

Evaluation of conjunctival swab as a mass-screening tool for molecular diagnosis of canine visceral leishmaniasis

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Abstract The canine visceral leishmaniasis (CVL) diagnosis is an important step of visceral leishmaniasis control program in Brazil once the dog is the main reservoir host of the disease. The aim of this study was to evaluate the conjunctival swab (CS) as a mass-screening tool for CVL molecular diagnosis in an endemic area classified as priority for the Brazilian Ministry of Health for surveillance action. A total of 1350 domiciled dogs were screened. The animals were evaluated by serological tests (enzyme-linked immunosorbent assay (ELISA) as screening and immunofluorescence antibody test (IFAT) for confirmation) and by CS associated to real-time PCR, using primers addressed to kinetoplast DNA (kDNA) minicircles and SYBR Green. Canine β -globin gene amplification was used to evaluate the sample DNA integrity. A subgroup of 484 animals was also submitted to clinical evaluation. Among the 1350 dogs screened, 369 (27.3 %) were positive by CS real-time PCR and 126 (9.3 %) tested positive by ELISA. Thirty-one percent (39/126) of the ELISA-positive dogs were confirmed by IFAT. CS real-time PCR was able to detect infection in dogs independently of the symptomatology degree ($p > 0.05$), while ELISA was more sensitive in the group of dogs that present three or more clinical signs related to CVL. The results demonstrated that CS real-time PCR was able to detect a higher number of infected dogs than ELISA and that the prevalence of canine infections has been underestimated by the serological assays. The use of sensitive

molecular diagnostic methods like CS real-time PCR, mainly in endemic areas, could greatly contribute to disease control.

Keywords Canine visceral leishmaniasis · Diagnosis · PCR · Real-time PCR · Conjunctival swab

Introduction

Visceral leishmaniasis (VL) is a zoonotic systemic disease caused by the intracellular protozoan parasite *Leishmania infantum* (= *Leishmania chagasi*). According to the World Health Organization (WHO), 90 % of all human cases occur in Bangladesh, Brazil, India, Nepal, and Sudan (WHO 2010). VL is a compulsory notification disease in Brazil, and in the last 5 years, more than 3000 cases per year were confirmed. The Brazilian Ministry of Health has instituted specific measures to control the disease dissemination, and these include early diagnosis and treatment of human cases, insect vector control, health education, and elimination of seropositive infected dogs (Ministério da Saúde 2013).

Dogs are the main domestic reservoir in urban areas, and some infected animals can develop overt clinical disease, whereas others remain as asymptomatic carriers without detectable clinical signs (Baneth et al. 2008; Solano-Gallego et al. 2009). Nevertheless, naturally infected asymptomatic dogs have been demonstrated to be easily infective to sand flies under experimental conditions (xenodiagnosis). The canine visceral leishmaniasis (CVL) prevalence in Brazil endemic areas ranges from 5.9 to 29.8 % (França-Silva et al. 2003; Malaquias et al. 2007; Rondon et al. 2008; Lopes et al. 2010), although the serological methods employed in

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the CVL detection exhibit low sensitivities and may underestimate the true value. Test accuracy is low, mainly for detection of asymptomatic dogs (de Paula et al. 2003; Almeida et al. 2005), reflecting in failures in control measures and the maintenance of infected dogs in endemic areas. The serological tests can also present false-positive results due to cross-reactions with other diseases like trypanosomiasis (Barbosa-De-Deus et al. 2002). In Brazil, serologic surveys have been accomplished using the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT) in dog screening-and-culling campaigns until 2012. Currently, a fast immunochromatographic test (Dual Path Platform (DPP)) was implemented as a screening test (Ministério da Saúde 2011). DPP has shown an excellent performance identifying 98 % of symptomatic dogs, but the efficacy for diagnosis of asymptomatic animals was only 47 % (Grimaldi et al. 2012).

Studies have shown that the CVL control actions have not achieved the expected impact. This negative outcome has been ascribed to delays in detecting and eliminating infected dogs, the tendency to replace infected dogs by susceptible puppies, and low sensitivity of the available serological methods (Rosário et al. 2005; Gomes et al. 2008).

