



Molecular diagnosis of canine visceral leishmaniasis: A comparative study of three methods using skin and spleen from dogs with natural *Leishmania infantum* infection



Levi Eduardo Soares Reis^{a,g}, Wendel Coura-Vital^{a,c,g}, Bruno Mendes Roatt^{b,g},
 Leoneide Érica Maduro Bouillet^a, Henrique Gama Ker^a,
 Rory Cristiane Fortes de Brito^a, Daniela de Melo Resende^a,
 Mariângela Carneiro^{c,d}, Rodolfo Cordeiro Giunchetti^e, Marcos José Marques^f,
 Cláudia Martins Carneiro^{a,b}, Alexandre Barbosa Reis^{a,b,g,*}

^a Laboratório de Pesquisas Clínicas, Programa de Pós-graduação em Ciências Farmacêuticas/CiPharma, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil

^b Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas/NUPEB, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil

^c Pós-graduação em Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

^d Laboratório de Epidemiologia de Doenças Infecciosas e Parasitárias, Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

^e Laboratório de Biologia das Interações Celulares, Departamento de Biologia Celular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

^f Laboratório de Parasitologia Básica, Departamento de Ciências Biológicas, Universidade Federal de Alfenas, Alfenas, Minas Gerais, Brazil

^g Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais – INCT-DT, CNPq, Brazil

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ABSTRACT

Polymerase chain reaction (PCR) and its variations represent highly sensitive and specific methods for *Leishmania* DNA detection and subsequent canine visceral leishmaniasis (CVL) diagnosis. The aim of this work was to compare three different molecular diagnosis techniques (conventional PCR [cPCR], seminested PCR [snPCR], and quantitative PCR [qPCR]) in samples of skin and spleen from 60 seropositive dogs by immunofluorescence antibody test and enzyme-linked immunosorbent assay. Parasitological analysis was conducted by culture of bone marrow aspirate and optical microscopic assessment of ear skin and spleen samples stained with Giemsa, the standard tests for CVL diagnosis. The primers L150/L152 and LINR4/LIN17/LIN19 were used to amplify the conserved region of the *Leishmania* kDNA minicircle in the cPCR, and snPCR and qPCR were performed using the DNA polymerase gene (DNA pol α) primers from *Leishmania infantum*. The parasitological analysis revealed parasites in 61.7% of the samples. Sensitivities were 89.2%, 86.5%, and 97.3% in the skin and 81.1%, 94.6%, and 100.0% in spleen samples used for cPCR, snPCR, and qPCR, respectively. We demonstrated that the qPCR method was the best technique to detect *L. infantum* in both skin and spleen samples. However, we recommend the use of skin due to the high sensitivity and sampling being less invasive.

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* Corresponding author at: Laboratório de Pesquisas Clínicas, Programa de Pós-graduação em Ciências Farmacêuticas/CiPharma, Escola de Farmácia, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil. Tel.: +55 31 35591694.

E-mail addresses: alexreis@nupeb.ufop.br, alexreisufop@gmail.com (A.B. Reis).

1. Introduction

Zoonotic visceral leishmaniasis caused by *Leishmania infantum* is the most severe and fatal form of leishmaniasis if untreated. The anthroponotic form is caused by *L. donovani* and causes a major disease burden globally. ZVL in Brazil is transmitted via sandfly (*Lutzomyia longipalpis*) bites during blood feeding (Lainson and Shaw, 1978) and the dogs are the main urban reservoirs of disease (Molina et al., 1994; Teixeira Neto et al., 2010). Canine visceral leishmaniasis (CVL) is a complex disease in which infection may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness (Reis et al., 2009; Solano-Gallego et al., 2009). In endemic areas, a susceptible fraction of infected dogs detected by serology and/or PCR – usually less than 50% – tends to progress toward clinical disease (Alvar et al., 2004; Coura-Vital et al., 2011). Recently several epidemiological studies demonstrated that serological tests detect fewer CVL cases compared with molecular methods, leading to an underestimate of the prevalence of infection (Oliva et al., 2006; Coura-Vital et al., 2011).

