

Polymerase chain reaction using conjunctival swab samples for detecting *Leishmania* DNA in dogs

Reação em cadeia da polimerase usando amostras de Swab de conjuntiva para detecção de DNA de *Leishmania* em cães

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How to cite: Magalhães KA, Pussi KF, de Araújo HK, do Carmo SB, Friozi E, Branquinho LS, et al. Polymerase chain reaction using conjunctival swab samples for detecting *Leishmania* DNA in dogs. *Braz J Vet Parasitol* 2021; 30(3): e009121. <https://doi.org/10.1590/S1984-29612021061>

Abstract

The dog is the main domestic reservoir of *Leishmania* and font of infection for the vector, constituting an important host for the transmission of the parasite to humans. Non-invasive collection of swab samples for leishmaniasis diagnosis has been a promising alternative. This study analyzed the positivity of polymerase chain reaction (PCR) for the diagnosis of canine leishmaniasis in conjunctiva samples. DNA extraction was performed using SDS 20% and PCR was performed using 13A/13B primers that amplify 120-bp of *Leishmania* kDNA. Of the 77 dogs analyzed, 50 (64.93%) had ocular changes: 25 (32.47%) dogs had periocular lesion, 41 (53.25%) dogs had purulent eye discharge, and 17 (22.08%) dogs had both signals. PCR was positive in 35 dogs (45.45%), and there was no significant difference between dogs with and without ocular signals ($p=0.4074$). PCR positivity was significant higher in dogs without periocular injury ($p=0.0018$). Conjunctive PCR, a less invasive, fast, and painless collection technique, is indicated to complement the diagnosis, especially in dogs without periocular injury, independent of the presence of purulent eye discharge.

Keywords: *Leishmania*, conjunctiva, PCR.

Resumo

O cão é o principal reservatório doméstico de *Leishmania* e também fonte de infecção para o vetor, constituindo um importante hospedeiro para a transmissão do parasita ao homem. A coleta não invasiva de amostras em swab para diagnóstico das leishmanioses tem sido uma alternativa promissora. Este estudo analisou a positividade da reação em cadeia da polimerase (PCR) para o diagnóstico de leishmaniose canina em amostras de conjuntiva. A extração do DNA foi realizada com SDS 20%. A PCR foi realizada com primers 13A/13B que amplificam 120-pb do kDNA de *Leishmania*. Dos 77 cães analisados, 50 (64,93%) tiveram alterações oculares; 25 (32,47%) cães tiveram uma lesão periocular; 41 (53,25%) tiveram secreção ocular purulenta e 17 (22,08%) cães tiveram ambos os sinais. A PCR foi positiva em 35 cães (45,45%) e não houve diferença significativa em cães com e sem sinais oculares ($p = 0,4074$). A positividade da PCR foi significativamente maior em cães sem lesão periocular ($p = 0,0018$). PCR em conjuntiva, uma técnica de coleta menos invasiva, rápida e indolor, é indicada para complementar o diagnóstico, principalmente em cães sem lesão periocular, independentemente da presença de secreção ocular purulenta.

Palavras-chave: *Leishmania*, conjuntiva, PCR.

Received May 15, 2021. Accepted June 1, 2021.

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Introduction

The second parasitic disease that causes most deaths worldwide is leishmaniasis complex. It is endemic in Asia, Africa, the American, and the Mediterranean region. In the American continent, it is mainly found in the tropical and subtropical regions (Horimoto & Costa, 2009). The transmission of leishmaniasis occurs through the bite of female dipterans of the subfamily Phlebotominae.

As dogs are considered the main domestic reservoirs, a reliable, accurate, and specific diagnostic method for detecting parasites is crucial (Molina et al., 1994). Parasitic DNA can be detected in a broad range of clinical specimens, such as bone marrow, lymph node aspiration, and liver, kidney, or other viscera biopsies (Perez, 2015). However, these samples are obtained by invasive and painful procedures.

Serological tests are widely used; however, these tests have limitations in diagnosis as they lack sensitivity and can generate false results in dogs with clinical disease. The combination of enzyme-linked immunosorbent assay (ELISA) and dual path platform (DPP™) significantly improved the sensitivity and specificity (Gomes et al., 2008). Molecular methods such as polymerase chain reaction (PCR) are highly sensitive and specific for detecting the parasite's DNA, regardless of the disease stage (Oliveira et al., 2006). The need for effective methods capable of detecting the parasite by using material from the less invasive collections allows the animals to be diagnosed more painlessly. Thus, the current study analyzes the positivity of conjunctival swab PCR in the diagnosis of Canine Leishmaniasis (LeishCan) in seropositive dogs for visceral leishmaniasis.

Material and Methods

This study was reviewed and approved by the Ethics Committee on the Use of Animals of the Universidade Federal da Grande Dourados (protocol number 27/2016). Written informed consent was obtained from the dog owners.

Dogs diagnosed with LeishCan in serological tests (DPP™ rapid test and ELISA) were sent to the Control Center of Zoonoses (CCZ) in the city of Campo Grande/Mato Grosso do Sul state for euthanasia from May to July 2016.

