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Accuracy of quantitative polymerase chain reaction in samples of frozen and paraffin-embedded healthy skin for the diagnosis of canine visceral leishmaniasis

[*Acurácia da reação em cadeia da polimerase quantitativa em amostras de pele íntegra congelada e parafinada no diagnóstico da leishmaniose visceral canina*]

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ABSTRACT

The purpose of the present work was to evaluate the accuracy of quantitative polymerase chain reaction (qPCR) performed on samples of fresh frozen tissue (FT) and formalin-fixed, paraffin-embedded (FFPE) healthy skin. This is a validation study conducted with samples from 46 dogs from an endemic area in Brazil. After sample collection, DNA extractions were conducted using commercial kits and qPCR was oriented to kinetoplast DNA (kDNA) targets of the *Leishmania infantum* species. The results obtained for the FFPE samples showed 63.6% sensitivity and 77.1% specificity, whereas those obtained for the FT samples showed 100% and 48.6%, respectively. Poor agreement was observed for the results of the qPCR technique with FT and FFPE samples. Our results suggest freezing as the most suitable conservation method for the formation of sample databases considering DNA recovery

Keywords: diagnosis, qPCR, canine visceral leishmaniasis

RESUMO

O objetivo deste trabalho foi avaliar a acurácia da PCR quantitativa (qPCR) realizada em amostras de pele íntegra congelada (FT) e parafinada (FFPE). Trata-se de um estudo de validação, com amostras provenientes de 46 cães de uma área endêmica no Brasil. Após as coletas de amostras, as extrações de DNA foram realizadas utilizando-se kits *comerciais, e a qPCR foi orientada para alvos do kDNA da espécie* Leishmania infantum*. Os resultados obtidos para as amostras FFPE foram 63,6% de sensibilidade e 77,1% de especificidade; para as amostras FT, 100% e 48,6%, respectivamente. A concordância dos resultados da técnica de qPCR com amostras FT e FFPE foi pobre. Os resultados sugerem que o congelamento é o método mais adequado de conservação para banco de amostras para recuperação de DNA.*

Palavras-chave: diagnóstico, qPCR, leishmaniose visceral canina

INTRODUCTION

Visceral leishmaniasis is a chronic infectious disease of public health importance worldwide, affecting both human beings and other species of wild and domestic mammals (Control…, 2010).

In Brazil, canine visceral leishmaniasis (CVL) is caused by *Leishmania (Leishmania) infantum* (sin. *L. chagasi)* (Kuls *et al*., 2011), and *Lutzomyia longipalpis* is its main vector (Brasil, 2006). Domestic dogs (*Canis familiaris*) have been considered its main reservoir and source of infection in urban areas; therefore, as a control measure, the euthanasia of dogs with positive diagnosis for CVL (Brasil, 2006) has been advocated.

Currently, the diagnosis of leishmaniasis is accomplished through the combination of clinical, laboratorial and epidemiological data, because the disease presents a wide spectrum of clinical manifestations, which complicates its identification (Gontijo and Melo, 2004).

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Polymerase chain reaction (PCR) -based techniques show high specificity and sensitivity, besides the ability to exponentially amplify DNA copies from a small sample amount (Mesquita *et al*., 2001) and different types of clinical materials (Almeida *et al*., 2013). Moreover, the immune status of dogs does not interfere with the test results, which allows the use of PCR in cases with inconclusive outcome by other techniques, as well as in cases of clinical (or immune) anergy or cross-reactivity in serological tests (Moreira *et al*., 2007; Lachaud *et al*., 2002). In addition, the PCR technique can be used in studies on the development of potential vaccines and monitoring of CVL treated animals (Fiuza *et al*., 2015; Nunes *et al*., 2016).

The quality of the DNA used in PCR is an important factor to obtain reliable results. Although DNA extraction requires a differentiated protocol, its extraction for PCR can be performed from fresh frozen tissues (FT) (Oliveira *et al*., 2005) and formalin-fixed, paraffin-embedded (FFPE) tissues (Roura *et al*., 1999). FT samples result in better quality DNA, while FFPE samples can result in partially degraded or impure DNA (Ferrer *et al*., 2007).