The polymerase chain reaction (PCR) has been shown to provide a rapid, specific, and sensitive technique for *Leishmania* detection and CVL diagnosis (Maia and Campino 2008; de Assis et al. 2010; de Queiroz et al. 2010). Recently, the real-time PCR was introduced for detection and typing of *Leishmania* (Schulz et al. 2003; Mary et al. 2004; Van der Meide et al. 2005) with the advantages of speed and reduced risk of sample contamination, since monitoring of amplification is conducted as the reaction proceeds. Studies have reported that real-time PCR has greater sensitivity than conventional PCR for CVL diagnosis and is reproducible in diagnostic routines (Francino et al. 2006). However, the use of noninvasive samples is very important for the diagnosis since they could be obtained outside of veterinary centers and could be applied to massive screenings of dogs. One useful sample is the conjunctival swab (CS) which is obtained using a sterile swab to sample the dogs' conjunctivas. The CS-PCR has been shown to be highly sensitive for CVL diagnosis in both symptomatic (Strauss-Ayali et al. 2004; Ferreira et al. 2008; Pilatti et al. 2009) and asymptomatic dogs (Leite et al. 2010). Although the high CS-PCR sensitivity and applicability for CVL molecular diagnosis have been confirmed by different research groups (Andrade and Melo 2014), field studies in wide heterogeneous populations including seronegative and seropositive animals are still lacking.

The aim of this study was to evaluate CS as a mass-screening tool for CVL molecular diagnosis by comparing the results of serological and molecular diagnosis. The CS samples were analyzed by a high-sensitivity real-time PCR protocol that uses primers addressed to kinetoplast DNA

(kDNA) minicircles. The study was performed in the North Region of Belo Horizonte City, capital of Minas Gerais State, an endemic area classified as priority by the Brazilian Ministry of Healthy for surveillance action.

Materials and methods

Ethical statement

This study was approved by the Committee of Ethics in Animal Experimentation of the Universidade Federal de Minas Gerais (UFMG) (protocol no. 001/2011) and by the City Council of Belo Horizonte (protocol no. 0344.0.000.410-11). All procedures were according to the guidelines established by the Brazilian Animal Experimental College (COBEA) and by the Brazilian Federal Law 11794 of the 2008. The owners of dogs enrolled in this project were informed of the research purposes. They were required to sign the informed consent form before sample and data collection.

Experimental design

The cross-sectional study was conducted between 2011 and 2012 in the north sanitary district of Belo Horizonte (19° 55' 15" S, 43° 56' 16" W), which covers an area of 34.32 km². According to demographic census performed by the Instituto Brasileiro de Geografia e Estatística (IBGE), the human population in this area was of 212,055 individuals (Belo Horizonte 2013). The canine population in the study area was estimated in 26,507 animals and the expected CVL prevalence between 5 and 10 %. A number of 1350 dogs were screened to obtain 120 ELISA-positive animals, in view to achieve a confidence interval of 95 % and an estimated precision of 1.5 %. The present study was accomplished in close collaboration with the Municipality Health Service of Belo Horizonte, and samples were collected during the annual canine serological survey conducted as part of the routine of CVL Control Program. The animals were evaluated by serological tests (ELISA as screening and IFAT for confirmation) and by CS associated to real-time PCR. Among the 1350 dogs screened, a subgroup of 484 animals was also submitted to clinical evaluation.

Sample collection

The collection of peripheral blood on filter paper for ELISA and IFAT assays was performed according to the Brazilian Ministry of Health guidelines (Ministério da Saúde 2013). CS samples were collected from both dogs' conjunctivas using sterile cotton swabs manufactured for bacteriological isolation. The cotton tips were broken and only the cotton parts were transferred to DNase-free sterile microtubes (Eppendorf®, Germany) and stored at -20 °C until use.

Clinical evaluation

A subgroup of 484 dogs was clinically assessed by a veterinary. The clinical signs observed or reported by the owner were documented in individual clinical records previously elaborated. These records also contained the serial number and corresponding codes from Municipality Health Service files, the animal's identification details, and a term of consent duly signed by the owner. All dogs sampled were photographed, and the photo was attached in the respective record in order to guarantee the information tracking.

The animals were classified in four different groups according to their clinical signs: (1) dogs without any apparent clinical sign (A); (2) dogs with clinical signs grade I (SI) that include animals showing up to two isolated clinical signs usually caused by CVL and there was no report of any other morbidity that could justify the symptoms; (3) dogs with clinical signs grade II (SII) that present three or more typical clinical signs for CVL without report of any other morbidity; and (4) dogs with clinical signs* (S*) showing at least one clinical sign related to CVL that however could have a different cause such as other grievances/morbidities previously diagnosed by a veterinary, physiological conditions, or determinants observed in the field, like animal's environment conditions or food availability, for example. It is relevant to mention that for dogs classified as S*, the CVL was also considered as a possible cause.