Some studies show that several types of biological samples can be used for the molecular diagnosis of CVL (Ferreira et al., 2008; Maia et al., 2009; de Almeida Ferreira et al., 2012). Studies comparing the DNA extraction methods (Demeke and Jenkins, 2010), types of primer pairs (Lachaud et al., 2002), target kinetoplasts (mitochondrial DNA) and target DNA specificity (Solcà et al., 2011) show a range in the performance of the molecular methods. However, few authors have compared different molecular techniques in various host tissues. In this context, there is no consensus about the best method and sample type for use in large-scale molecular diagnosis of CVL in an endemic area. The main goal of this work was to compare the performance of three different molecular diagnostic techniques (conventional PCR [cPCR], quantitative PCR [qPCR], and seminested PCR [snPCR]) using skin and spleen tissue from dogs naturally infected by *L. infantum* in an endemic area and exhibiting different clinical forms of CVL.

2. Materials and methods

2.1. Clinical samples

The study involved 60 seropositive dogs (*Canis familiaris*) naturally infected by *L. infantum*. The samples were collected at the Zoonotic Disease Control Center of the Belo Horizonte, Minas Gerais, Brazil. In Brazil, all seropositive dogs must be euthanized, and skin and spleen samples were collected after euthanasia. The biopsy of the ear skin and spleen were collected using a sterile scalpel. The Laboratory of Zoonosis of the Prefeitura Municipal de Belo Horizonte conducted serological examination of these animals, using enzyme-linked immunosorbent assay and immunofluorescence antibody test (Biomanguinhos, Rio de Janeiro, RJ, Brazil). To confirm the CVL infection by the *L. infantum*, the Dual-Path Platform (DPP® CVL, Biomanguinhos, Rio de Janeiro, RJ, Brazil) composed of specific recombinant proteins (rK26 and rK39) were employed (Grimaldi et al., 2012) along with the PCR-RFLP to identify *L.*

infantum (data not shown) as described previously (Volpini et al., 2004; de Andrade et al., 2006; Coura-Vital et al., 2011). All dogs with positive ELISA, IFAT, DPP® and PCR-RFLP/*L. infantum* were included in this study. Based on the presence of clinical signs of CVL, the dogs were divided into three groups: asymptomatic dogs ($n=20$), with no signs suggestive of disease; oligosymptomatic dogs ($n=22$), presenting one to three signs; and symptomatic dogs ($n=18$), with more than three signs.

2.2. DNA extraction

Skin and spleen samples were stored frozen at -80°C for subsequent analyses. Good laboratory practice was used to avoid DNA cross-contamination, and negative controls were included during all DNA extraction procedures and in the performance of molecular techniques. Total genomic DNA was extracted from approximately 20 mg of tissue (skin or spleen). DNA was extracted using Wizard™ Genomic DNA purification kits (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and quality of DNA obtained from tissues was determined with a spectrophotometer (NanoVue Plus, GE Healthcare Products, Piscataway, NJ, USA).

2.3. Molecular methods

2.3.1. Conventional PCR

The primers L150/L152 were used to amplify the conserved region of the *Leishmania* kDNA minicircle, a 120-bp fragment, and are shown in Table 1 (Degraeve et al., 1994). The reaction mixture consisted of $1\times$ buffer (10 mM Tris-HCl, 50 mM KCl [pH 8.8]), 1.5 mM MgCl_2 , 0.2 mM dNTP, 1.0 pmol of each primer, 0.76 U of Taq polymerase (Fermentas, USA), 1.0 μL DNA, and MilliQ water to a final volume of 12.5 μL /well (MicroAmp® Fast Optical 96-well, Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed in a 96-well Verit Thermal Cycler (Applied Biosystems) using the following program: initial denaturation at 96°C for 6 min, followed by 40 cycles of 30 s at 93°C , 30 s at 64°C , and 30 s at 72°C , with a final extension at 72°C for 7 min.

2.3.2. Seminested PCR

Primers LINR4, LIN17, and LIN19 were used to amplify the conserved region of the *Leishmania* kDNA minicircle, a 700-bp fragment, and are shown in Table 1 (Aransay et al., 2000). The combination of primers LINR4, LIN17, and LIN19 was used in a snPCR technique. The first amplification reaction was carried out in a 5 μL volume containing 0.95 μL of DreamTaq Green Mix $2\times$ (Fermentas, USA), 1 mM LINR4, 0.2 mM LIN17, 2.5 μL of DNA extract, and 0.95 μL of MilliQ water overlaid with mineral oil. The mixture was incubated in a Thermal Cycler (Applied Biosystems) using the following program: initial denaturation at 94°C for 5 min, followed by 17 cycles of 30 s at 94°C , 30 s at 52°C , and 30 s at 72°C , with a final extension at 72°C for 10 min. The second seminested reaction was carried out with the addition of 45 μL of a solution containing 20.25 μL of DreamTaq Green Mix $2\times$ (Fermentas, USA), 1 mM LIN19, and 20.25 μL of MilliQ to a total volume of the first solution amplified.