However, FFPE samples can be stored without cooling, which facilitates their transportation and storage in locations with poor infrastructure (Laskay *et al.,* 1995). In this study, we evaluated the accuracy of quantitative polymerase chain reaction (qPCR) conducted with FT and FFPE samples of healthy skin, collected from the scapular region of dogs, for *L. infantum* DNA detection.

MATERIALS AND METHODS

The sample bank comprised dogs from a CVL endemic area in Belém, Para state, Brazil. The animals were submitted to biopsy of healthy skin in order to perform reference techniques for CVL diagnosis. Dogs which tested positive in at least one of the following tests were considered infected with *Leishmania*: parasitological culture (Silva *et al*., 2011) followed by isoenzymatic characterization (Cupolillo *et al*., 1994); immunohistochemistry (IHC) (Quintella *et al*., 2009); and microscopy identification after hematoxylin and eosin staining (HE) (Carson and Hladick, 2009).

This is a diagnostic validation study using a convenience sample. We used two fragments - one prepared with FFPE and another with FT - of healthy skin collected from the scapular region, with a three-millimeter (mm) punch, of each of the 46 dogs surveyed. The protocol for obtaining the clinical samples was approved by the Ethics Committee on Animal Use - CEUA/Fiocruz (N. L-038/08).

We used the guideline of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin *et al*., 2009). All phases of qPCR were performed in a reference center for CVL.

Five 5µm-thick histological sections were obtained from each block of FFPE samples using a microtome (Leica Microsystems RM 2025). To prevent contamination between samples, the microtome was cleaned with 70% ethanol and had its disposable blade replaced at the start of every new sample processing.

DNA extraction was performed for each tissue using a specific kit, in accordance with the manufacturers' protocols, aiming to achieve the best DNA yield results.

Deparaffinization and DNA extraction were conducted using a QIAamp® DNA FFPE Tissue kit (Qiagen, California, USA), whereas an Illustra™ Tissue & Cells Genomicprep Mini spin kit (GE Healthcare, UK) was used for the extraction and purification of DNA from FT samples. The manufacturers' recommendations were followed for both processes.

DNA quantification was conducted by fluorimetry using a Qubit® 2.0 Fluorometer (Invitrogen®, California, USA) platform together with a Qubit® dsDNA HS Assay (Invitrogen®, California, USA) reagent specific for double-stranded DNA, following the manufacturers' recommendations.

Standardized tests using culture of promastigote forms of *L. infantum* and sample of negative dogs were performed to determine reaction quality. The standard curve was made with a DNA sample obtained from the culture of promastigote forms of *L. infantum* (MHOM/BR/1974/PP75) in exponential growth phase. The DNA of 1 x 10^6 parasites was

extracted using a DNeasy® Blood & Tissue kit (Qiagen® California, USA), following the manufacturer's recommendations.

Considering that the DNA extraction method showed 100% yield, the resulting DNA sample from $1x10^6$ parasites eluted in 200µL buffer solution presented a concentration of 5,000 genome equivalents (gEq) of *L. infantum*/µL. Serial decimal dilutions of this DNA sample were performed upon assembly of the reaction plate. Each point of the curve was built in triplicate, using 5µL of each dilution. We used dilutions relating to 500, 50, 5, 0.5 and 0.05gEq of *L. infantum*/µL, which represent the points 2,500, 250, 25, 2.5, 0.25gEq of *L. infantum*/qPCR reaction. Standard curves were established for each reaction plate to determine the number of genome equivalents (gEq) of *L. infantum* in the DNA samples assessed in the study.

After extraction, the DNA samples in triplicate were submitted to amplification by qPCR using a StepOne™ (Applied Biosystems® CA, USA) platform and a TaqMan® (TaqMan® MGB Applied Biosystems®, CA, USA) hydrolysis probe. The probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-NFQ-MGB) and the indicators LEISH-1 (5'-AACTTTTCTGGTCCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3') were designed to recognize conserved regions of kDNA of *L. Infantum* (Francino *et al*., 2006).