Serological tests

The serological tests were executed by the Laboratory of Zoonotic Disease Control Department (LABZOO) of Municipality Health Service of Belo Horizonte. According to the Brazilian Ministry of Health guidelines (Ministério da Saúde 2013), two serological tests were used: enzyme-linked immunosorbent assay (ELISA-*EIE*—canine visceral leishmaniasis produced by Bio-Manguinhos/Fiocruz, Brazil) and the immunofluorescence antibody test (IFAT-*IFI*—canine visceral leishmaniasis produced by Bio-Manguinhos/Fiocruz, Brazil). ELISA was used as the screening test and IFAT as the confirmatory assay. The consolidated serological result was considered positive when ELISA and IFAT were simultaneously reagent ($\geq 1:80$). The results were considered indeterminate when ELISA was reagent, and IFAT showed fluorescence at sera dilution of 1:40.

DNA extraction

The cottons from right and left conjunctivas of the same animal were mixed constituting a unique sample. The DNA purification from cotton swabs was carried out by the phenol-chloroform-isoamyl alcohol method. Each cotton received 600 μL of lysis buffer (50 mmol/L Tris, 50 mmol/L NaCl,

and 10 mmol/L EDTA, pH 8.0) containing proteinase K (250 $\mu\text{g}/\text{mL}$) and Triton X-100 (1 %). After the incubation (2 h at 56 °C), the solution was eluted by inserting the cotton inside a 5-mL syringe. The plunger was pushed and the eluate was recovered in the same Eppendorf tube used for the lysis step. Then, 500 μL of 75 % Tris-saturated phenol (Sigma-Aldrich®) and 25 % chloroform-isoamyl alcohol (Sigma-Aldrich®) were added. The organic phase was separated from the aqueous phase by centrifugation at 12,000 $\times g$ for 5 min, and the organic material was transferred to a new microtube. The extraction was repeated with 500 μL of 50 % phenol, 50 % chloroform-isoamyl alcohol, and once with 100 % chloroform-isoamyl alcohol. The DNA precipitation was performed with one volume of isopropanol-sodium acetate, followed by washing with 75 % ethanol. The DNA pellet was suspended in 60 μL of autoclaved ultrapure water. DNA preparations were kept at -20 °C until being used.

Real-time PCR assay

Real-time PCR was performed according to de Paiva Cavalcanti et al. (2009) with some changes (Carvalho Ferreira et al. 2014). *L. infantum*-specific primers (Linf.1-23F: 5'-TCCCAAACCTTTTCTGGTCCT-3' and Linf.1-154R: 5'-TTACACCAACCCCCAGTTTC-3') that amplify a 132-bp fragment (T_m 81 °C) of kDNA were used. PCR was carried out in a final volume of 12.5 μL containing 3.0 pmol of each primer, 6.25 μL of 2 \times SYBR Green reaction master mix® (Applied Biosystems®, USA), and 2.0 μL of DNA with a final concentration around 20 ng/ μL . Reactions were processed and analyzed in a StepOne™ System (Applied Biosystems®). The reaction was performed with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of amplification (95 °C/15 s, 60 °C/1 min). Nontemplate controls and quantitative standards were included. Standard curves were prepared using dilutions of *L. infantum* (MHOM/1973/BH46) DNA. The dissociation curve analysis was performed to validate the positive samples. Five percent of the samples were chosen randomly and reanalyzed to verify the reproducibility.

The amplification of the canine housekeeping gene β -globin was used to evaluate the sample DNA integrity. β -Globin-specific primers (β -globin: 5' CAA CTT CAT CCA CGT TCA CC 3' and β -globin 01: 5' ACA CAA CTG TGT TCA CTA GC 3') (Greer et al. 1991) that amplify a 118-bp fragment (T_m 79 °C) of β -globin gene were used. PCR was carried out in a final volume of 12.5 μL containing 3.0 pmol of each primer, 6.25 μL of 2 \times SYBR Green reaction master mix® (Applied Biosystems®, USA), and 2.0 μL of DNA with a final concentration around 20 ng/ μL . Reactions were processed and analyzed in a StepOne™ System (Applied Biosystems®). The reaction was performed with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of

amplification (95 °C/15 s, 60 °C/1 min). Nontemplate controls and quantitative standards were included. Samples that were positive for β -globin were validated, whereas negative samples were excluded from the study.