Table 1
Sequences of primers used for molecular methods.

Method ^a	Target	Primer sequence (5'–3')	Product (bp)
cPCR	L150/L152	Forward: GGG (G/T)AG GGG CGT TCT (G/C)CG AA Reverse: (G/C)(G/C)(G/C)(A/T)CT AT(A/T) TTA CAC CAA CCC C	120
snPCR	LINR4 LIN17 LIN19	Forward: GGG GTT GGT GTA AAA TAG GG Reverse: TTT GAA CGG GAT TTC TG Reverse: CAG AAC GCC CCT ACC CG	700
qPCR	DNA Pol α	Forward: TGT CGC TTG CAG ACC AGA TG Reverse: GCA TCG CAG GTG TGA GCA C	90
qPCR	G3PDH	Forward: TCA ACG GAT TTG GCC GTA TTG G Reverse: TGA AGG GGT CAT TGA TGG CG	90

^a cPCR, conventional PCR; snPCR, seminested PCR; qPCR, quantitative PCR.

The mixture was incubated using the following program: initial denaturation at 94 °C for 5 min, followed by 33 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C, with a final extension at 72 °C for 10 min. Five microliters of the amplification reaction product was resolved on a 1.5% agarose gel and visualized under UV transillumination.

2.3.3. Quantitative PCR

In order to quantify parasite burdens, we used primers that amplified a 90-bp fragment of a single-copy of DNA polymerase gene (DNA pol α) from *L. infantum* (Bretagne et al., 2001). PCR was carried out in a final volume of 10 μ L containing 5 pmol forward and reverse primers, SybrGreen reaction master mix (Applied Biosystems, USA), and 2 μ L of template DNA. PCR conditions were as follows: an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Standard curves were prepared for each run using known quantities of pGEM[®] T plasmids (Promega, USA) containing the insert of interest. In order to verify the integrity of the samples, the same procedure was carried out for the G3PDH gene (90-bp fragment). Reactions were processed and analyzed in an ABI Prism 7500-Sequence Detection System (Applied Biosystems, USA).

2.4. Standard tests

Two methods were used as the standard tests for diagnosis, parasitological examination and bone marrow culture. Parasitological examinations involved the analysis of Giemsa-stained slides containing skin and spleen sections for the detection of amastigote forms and bone marrow culture in Novy-MacNeal-Nicolle-liver infusion tryptose (NNN-LIT) medium supplemented with 20% fetal bovine serum.

Table 2
Comparison of diagnostic methods and clinical manifestations.

Clinical forms	Diagnostic test positivity rate n ^a (%)						Parasitological tests ^b
	Skin			Spleen			
	cPCR	snPCR	qPCR	cPCR	snPCR	qPCR	
Asymptomatic	12/20 (60.0)	9/20 (45.0)	18/20 (90.0)	11/20 (55.0)	13/20 (65.0)	20/20 (100.0)	9/20 (45.0)
Oligosymptomatic	15/22 (68.2)	18/22 (81.8)	20/22 (90.9)	17/22 (77.3)	20/22 (90.9)	22/22 (100.0)	14/22 (63.6)
Symptomatic	17/18 (94.4)	16/18 (88.9)	18/18 (100.0)	16/18 (88.9)	18/18 (100.0)	18/18 (100.0)	14/18 (77.8)
Total	44/60 (73.3)	43/60 (71.7)	56/60 (93.3)	44/60 (73.3)	51/60 (85.0)	60/60 (100.0)	37/60 (61.7)

^a Number of positive dogs.

^b Bone marrow aspirate in culture medium NNN/LIT and direct visualization of amastigotes of the parasite.

2.5. Statistical analyses

The sensitivity and specificity of the molecular methods were assessed by comparison with results from the parasitological examinations and culture for *L. infantum* parasite detection. The calculations of sensitivity was performed using Stata software (version 11.0, Stata Corporation, College Station, TX, USA).

2.6. Ethical approval

The study was approved by the Committees of Ethics in Animal Experimentation of the Universidade Federal de Ouro Preto (protocol number 083/2007), the Universidade Federal de Minas Gerais (protocol number 020/2007), and the City Council of Belo Horizonte (protocol number 001/2008). All procedures in this study were according to the guidelines set by the Brazilian Animal Experimental College (COBEA), Federal Law number 11794.