The reactions were performed with Universal Mastermix (Applied Biosystems, CA, USA) reagent, according to the manufacturer's instructions, in a final volume of 25µL, containing 5µL of DNA sample, 1.35µML of the indicators LEISH-1 and LEISH-2, 0.5µM of the probe, and 2µL of bovine albumin serum (BSA) (Sigma-Aldrich®) at 5µg/µL concentration.

The qPCR methodology is reported in the paper by Solcà *et al*., (2014), briefly, the reactions were conducted in a 48-well plate (Applied Biosystems), sealed with adhesive film **(**Applied Biosystems), with the following DNA amplification protocol: 1 denaturation cycle at 50°C for 2 minutes, 1 denaturation cycle at 95°C for 10 minutes, and 40 cycles of denaturation at 95°C for 15 seconds and of annealing/extension at 60°C for 1 minute.

In each amplification plate, we used positive (standard curve of *L. infantum* culture) and negative (ultrapure water) controls of reaction, and threshold set at 0.1, to determine the quantification cycle (Cq). The cutoff point and the detection limit were determined after qPCR reaction standardization under laboratory conditions. Samples with Cq lower than 37 cycles were considered positive for *L. infantum* DNA, whereas samples with Cq greater than 37 cycles were considered undetectable. The detection limit was 0.0025 GEQ of L. infantum/reaction.

The results were then normalized using the concentration of DNA present in the 5µL of sample as the denominator.

In order to identify possible false-negative results caused by the presence of inhibitors in the reaction or by degraded DNA sample, a test was conducted on all samples undetected by qPCR. We used the Taqman® Gene Expression Assay system (Applied Biosystems, CA, USA), which contains a primer pair and a probe preset for the realization of qPCR, for the amplification of a segment of the canine constitutive gene that encodes the β-actin protein subunit (REF: Cf03023880_ g1, Applied Biosystems, CA, USA), in a final total reaction volume of 25µL, containing 2µL of DNA and 23µl of reaction mixture composed of 12.5µL of Universal Mastermix, 1.25µL of the primers and probe solution, and 9.25µL of ultrapure water. In this phase, the result was qualitative, considered to be detectable or undetectable. DNA samples that showed amplification were considered positive, thus containing intact and inhibitor-free DNA.

The results obtained were organized into a database and processed by the software program Statistical Package for the Social Science (SPSS), version 16.0. The median, minimum and maximum of DNA quantification were obtained and compared according to the method of conservation (FT and FFPE) by the Wilcoxon test. The results of the qPCR were compared, and the degree of agreement between the paired tissue samples preserved in FT or FFPE was determined by the Kappa coefficient. The degree

of agreement was classified according Landis and Koch (1977). The values for sensitivity, specificity and confidence intervals of the tests were calculated using the reference standard (positive in at least one of the diagnostic tests: parasitological culture, IHC, or HE staining). The Mann-Whitney nonparametric test was used for comparison of genomic equivalents (parasitic load) in detectable FT and FFPE samples. Results were considered statistically significant at $P<0.05$.

RESULTS

Of the 46 dogs included in the study, 23.9% tested positive in at least one of the three techniques used as standard diagnostic reference of CVL. Regarding the results of DNA quantification by fluorimetry, we observed that the median of DNA concentration in the FFPE skin samples (0.1540ng/µL, min: 0.0005 and max: 1.5400) was significantly lower ($P<0.001$) than that observed in the FT samples (6.9600ng/µL, min: 0.0005 and max: 77.7000); we also observed a large variation between the minimum and maximum DNA concentration values, mainly in the FT specimens.

Sensitivity, specificity, accuracy, negative and positive predictive values of qPCR were calculated using the results obtained with FT and FFPE samples of healthy skin. The results are shown in Table 1.