Statistical analysis

The frequencies of positive results obtained from all the clinical samples were compared using the Pearson χ^2 test with a 5 % significance level. The difference between the results was considered significant with P value <0.05. The kappa index (κ) was used to evaluate the agreement between tests, and it was interpreted in accordance with Landis et al. (1977).

Results

All animals were screened by ELISA and CS real-time PCR. IFAT was used as a confirmatory assay for ELISA-positive dogs, in accordance with the guidelines of the Brazilian Health Ministry. Of the 1350 dogs screened, 369 (27.3 %) were positive by CS real-time PCR and 126 (9.3 %) tested positive by ELISA. Among the ELISA-positive dogs, 31.0 % (39/126) were confirmed by IFAT. The reproducibility of CS real-time PCR assay was 95.7 %.

When ELISA and CS real-time PCR results were compared, it was observed that 28.5 % (105/369) of dogs positive in the CS real-time diagnosis were positive in the ELISA assay, while 2.1 % (21/981) of CS real-time PCR-negative dogs were positive by ELISA. By the other side, 83.3 % (105/126) of the ELISA-positive dogs tested positive in the CS real-time PCR, whereas 21.6 % (264/1224) of the ELISA-negative animals were positive by CS real-time PCR (Table 1).

The IFAT was performed for the 126 ELISA-positive samples. Among the IFAT-positive dogs, 87.2 % (34/39) were simultaneously positive in the CS real-time PCR. Five animals were negative in the CS real-time PCR but IFAT-positive.

Table 1 Comparison between ELISA and CS real-time PCR in dogs evaluated for CVL

Diagnosis			
CS real-time PCR	ELISA		
	(+) <i>n</i> (%)	(-) <i>n</i> (%)	Total <i>n</i> (%)
(+)	105 (7.8)	264 (19.6)	369 (27.3)
(-)	21 (1.6)	960 (71.1)	981 (72.7)
Total	126 (9.3)	1224 (90.7)	1350 (100)

+ positive, - negative, *n* number of animals

Sixty-eight animals that test positive in the CS real-time PCR assay were negative for IFAT (Table 2).

Fair agreement was observed when the CS real-time PCR was compared with ELISA ($\kappa=0.28$) and IFAT ($\kappa=0.23$). No agreement was observed when the ELISA and IFAT were compared ($\kappa=0$). The association between ELISA/CS real-time PCR, IFAT/CS real-time PCR, and ELISA/IFAT was not significant ($p>0.05$).

A subgroup of 484 animals was submitted to clinical diagnosis. When the clinical signs were evaluated in the 52/484 (10.7 %) ELISA-positive dogs, it was observed that 73.1 % of dogs presented clinical signs; 55.7 % of them were included in the SII group (Table 3). The skin disorders were the main clinical signs found at the time of assessment (53.8 %). The second most frequent type of clinical manifestation was lymphadenopathy (42.3 %), followed by onychogryphosis (38.5 %); hair opacity (34.6 %); apathy (30.8 %); weight loss (26.9 %); cachexia (26.9 %); alopecia (26.9 %); pale mucus membranes (23.1 %); ocular disorders (21.2 %); locomotion disorders (9.6 %); and gastrointestinal disorders such as diarrhea, vomiting, or melena (5.8 %) (Table 4).

In the group of 24 animals with serological diagnosis confirmed by IFAT, 83.3 % of dogs were symptomatic being 66.7 % of SII group (Table 3). Skin disorders were also the main clinical signs found (66.7 %). Secondly, apathy, lymphadenopathy, onychogryphosis, and weight loss (45.8 % each) were observed. The other types of clinical manifestations were cachexia (41.7 %), hair opacity (37.5 %), pale mucus membranes (33.3 %), alopecia (29.2 %), ocular disorders (16.7 %), locomotion disorders (16.7 %), and gastrointestinal disorders (8.3 %).

By CS real-time PCR were found 156/484 (32.2 %) positive dogs. In this group, 35.3 % of animals were asymptomatic (A), 33.3 % fitted to the SII group, and 26.9 % to the SI, and 4.5 % were included in the S*(Table 3). The skin disorders were again the main clinical signs found (32.9 %). The second type of clinical manifestation was lymphadenopathy (29.1 %),