3. Results

3.1. Comparison of diagnostic methods in different tissues according to clinical manifestations

In order to compare molecular diagnostic methods in different tissues, we performed cPCR, snPCR, and qPCR in skin and spleen samples from naturally infected dogs with different clinical forms of CVL. Parasite DNA was detected in 73.3% (44/60) of the samples from both tissues (skin and spleen) using cPCR, while snPCR showed a positivity of 71.7% (43/60) and 85.0% (51/60) for the skin and spleen, respectively. With qPCR, parasite DNA was detected in 93.3% (56/60) and 100% (60/60) of the skin and spleen samples, respectively. Based on parasitological tests, the

Table 3
Molecular diagnostic methods performance analysis.^a

Tissue	Molecular methods	TP	FN	Sensitivity (%) (95% CI)
Skin	cPCR	33	4	89.2 (75.3–95.7)
	snPCR	32	5	86.5 (72.0–94.1)
	qPCR	36	1	97.3 (86.2–99.5)
Spleen	cPCR	30	7	81.1 (65.8–90.5)
	snPCR	35	2	94.6 (82.3–98.5)
	qPCR	37	0	100 (90.6–100.0)

^a CI, confidence interval; TP, true positive; FN, false negative.

parasite was detected in 61.7% of samples used (Table 2). The positivity was 33.3% (20/60) in bone marrow culture samples, 8.3% (5/60) in skin samples, and 55% (33/60) in spleen samples based on optical microscopy (data not shown). Positivity was higher with molecular methods compared with parasitological tests. Furthermore, it was observed with all diagnostic methods that the positivity increased according to disease progression.

3.2. Evaluation of molecular diagnostic methods' performance

Sensitivity for each test was estimated using positive samples according to at least one parasitological test, as the basis for comparison ($n = 37$). The number of samples which were true positive (TP) and false negative (FN), as well as the sensitivity values in each tissue are shown in Table 3.

4. Discussion

In the present study, we compared the positivity of parasitological and molecular methods using skin and spleen samples from dogs naturally infected by *L. infantum* that exhibited different clinical forms of CVL (asymptomatic, oligosymptomatic, and symptomatic). The primers used for cPCR and snPCR target the kDNA of *Leishmania* and have a high sensitivity due to high copy number (around 10,000 copies per parasite) (Nicolas et al., 2002). In qPCR, the target was the genomic sequences for DNA polymerase α (Pol α) of *L. infantum*, which is a single-copy-number gene that showed good results for CVL diagnosis (de Almeida Ferreira et al., 2012). In the present study we demonstrated that the qPCR method performed the best in skin and spleen samples, showing the highest sensitivity in comparison with cPCR and snPCR.

The use of qPCR provides the ability to perform very sensitive, accurate, and reproducible measurements of specific DNA present in a sample (Mortarino et al., 2004; Francino et al., 2006). Its high sensitivity and specificity have been reported using many different tissue types, including blood, lymph node, and bone marrow (Rodríguez-Cortés et al., 2007; Belinchón-Lorenzo et al., 2013). Regardless of the clinical form, we found that qPCR yielded the highest positivity for detecting CVL infection. Spleen positivity was higher than that of the skin, but in both tissues positivity increased with disease progression. It has previously been reported that asymptomatic dogs present low tissue parasitism, while symptomatic dogs

have high parasite loads in various tissues such as skin, bone marrow, liver, lymph nodes, and spleen (Reis et al., 2009). These findings may be associated with a distinct CVL clinical spectrum and tissue parasitic load as demonstrated by Alves et al. (2009), who also found a positive correlation between symptoms and high parasite density in tissues. The difference in the positivity between the skin and spleen is probably related to the individual immune response, basically the relationship with disease progression, and the fact that the parasites do not distribute themselves evenly in all tissues (Maia and Campino, 2008).

In the present study, we compared the molecular diagnostic methods with parasitological tests; we noted that the latter showed lower positivity among the seropositive dogs. These data reinforce previous demonstrations that molecular tests are better for detecting CVL infection compared with parasitological tests (Maia et al., 2009; Manna et al., 2009).