Table 1. Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of Quantitative Polymerase Chain Reaction (qPCR) using DNA samples of FT and FFPE skin

Healthy skin samples	Sensitivity	Specificity	PPV	NPV	Accuracy
FFPE	63.6% (43-90%)	77.1% (90-100%)	46.7%	87.1%	73.9%
FT	100% (62-98%)	48.6% (75-96%)	37.9%	100%	60.9%

Note: FFPE: formalin-fixed, paraffin-embedded sample. FT: frozen tissue. PPV: positive predictive value. NPP: negative predictive value. REFERENCE STANDARD: parasitological culture, IHC, HE.

When comparing the 46FT and FFPE samples by qPCR, we observed that 24 of them showed concordant results (11 detectable and 13 undetectable) and 22 presented discordant results (18 detectable on FT and undetectable on FFPE; and 4 detectable on FFPE and undetectable on FT).

The complete agreement of the results of the samples for qPCR (detectable/undetectable) was 52.2%. The Kappa index presented a degree of 0.12, which is a poor degree of agreement.

The result of the medians of genome equivalents (gEq) of *L. infantum* (parasitic load) in the qPCR was 5.463gEq (min: 0.431 max: 1687515.00) for the 29 FT detectable samples and 1.421 gEq (min: 0.616 max: 29.030) for the 14 FFPE detectable samples ($P=0.001$) (Table 2).

The medians of genome equivalents (gEq) of *L. infantum* (parasitic load) in concordant and discordant samples between the reference standard and qPCR are also shown in Table 2. However, no statistically significant differences were observed.

Table 2. Medians of gEq of *L. infantum* (parasitic load) obtained by Quantitative Polymerase Chain Reaction (qPCR) and the results of reference standard in FFPE and FT skin samples

Healthy skin samples	(+) qPCR (-) reference standard (Discordant)	(+) qPCR (+) reference standard (Concordant)	Total
FFPE[a]	0.933gEq (n=7)	3.141gEq (n=7)	1.421gEq (n=14)
FT[b]	3.366gEq (N=18)	517.967gEq (n=11)	5.463gEq (n=29)

Note: FFPE: formalin-fixed, paraffin-embedded sample. FT: frozen tissue. REFERENCE STANDARD: parasitological culture, IHC, HE. +: positive, -: negative. (a) discordant FFPE *versus* concordant FFPE skin samples, Mann-Whitney test, $P=0.209$; (b) discordant FT *versus* concordant FT skin samples, Mann-Whitney test, $P=0.084$.

All 17 FT negative samples showed amplification of the gene for β-actin, and six FFPE negative samples (12%, n=31) showed no amplification.

DISCUSSION

In this study, fragments of healthy skin collected from the scapular region of dogs were used as biological samples for the diagnosis of CVL by specific qPCR targeting *L. infantum* kDNA sequence. The skin has proved to be a good site for parasitological confirmation (Madeira *et al*., 2009), and it is an important indicator, considering that the transmission of infection occurs during the blood feeding of the insect vector. Furthermore, the material collected from the same place on the skin can be used to perform other diagnostic tests, as the three used in this study as reference standard (parasitological culture, HE, and IHC), with no need to increase the number of biological specimens collected from the animal (Xavier *et al*., 2006).

With the extraction of DNA from FT samples, it was possible to obtain samples with a higher mean concentration of DNA compared with those prepared with FFPE tissue (Isola *et al.,* 1994).

This outcome contradicts authors who have found good results with human FFPE samples (Vilanova-costa *et al*., 2008). One of the limiting factors for DNA recovery from FFPE tissues is the incubation time for fixation for later paraffinization (Ferrer *et al*., 2007). In our study, the residence time in formalin was 2-10 days. In the specific literature, studies show that acid fixatives interfere with the quality of nucleic acids, contributing to DNA degradation (Ben-ezra *et al*., 1991; Greer *et al*., 1991), and that samples fixed and stored for more than six months present undefined or poor results in PCR owing to DNA degradation (Ferrer *et al*., 2007).