Table 2 Comparison between IFAT and CS real-time PCR in dogs evaluated for CVL

Diagnosis				
CS real-time PCR	IFAT			
	(+) <i>n</i> (%)	(-) <i>n</i> (%)	In <i>n</i> (%)	Total <i>n</i> (%)
(+)	34 (27.0)	68 (54.0)	3 (2.4)	105 (83.3)
(-)	5 (4.0)	14 (11.1)	2 (1.6)	21 (16.7)
In	0 (0)	0 (0)	0 (0)	0 (0)
Total	39 (31.0)	82 (65.1)	5 (4.0)	126 (100)

+ positive, - negative, *In* indeterminate, *n* number of animals

Table 3 Clinical status in ELISA, IFAT, and CS real-time PCR-positive groups

Clinical	ELISA <i>n</i> (%)	IFAT <i>n</i> (%)	CS real-time PCR <i>n</i> (%)
A	14 (26.9)	4 (16.7)	55 (35.3)
SI	8 (15.4)	4 (16.7)	42 (26.9)
SII	30 (57.7)	16 (66.7)	52 (33.3)
S*	0 (0)	0 (0)	7 (4.5)
Total	52 (100)	24 (100)	156 (100)

A dogs without any apparent clinical sign, SI dogs with clinical signs grade I, SII dogs with clinical signs grade II, S* dogs with clinical signs*, *n* number of animals

followed by onychogryphosis (27.8 %), hair opacity (19.0 %), alopecia (15.8 %), apathy (14.6 %), pale mucus membranes (13.9 %), ocular disorders (12.7 %), cachexia (12.7 %), weight loss (12.0 %), gastrointestinal disorders (4.4 %), and locomotion disorders (4.4 %) (Table 4).

Discussion

The present work was performed to evaluate the CS real-time PCR performance for CVL molecular diagnosis in a routine epidemiological survey. This is the first study investigating the efficacy of these procedures in a large population of dogs living in an endemic area of intense VL transmission.

The results showed that the prevalence of canine *L. infantum* infection determined by CS real-time PCR was

Table 4 Distribution of clinical signs in relation to diagnostic methods evaluated

Clinical signs	ELISA		IFAT		CS real-time PCR	
	(+)	%	(+)	%	(+)	%
Alopecia	14	26.9	7	29.2	25	15.8
Apathy	16	30.8	11	45.8	23	14.6
Cachexia	14	26.9	10	41.7	20	12.7
Gastrointestinal disorders	3	5.8	2	8.3	7	4.4
Pale mucus membranes	12	23.1	8	33.3	22	13.9
Locomotion disorders	5	9.6	4	16.7	7	4.4
Lymphadenopathy	22	42.3	11	45.8	46	29.1
Ocular disorders	11	21.2	4	16.7	20	12.7
Onychogryphosis	20	38.5	11	45.8	44	27.8
Hair opacity	18	34.6	9	37.5	30	19.0
Skin disorders	28	53.8	16	66.7	52	32.9
Weight loss	14	26.9	11	45.8	19	12.0
<i>n</i>	52		24		158	

+ positive, *n* number of animals evaluated

higher than that reported by the serological methods. The finding of a large number of dogs that tested negative in the serological assays but were positive for the CS real time-PCR was expected in view of the molecular assay higher sensitivity. False-positive results due to serological assays cross-reactivity with other trypanosomatids, mainly with *Leishmania braziliensis* and *Trypanosoma* ssp. (Viol et al. 2012), could explain the small group of dogs that tested positive in the serological assays but was CS real-time PCR-negative, since this test was *L. infantum*-specific.

Coura-Vital et al. (2011) studied prevalence and risk factors associated with *L. infantum* infection in a sample of 1443 dogs of Belo Horizonte City. This study used blood samples and conventional kDNA PCR for diagnosis. They obtained a positivity of 24.7 %, which was very similar to the results obtained in the present study using CS real-time PCR (27.3 %). Prevalence obtained by ELISA (9.4 %) was also equivalent of the present work (9.3 %). Other studies realized in different endemic areas point in the same direction: the prevalence of canine *L. infantum* infection determined by molecular tests was higher than verified by serological methods (Solano-Gallego et al. 2011; Lachaud et al. 2002; Wang et al. 2011). However, these studies used blood or invasive samples like skin biopsies and bone marrow.

The sensitivity and utility of whole blood or buffy coat for CVL screening are still contradictory. Unlike its performance in human medicine, PCR-based *Leishmania* detection in CVL using blood samples showed result variability and is considered of little diagnostic value (Reale et al. 1999). Problems related to DNA preparation, high frequency of PCR inhibitors in dog blood, low sensitivity, and variations of the parasite load in the course of infection have been reported (Strauss-Ayali et al. 2004; Ferreira et al. 2012; Geisweid et al. 2013; Ikononopoulos et al. 2003).