Considering the results obtained when the skin samples were used, the best performance by a molecular diagnostic method was qPCR followed by cPCR and snPCR; qPCR showed the highest sensitivity. The skin of infected dogs is a reservoir for the parasite and may represent an important role in the infection of sand flies and the transmission of the parasite to other dogs and to humans (Giunchetti et al., 2006; Michalsky et al., 2007). From the localized cutaneous infection, the parasite can be disseminated via lymphatic or blood vessels, infecting macrophages of the bone marrow, lymph node, liver, and spleen, as well as kidneys and gastrointestinal tract (Tryphomas et al., 1977; Keenan et al., 1984).

The spleen samples showed the best performance with qPCR, while snPCR had the second best performance in this tissue. The spleen is considered to be one of the main organs affected in ongoing CVL because it is a site of cell activation during infection (Keenan et al., 1984). However, the difference in sensitivity of qPCR for spleen and skin samples was very small, thus justifying the use of the skin as a main biological material for CVL diagnosis. It is important to note that obtaining skin samples is less invasive than spleen samples. One of the major concerns about using spleen aspiration is the risk of severe bleeding and even death (Léveillé et al., 1993). This risk can be controlled by using abdominal ultrasonography (Watson et al., 2010). Without an abdominal ultrasound, however, a professional veterinarian with expertise and deep knowledge of canine anatomy is needed for needle aspiration of the spleen (Barrouin-Melo et al., 2004); this prevents its use on a large scale. The interpretation of PCR results should be made cautiously in clinically healthy dogs, since the PCR only permits to identify the presence of parasite DNA (Solano-Gallego et al., 2009). In a prospective study it was observed that subpatent infections (detected by PCR) may or may not progress to a stage of active CVL infection and in some cases they may convert to negative (Foglia Manzillo et al., 2013).

The use of molecular techniques on non-invasive samples, as skin biopsies, skin swabs, hair, nasal and oral swabs and conjunctival swab has shown potential results for use in the diagnosis of CVL in endemic areas (Belinchón-Lorenzo et al., 2013; Ferreira et al., 2013). The high

positivity found in our work by qPCR on the ear skin samples, suggesting that the combination of molecular diagnosis with other biological noninvasive samples, would be useful in massive screening of dogs infected.

Quantitative PCR has advantages over cPCR and snPCR because the reaction is done in fewer steps, with simple protocols. Amplification and detection of amplified material need not be displayed on a gel (agarose or polyacrylamide), thus reducing the risk of contamination by the handler under the conditions in which the reaction occurs.

Currently, molecular techniques are not considered to be labor intensive, and their costs have been declining due to high use in clinical laboratory diagnostics. There are already several reagent brands on the market, and several procedures for DNA extraction have been standardized; there is also a range of amplification protocols available (Quaresma et al., 2009). These factors have contributed to simplifying the methods, reducing costs, and obtaining results rapidly, making molecular techniques more accessible in research laboratories.

5. Conclusion

Among the three molecular techniques assessed for skin and spleen samples, we conclude that the best technique for the molecular diagnosis of CVL in dogs naturally infected with *L. infantum* is qPCR using skin samples. We recommend skin biopsies due to the high sensitivity as well as the skin being easier and less invasive to access compared with the spleen.

Conflict of interest

None of the authors has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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References

Alvar, J., Canavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. *Adv. Parasitol.* 57, 1–88.

Alves, C.F., de Amorim, I.F.G., Moura, E.P., Ribeiro, R.R., Alves, C.F., Michalick, M.S., Kalapothakis, E., Bruna-Romero, O., Tafuri, W.L., Teixeira, M.M., Melo, M.N., 2009. Expression of IFN-gamma, TNF-alpha, IL-10 and TGF-beta in lymph nodes associates with parasite load and clinical form of disease in dogs naturally infected with *Leishmania (Leishmania) chagasi*. *Vet. Immunol. Immunopathol.* 128, 349–358.

de Andrade, H.M., Reis, A.B., dos Santos, S.L., Volpini, A.C., Marques, J.M., Romanha, A.J., 2006. Use of PCR-RFLP to identify *Leishmania* species in naturally infected dogs. *Vet. Parasitol.* 140, 231–238.

Aransay, A.M., Scoulica, E., Tselentis, Y., 2000. Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. *Appl. Environ. Microbiol.* 66, 1933–1938.

Barrouin-Melo, S.M., Larangeira, D.F., Trigo, J., Aguiar, P.H.P., Dos-Santos, W.L.C., Pontes-de-Carvalho, L., 2004. Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of *Leishmania chagasi* infection in dogs. *Mem. Inst. Oswaldo Cruz* 99 (2), 195–197.