The deparaffinization process and the different phases of extraction can also cause fragmentation, loss of DNA, and inhibition of the reaction (Dietrich *et al*., 2013). The efficiency of the DNA extraction technique is also related to the method used (Fernandes *et al*., 2004). In our study, we did not assess the efficiency of the techniques used for DNA extraction, but we tried to use appropriate methodologies and reagents for each type of biological sample. However, because of the low concentration of DNA in some samples, it was not possible to repeat some analyses, particularly those that showed high standard deviation in triplicate, probably due to the same fact.

The low DNA concentration extracted from FFPE samples has prevented the use of a single, predefined concentration in qPCR assays for the different samples. Therefore, we used a fixed volume of DNA sample per reaction, irrespective of its concentration. For this reason, it is possible that the validity of qPCR may have been compromised, because the lower the concentration of DNA in this reaction, the lower the chance of detection of the target DNA sequence.

To overcome such limitations and increase the sensitivity of the PCR technique for detection of the parasite genome in FFPE samples, the use of a larger number of sections of FFPE tissue for DNA extraction has been suggested, considering that the number of parasites per section is small (Roura *et al*., 1999). In the present study, we used five 5mm-thick slices of FFPE healthy skin samples for DNA extraction, whereas FT samples were prepared with 3mm punches on the skin. Because the obtained DNA samples were eluted in 50uL buffer solution, in both cases, the differences between the mass of FT and FFPE tissue subjected to DNA extraction may have contributed to the large difference in the mean final concentration values of DNA obtained from these two sources of material.

The difficulties found in DNA amplification by PCR may also be related to the presence of inhibitors of DNA polymerase and to the small initial number of molecules containing target sequences with sufficient integrity for amplification. However, the process of PCR inhibition can be reversed with adaptations of the technique, such as the use of a higher concentration of polymerase and dNTP, and increased extension time (Dietrich *et al*., 2013).

The qPCR for detection of *L. infantum* using FT samples presented high sensitivity but low specificity. Other authors have found positivity of approximately 100% with PCR (Queiroz *et al*., 2010).

The low specificity of qPCR found in the present study may have resulted from the low sensitivity of the techniques used as standard diagnostic reference (Schubach *et al*., 2014; Solcá *et al*., 2014). In our study, qPCR was able to detect *Leishmania* DNA in samples with parasite load below the limit required to obtain positive results in parasitological culture, HE, and IHC. The median for gEq/ng of DNA in the FT samples was significantly higher compared with that of the FFPE samples. Four samples of FFPE tissue showed undetectable results in qPCR and were positive in the reference standard and FT qPCR. In addition, low parasite load may be a predictive factor for false-negative results on FFPE tissue (Frickmann *et al*., 2013).

In this study, six FFPE samples showed undetectable results in the DNA quality test (qPCR for β-actin). However, there was not sufficient sample volume for subsequent analysis of DNA degradation in any of the cases.

Analysis of the medians of genome equivalents (gEq) of *L. infantum* (Table 2) showed that samples that are negative in the reference standard but positive in qPCR tend to present lower parasitic load (median of gEq/ng of DNA), although not statistically significant due to the wide dispersion of data. This fact suggests that positive results in the reference standard require levels of parasitic load higher than the minimum necessary for detection by qPCR.

The qPCR technique presented 100% sensitivity on FT samples, but low specificity was observed for this technique owing to the low parasitic detection by reference standards; these findings suggest that qPCR on FT samples should be used as the reference standard for CVL. Thus, the use of qPCR would allow better monitoring of the parasitic load of treated dogs (Manna *et al*., 2008), considering that the immune status of dogs does not interfere with the result (Moreira *et al*., 2007).

CONCLUSION

Our results suggest freezing as the most suitable conservation method for the formation of sample databases considering DNA recovery.

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REFERENCES

ALMEIDA, A.B.; SOUSA, V.R.; GASPARETTO, N.D. *et al*. Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples. *Diagn. Microbiol. Infect. Dis*., v.76, p.321-324, 2013.

BEN-EZRA, J.; JOHNSON, D.A.; ROSSI, J. *et al*. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. *J. Histochem. Cytochem.,* v.39, p.351-354, 1991.