Despite to the high positive indices for PCR using skin samples of dogs (Xavier et al. 2006), the collection of this sample is painful, bloody, and invasive, requiring local anesthesia and aseptic manipulation. PCR performed using bone marrow (Fisa et al. 2001) and lymph node aspirates (Fisa et al. 2001; Almeida et al. 2013) also has shown high sensitivity, but again, the procedure of sampling is invasive, offering risk of infection for the animal and demands very-well-trained personnel. These constraints make these samples unsuitable for large-scale surveys. The use of DNA purified from CS for PCR-based diagnosis of CVL was introduced in order to reduce the need for invasive procedures (Strauss-Ayali et al. 2004; Ferreira et al. 2008). Contextually, Lombardo et al. (2012) and Ferreira et al. (2012) verified that CS has similar sensitivity to bone marrow and lymph nodes samples, respectively.

Carvalho Ferreira et al. (2014) pointed that CS real-time PCR was a very sensitive approach to detect *L. infantum* in dogs. CS real-time PCR was positive for 96.7 % of dogs

without clinical signs and in 100 % of the symptomatic animals. This study enrolled 60 infected dogs which were simultaneously positive in the parasitological and serological assays. The high positivity found in the present work with 1350 animals, including seronegative dogs, confirms these results. The study demonstrated that a large number of CS real-time PCR-positive dogs, with or without VL clinical signs, had negative serological diagnosis, showing that the use of CS real-time PCR in endemic areas would have impact in disease control.

The CS real-time PCR if used as a screening tool would identify a greater number of infected dogs. Nevertheless, this test should necessarily be associated with a confirmatory assay as DPP, for example, since some CS real-time PCR-positive dogs with negative serology may convert to CS real-time PCR-negative in the future without seroconversion (data not showed). It seems that in these cases, the CS real-time PCR was able to detect initial transient infections that were controlled by the animals with consequent reduction of the parasite burden beyond to the detection limit of the assay. This finding is in agreement with studies showing that a percentage of infected asymptomatic dogs may evolve to spontaneous cure (Baneth et al. 2008; Solano-Gallego et al. 2009). The combination of more than one diagnostic technique is recommended for CVL diagnosis (Morales-Yuste et al. 2012). In this context, a CS real-time PCR-positive dog with negative serology should be indicated as an animal to be monitored, since this group of dogs has high probability of seroconversion. Coura-Vidal et al. (2013) showed the importance of a PCR-positive test as a factor associated with seroconversion by *L. infantum*, demonstrating that PCR-positive dogs had twice the risk to become ELISA-positive in relation to PCR-negative ones. In this perspective, CS real-time PCR is a valuable tool to anticipate actions to control CVL in endemic areas.

The use of a rapid serological test associated with a confirmatory molecular assay has been indicated as necessary to detect all infected dogs, both asymptomatic and symptomatic (Veras et al. 2014). The value of CS real-time PCR as a confirmatory diagnostic test should also be considered. Among the ELISA-positive dogs, 30.9 % (39/126) were confirmed by IFAT while 83.3 % (105/126) were positive by CS real-time PCR. Nowadays, the costs of molecular tests are still higher in relation to serological assays and the use of molecular diagnosis as a confirmatory test can be more feasible at first.

It was observed that CS real-time PCR was able to detect infection in dogs independently of the symptomatology degree ($p > 0.05$) while ELISA was more sensitive in SII dogs, demonstrating the CS real-time PCR capacity to identify infected asymptomatic dogs, a drawback frequently reported for the serologic assays. The high frequency of skin disorders observed in CVL-positive animals was compatible with the findings of Almeida et al. (2005) that evaluated the clinical

signs in naturally infected dogs living in endemic areas. The major CVL clinical signs seen were emaciation and skin ulcers (80 %), followed by onychogryphosis and conjunctivitis (73 %). Freitas et al. (2012) evaluated naturally infected dogs with positive serology and observed cachexia as the most frequent clinical sign. Interestingly, in the present study, ocular abnormality was not a main clinical sign found in the group of CS real-time PCR-positive dogs.

In conclusion, the results demonstrated that CS real-time PCR was able to detect a higher number of infected dogs in relation to ELISA and would be a useable tool for routine screening in leishmaniasis control programs. The results of this study contribute to make molecular assays available in endemic areas, and these procedures can have a strong impact on disease control.

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Conflict of interest The authors declare that no competing interests exist.

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