Belinchón-Lorenzo, S., Iniesta, V., Parejo, J.C., Fernández-Cotrino, J., Muñoz-Madrid, R., Soto, M., Alonso, C., Gómez Nieto, L.C., 2013. Detection of *Leishmania infantum* kinetoplast minicircle DNA by real time PCR in hair of dogs with leishmaniosis. *Vet. Parasitol.* 192, 43–50.

Bretagne, S., Durand, R., Olivi, M., Garin, J.F., Sulahian, A., Rivollet, D., Vidaud, M., Deniau, M., 2001. Real-time PCR as a new tool for quantifying *Leishmania infantum* in liver in infected mice. *Clin. Diagn. Lab. Immunol.* 8, 828–831.

Coura-Vital, W., Marques, M.J., Veloso, V.M., Roatto, B.M., Aguiar-Soares, R.D.D.O., Reis, L.E.S., Braga, S.L., Morais, M.H., Reis, A.B., Carneiro, M., 2011. Prevalence and factors associated with *Leishmania infantum* infection of dogs from an urban area of Brazil as identified by molecular methods. *PLoS Negl. Trop. Dis.* 5, e1291.

de Almeida Ferreira, S., Leite, R.S., Ituassu, L.T., Almeida, G.G., Souza, D.M., Fujiwara, R.T., de Andrade, A.S.R., Melo, M.N., 2012. Canine skin and conjunctival swab samples for the detection and quantification of *Leishmania infantum* DNA in an endemic urban area in Brazil. *PLoS Negl. Trop. Dis.* 6, e1596.

Degrave, W., Fernandes, O., Campbell, D., Bozza, M., Lopes, U., 1994. Utilization of molecular probes and PCR for detection and typing of *Leishmania* – a mini-review. *Mem. Inst. Oswaldo Cruz.* 89, 463–469.

Demeke, T., Jenkins, G.R., 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal. Bioanal. Chem.* 396, 1977–1990.

Ferreira, S.A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2008. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. *Vet. Parasitol.* 152, 257–263.

Ferreira, S.A., Almeida, G.G., Silva, S.O., Vogas, G.P., Fujiwara, R.T., Andrade, A.S.R., Melo, M.N., 2013. Nasal, oral and ear swabs for canine visceral leishmaniasis diagnosis: new practical approaches for detection of *Leishmania infantum* DNA. *PLoS Negl. Trop. Dis.* 7 (4), e2150.

Foglia Manzillo, V., Di Muccio, T., Cappiello, S., Scalone, A., Paparcone, R., Fiorentino, E., Gizzarelli, M., Gramiccia, M., Gradoni, L., Oliva, G., 2013. Prospective study on the incidence and progression of clinical signs in naïve dogs naturally infected by *Leishmania infantum*. *PLoS Negl. Trop. Dis.* 7, e2225.

Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Vet. Parasitol.* 137, 214–221.

Giunchetti, R.C., Mayrink, W., Genaro, O., Carneiro, C.M., Correia-Oliveira, R., Martins-Filho, O.A., Marques, M.J., Tafuri, W.L., Reis, A.B., 2006. Relationship between canine visceral leishmaniasis and the *Leishmania (Leishmania) chagasi* burden in dermal inflammatory foci. *J. Comp. Pathol.* 135, 100–107.

Grimaldi, G., Teva, A., Ferreira, A.L., Dos Santos, C.B., Pinto, I.de-S., de-Azevedo, C.T., Falqueto, A., 2012. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.* 106, 54–59.

Keenan, C.M., Hendricks, L.D., Lightner, L., Johnson, A.J., 1984. Visceral leishmaniasis in the German shepherd dog. *II. Pathology. Vet. Pathol.* 21 (1), 80–86.

Lachaud, L., Chabbert, E., Dubessay, P., Dereure, J., Lamothe, J., Dedet, J.P., Bastien, P., 2002. Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. *Parasitology* 125 (Pt. 3), 197–207.

Lainson, R., Shaw, J.J., 1978. Epidemiology and ecology of leishmaniasis in Latin-America. *Nature* 273, 595–600.

Léveillé, R., Partington, B.P., Biller, D.S., Miyabayashi, T., 1993. Complications after ultrasound-guided biopsy of abdominal structures in dogs and cats: 246 cases (1984–1991). *J. Am. Vet. Med. Assoc.* 203, 413–415.