BRASIL. Ministério da Saúde. Manual de vigilância e controle da leishmaniose visceral. Brasília, 2006. Disponível em: <https://www.icict.fiocruz.br/sites/www.icict.fiocruz.br/files/Manual_Vigilancia_Controle_Leishmaniose_Visceral.pdf>. Acessado em: jul. 2016.

BUSTIN, S.A.; BENES, V., GARSON, J.A. *et al*. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem*., v.55, p.611-622, 2009.

CARSON, F.L.; HLADICK, C. *Histotechnology*: a self instructional text. 3.ed. Chicago: ASCP Press, 2009. [403p.].

CONTROL of the leishmaniasis. Geneva: World Health Organization, 2010. (Technical report series 949). Avaliable in: <http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng.pdf>. Accessed in: 02. Jul. 2016.

CUPOLILLO, E.; GRIMALDI, J.G.; MOMEN, H. A general classification of new world Leishmania using numeral zymotaxomomy. *Am. J. Trop. Med. Hyg.,* v.50, p.296-311, 1994.

DIETRICH, D.; UHL, B.; SAILER, V. *et al*. Improved PCR performance using template DNA from formalin-fixed and paraffin-embedded tissues by overcoming PCR inhibition. *PLoS. One*, v.8, 2013.

FERNANDES, J.V.; MEISSNER, R.V.; FERNANDES T.A.A.M. *et al*. Comparação de três protocolos de extração de DNA a partir de tecido fixado em formol e incluído em parafina. *J. Bras. Patol. Med. Lab*. v.40, 2004.

FERRER, I.; ARMSTRONG, J.; CAPELLARI, S. *et al*. Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: a BrainNet Europe Study. *Brain Pathol*., v.17, p.297-303, 2007.

FIUZA, J.A.; GANNAVARAM, S.; SANTIAGO, H.C. *et al*. Vaccination using live attenuated Leishmania donovani centrin deleted parasites induces protection in dogs against *Leishmania infantum*. *Vaccine*, v.33, p.280-288, 2015.

FRANCINO, O.; ALTET, L.; SANCHEZ-ROBERT, E. *et al*. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniasis. *Vet. Parasitol.* v.137, p.214-221, 2006.

FRICKMANN, H.; TENNER-RACZ, K.; EGGERT, P. *et al*. Influence of parasite density and sample storage time on the reliability of Entamoeba histolytica-specific PCR from formalin-fixed and paraffin embedded tissues. *Diagn. Mol. Pathol.*, v.22, p.236-234, 2013.

GONTIJO, C.M.F.; MELO, M.N. Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. *Rev. Bras. Epidemiol.*, v.7, p.338-349, 2004.

GREER, C.E.; LUND, J.K.; MANOS, M.M. PCR Amplification from Paraffin-embedded tissues: recommendations on fixatives for long-term storage and prospective studies. *Genome Res.*, v.1, p.46-50, 1991.

ISOLA, J.; DEVRIES, S.; CHU, L. *et al*. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. *Am. J. Pathol.*, v.145, p.1301-1308, 1994.

KUHLS, K.; ALAM, M.Z.; CUPOLILLO, E. *et al*. Comparative microsatellite typing of New World *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. *PLos Neglect. Trop. Dis.*, v.5, n.6, 2011.

LACHAUD, L.; CHABBERT, E.; DUBESSAY, P. *et al.* Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers. *Parasitology*, v.125, p.197-207, 2002.

LANDIS, J.R.; KOCH, G.G. The measurement of observer agreement for categorical data. *Biometrics*, v.33, p.159-174, 1977.

LASKAY, T.; MIKÓ, T.L.; NEGESSE, Y. *et al.* Detection of cutaneous Leishmania infection in paraffin-embedded skin biopsies using the polymerase chain reaction. *Trans. R. Soc. Trop. Med. Hyg*., v.89, p.273-275, 1995.

