et al.*** Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *Journal of Clinical Microbiology* 2002; **40**: 210–215.
13. **Maia C, Campino L.** Methods for diagnosis of canine leishmaniasis and immune response to infection. *Veterinary Parasitology* 2008; **158**: 274–287.
14. **Strauss-Ayali D, *et al.*** Polymerase chain reaction using noninvasively obtained samples, for the detection of *Leishmania infantum* DNA in dogs. *Journal of Infectious Diseases* 2004; **189**: 1729–1733.
15. **Ferreira SA, *et al.*** Canine skin and conjunctival swab samples for the detection and quantification of *Leishmania infantum* DNA in an endemic urban area in Brazil. *PLoS Negected Tropical Diseases* 2012; **6**: 1–9.
16. **Leite RS, *et al.*** PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab sample. *Veterinary Parasitology* 2010; **170**: 201–206.
17. **Francino O, *et al.*** Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Veterinary Parasitology* 2006; **137**: 214–221.
18. **Cohen J.** A coefficient of agreement for nominal scales. *Educational and Psychological Measurement* 1960; **20**: 37–46.
19. **McNemar Q.** Note on the sampling error of the difference between correlated proportions or percentages. *Psychometrika* 1947; **12**(2): 153–157.
20. **Altman DG.** *Practical Statistics for Medical Research*. London: Chapman and Hall, 1991, p. 611.
21. **Byrt CJ, *et al.*** Prevalence, and κ. *Journal of Clinical Epidemiology* 1993; **45**: 423–429.
22. **Looney SW, Hagan JL.** Statistical methods for assessing biomarkers and analyzing biomarker data. In: Rao CR, Miller JP, Rao DC eds. *Handbook of Statistics: Epidemiology and Medical Statistics*, vol. **27**. Amsterdam: North Holland, 2008, pp. 116–147.
23. **Sahai H, Khurshid A.** *Statistics in Epidemiology: Methods, Techniques, and Applications*. Boca Raton: CRC Press Inc., 1995, p. 352.
24. **Bossuyt PM, *et al.*** Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *Clinical Chemistry* 2003; **49**: 1–6.
25. **Bossuyt PM, *et al.*** The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Clinical Chemistry* 2003; **49**: 7–18.
26. **Oliva G, *et al.*** Incidence and time course of *Leishmania infantum* infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. *Journal of Clinical Microbiology* 2006; **44**: 1318–1322.
27. **Michalsky EM, *et al.*** Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to

Lutzomyia longipalpis phlebotomine sand flies. *Veterinary Parasitology* 2007; **147**: 67–76.

28. **Verçosa BL, *et al.*** Transmission potential, skin inflammatory response, and parasitism of symptomatic and asymptomatic dogs with visceral leishmaniasis. *BMC Veterinary Research* 2008; **4**: 45. doi: 10.1186/1746-6148-4-45.
29. **Solano-Gallego L, *et al.*** Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. *Veterinary Parasitology* 2009; **165**: 1–18.
30. **Lombardo G, *et al.*** Detection of *Leishmania infantum* DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. *Veterinary Parasitology* 2012; **184**: 10–17.
31. **Feinstein AR, Cicchetti DV.** High agreement but low κ. I. The problems of two paradoxes. *Journal of Clinical Epidemiology* 1989; **43**: 543–549.
32. **Grimaldi G, *et al.*** Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2012; **106**: 54–59.
33. **Aschar M, *et al.*** Value of the oral swab for the molecular diagnosis of dogs in different stages of infection with *Leishmania infantum*. *Veterinary Parasitology* 2016; **225**: 108–113.