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. *Vet. Parasitol.* 158, 274–287.

- Maia, C., Ramada, J., Cristóvão, J.M., Gonçalves, L., Campino, L., 2009. **Diagnosis of canine leishmaniasis: conventional and molecular techniques using different tissues.** *Vet. J.* 179, 142–144.
- Manna, L., Reale, S., Vitale, F., Gravino, A.E., 2009. **Evidence for a relationship between *Leishmania* load and clinical manifestations.** *Res. Vet. Sci.* 87, 76–78.
- Michalsky, E.M., Rocha, M.F., Rocha Lima, A.C.V.M.D.A., et al., 2007. **Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotomine sand flies.** *Vet. Parasitol.* (1/2), 67–76.
- Molina, R., Amela, C., Nieto, J., San-Andrés, M., González, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994. **Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*.** *Trans. R. Soc. Trop. Med. Hyg.* 88, 491–493.
- Mortarino, M., Franceschi, A., Mancianti, F., Bazzocchi, C., Genchi, C., Bandi, C., 2004. **Quantitative PCR in the diagnosis of *Leishmania*.** *Parassitologia* 46, 163–167.
- Nicolas, L., Prina, E., Lang, T., Milon, G., 2002. **Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues.** *J. Clin. Microbiol.* 40, 1666–1669.
- Oliva, G., Scalone, A., Manzillo, V.F., Gramiccia, M., Pagano, A., Muccio, T.D., Gradoni, L., 2006. **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.** *J. Clin. Microbiol.* 44, 1318–1322.
- Quaresma, P.F., Murta, S.M.F., Ferreira, E.D.C., da Rocha-Lima, A.C.V.M., Xavier, A.A.P., Gontijo, C.M.F., 2009. **Molecular diagnosis of canine visceral leishmaniasis: identification of *Leishmania* species by PCR-RFLP and quantification of parasite DNA by real-time PCR.** *Acta Trop.* 111, 289–294.
- Reis, A.B., Martins-Filho, O.A., Teixeira-Carvalho, A., Giunchetti, R.C., Carneiro, C.M., Mayrink, W., Tafuri, W.L., Corrêa-Oliveira, R., 2009. **Systemic and compartmentalized immune response in canine visceral leishmaniasis.** *Vet. Immunol. Immunopathol.* 12, 87–95.
- Rodríguez-Cortés, A., Ojeda, A., López-Fuertes, L., Timón, M., Altet, L., Solano-Gallego, L., Sánchez-Robert, E., Francino, O., Alberola, J., 2007. **A long term experimental study of canine visceral leishmaniasis.** *Int. J. Parasitol.* 37, 683–693.
- Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. **Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniasis.** *Vet. Parasitol.* 165, 1–18.
- Solcà, M.D.S., Guedes, C.E.S., Nascimento, E.G., Oliveira, G.G.D.S., Dos Santos, W.L.C., Fraga, D.B.M., Veras, P.S.T., 2011. **Qualitative and quantitative polymerase chain reaction (PCR) for detection of *Leishmania* in spleen samples from naturally infected dogs.** *Vet. Parasitol.* 184, 133–140.
- Teixeira Neto, R.G., Giunchetti, R.C., Carneiro, C.M., Vitor, R.W.D.A., Coura-Vital, W., Quaresma, P.F., Ker, H.G., de Melo, L.A., Gontijo, C.M., Reis, A.B., 2010. **Relationship of *Leishmania*-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis.** *Vet. Parasitol.* 169, 248–257.
- Tryphomas, L., Zawdzka, Z., Bernard, M.A., Janzen, E.A., 1977. **Visceral leishmaniasis in a dog: clinical hematological and pathological observations.** *Can. J. Comp. Med.* 41, 1–12.
- Volpini, A.C., Passos, V.M., Oliveira, G.C., Romanha, A.J., 2004. **PCR-RFLP to identify *Leishmania* (*Viannia*) *braziliensis* and *L. (Leishmania)* *amazonensis* causing American cutaneous Leishmaniasis.** *Acta Trop.* 90, 31–37.
- Watson, A.T., Penninck, D., Knoll, J.S., Keating, J.H., Sutherland-Smith, J., 2010. **Safety and correlation of test results of combined ultrasound-guided fine-needle aspiration and needle core biopsy of the canine spleen.** *Vet. Radiol. Ultrasound* 52, 317–322.