MADEIRA, M.F.; SOUSA, M.A.; BARROS, J.H. *et al*. *Trypanosoma caninum* n. sp. (Protozoa: Kinetoplastida) isolated from intact skin of a domestic dog (Canis familiaris) captured in Rio de Janeiro, Brazil. *Parasitol*ogy, v.136, p.411-423, 2009.

MANNA, L.; GRAVINO, A.E.; PICILLO, E. *et al*. Leishmania DNA quantification by real-time PCR in naturally infected dogs treated with miltefosine. Animal biodiversity and emerging diseases. *Ann. N.Y. Acad. Sci*., v.1149, p.358-360, 2008.

MESQUITA, R.A. ANZAI, E.K.; OLIVEIRA, R.N.; NUNES, F.D. *et al*. Avaliação de três métodos de extração de DNA de material parafinado para amplificação de DNA genômico pela técnica da PCR. *Pesqui. Odontol. Bras.*, v.15, p.314-319, 2001.

MOREIRA, M.A.; LUVIZOTTO, M.C.; GARCIA, J.F. *et al*. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. *Vet. Parasitol.*, v.145, p.245-252, 2007.

NUNES, J.B.; LAURENTI, M.D.; KANAMURA, H.Y. *Leishmania infantum* infection in dogs from the southern region of Minas Gerais state, Brazil. *Rev. Inst. Med. Trop*. v.58, p.75, 2016.

OLIVEIRA, F.S.; PIRMEZ, C.; PIRES, M.Q. *et al*. PCR-based diagnosis for detection of *Leishmania* in skin and blood of rodents from an endemic area of cutaneous and visceral leishmaniasis in Brazil. *Vet. Parasitol*., v.129, p.219-227, 2005.

QUEIROZ, N.M.G.P.; ASSIS, J.; OLIVEIRA, T.M.F. *et al.* Diagnóstico da leishmaniose visceral canina pelas técnicas de imunoistoquímica e PCR em tecidos cutâneos sem associação com a RIFI e ELISA-teste. *Rev. Bras. Parasitol. Vet.*, v.19, p.32-38, 2010.

QUINTELLA, L.P.; CUZZI, T.; MADEIRA, M.F. *et al.* Immunoperoxidase technique using an anti-*Leishmania (L.) chagasi* hyperimmune serum in the diagnosis of culture confirmed American Tegumentary Leishmaniasis. *Rev. Inst. Med. Trop. São Paulo*, v.51, p.83-86, 2009.

ROURA, X.; FONDEVILA, D.; SANCHEZ, A.; *et al*. Detection of *Leishmania* infection in paraffin-embedded skin biopsies of dogs using polymerase chain reaction. *J Vet Diagn Invest*, v. 11, n, 4, p.385-7, 1999.

SCHUBACH, E.Y.P.; FIGUEIREDO, F.B.; ROMERO, G.A.S. Accuracy and reproducibility of a rapid chromatographic immunoassay for the diagnosis of canine visceral leishmaniasis in Brazil. *Trans. R. Soc. Trop. Med. Hyg.,* v.108, p.568-574, 2014.

SILVA, D.A.; MADEIRA, M.F.; TEIXEIRA, A.C. *et al*. Laboratory tests performed on Leishmania seroreactive dogs euthanized by the leishmaniasis control program. *Vet. Parasitol.*, v.179, p.257-261, 2011.

SOLCÀ, M.S.; BASTOS, L.A.; GUEDES, C.E. *et al*. Evaluating the accuracy of molecular diagnostic testing for canine visceral leishmaniasis using latent class analysis. *PLoS One*, v.9, n.7, 2014.

VILANOVA-COSTA, C.; NÓBREGA, J.B.; SOUTO, R.; CRUZ, A.D. Extração e purificação de DNA em material biológico parafinado. *Estudos*, v.35, p.143-152, 2008.

XAVIER, S.C.; ANDRADE, H.M.; MONTE, S.J.H. *et. al*. Comparison of paraffin-embedded skin biopsies from different anatomical regions as sampling methods for detection of *Leishmania* infection in dogs using histological, immunohistochemical and PCR methods. *BMC. Vet. Res.*, v.2, p.17, 2006.