The veterinarian at CCZ, before the collection of samples, performed the evaluation of clinical signals and the characterization of the animals regarding gender (male and female), age [puppies (up to one year old) and young adults (2–4 years old)], and size [small (below 40 centimeter), medium (40 to 60 centimeter), and large (over 60 centimeter)]. Silveira et al. (2018) analyzed the dogs, along with the samples collected from other animals in the same place and period and reported the data. The samples were collected from the conjunctiva of the right and left eye of each dog using a sterile cotton swab.

The DNA was obtained using SDS 20% and resuspended in 50 µL of TE buffer (10 mM TRIS; 1 mM EDTA; pH 8.0) according to Neitzke-Abreu et al. (2020). For each extraction, a positive control [dog blood without leishmaniasis plus 10⁴ promastigotes *Leishmania (Leishmania) infantum*] and a negative amplification control (dog blood without leishmaniasis) were included.

After obtaining the DNA, the samples were subjected to PCR using primers 13A (5-GTG GGG GAG GGG CGT TCT-3) and 13B (5-ATT TTA CAC CAA CCC CCA GTT-3) (Rodgers et al., 1990), which were used to amplify 120-bp of *Leishmania* spp. A negative control (water) and two positive controls [DNA *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*] were added.

Samples with negative PCR results for *Leishmania* were subjected to a new reaction using β-actin F (5'-CTT CTA CAA CGA GCT GCG CG-3') and β-actin R (5'-TCA TGA GGT AGT CGG TCA GG-3') primers described by Silveira et al. (2018), were directed to amplify a 307-bp fragment present in the β-actin gene *Canis familiaris*.

The proportions were analyzed using OpenEpi version 3.01, with a confidence interval (CI) of 95% and the Chi-square test was used to compare PCR positivity.

Results

Seventy-seven dogs of both genders and different ages and sizes were enrolled in this study. Of the dogs analyzed, 64.93% (50/77) (CI 95%, 53.80–74.65) had eye signals, 32.47% (25/77) (CI 95%, 23.06–43.54) had periocular injury, 53.25% (41/77) (CI 95%, 42.22–63.97) had purulent eye discharge and 22.08% (17/77) (CI 95%, 14.27–32.54) had periocular lesion with purulent eye discharge.

PCR was positive in 45.45% (35/77) (CI 95%, 34.81–56.53). There was no significant difference in PCR positivity in females [53.49% (23/43) (CI 95%, 38.66–67.92)] and males [36.67% (11/30) (CI 95%, 21.02–54.78)] ($p = 0.1563$),

and one dogs (1/4) with positive PCR had no information about gender. Regarding size, PCR was positive in 66.67% (6/9) (CI 95%, 33.24–90.73) of large dogs, 29.03% (9/31) (CI 95%, 15.18–46.64) in medium dogs, and 57.14% (20/35) (CI 95%, 40.50–72.65) in small dogs. There was a significant difference between medium and large dogs ($p = 0.0172$), and between medium and small dogs ($p = 0.0216$), while between small and large dogs there was no significant difference ($p = 0.3513$). Regarding age, PCR was positive in 47.06% (8/17) (CI 95%, 24.78–70.27) of puppies, in 44.83% (26/58) (CI 95%, 32.44–57.72) of young adults ($p = 0.8709$), and in one dog (1/2) with positive PCR had no age identification.

There was no significant difference in the results of PCR in dogs with or without ocular signals ($p = 0.4074$). PCR positivity for dogs with some ocular signal was 42.0% (21/50) (CI 95%, 28.96–55.95), positivity in dogs with periocular injury was 20.0% (5/25) (CI 95%, 8.86–39.13), dogs with purulent secretion were 46.34% (19/41) (CI 95%, 32.06–61.25) and dogs with both signals were 23.53% (4/17) (CI 95%, 9.56–47.26). Only one dog presented with corneal opacity and was negative for PCR. Considering only periocular injury, the positivity of the PCR was significant higher in dogs without periocular injury ($p = 0.0018$). In addition, 58.0% (29/50) (CI 95%, 44.05–71.04) dogs had eye signals but had negative PCR results, 51.85% (14/27) (CI 95%, 33.31–70.02) dogs had no eye signals but had positive PCR, and 48.15% (13/27) (CI 95%, 29.98–66.69) dogs had no eye signals and negative PCR.

Discussion

Although our results demonstrated no significant difference in PCR in dogs with eye signals, studies have demonstrated a significant association with the presence of clinical signs and positive PCR in conjunctiva samples in swab, blood, bone marrow and skin (Ferreira et al., 2012; Pereira et al., 2016). Dermatological changes, weight loss, lymphadenopathy, onychogryphosis and hepatosplenomegaly have been reported in all dogs with a positive result in conjunctive PCR. Ocular abnormalities in LeishCan are common (Penã et al., 2000; Brito et al., 2010; Baneth et al., 2008) and result from the immune response due to infection *Leishmania*, as the conjunctiva is one of the first lymphoid centers assets (Bruto et al., 2010). The report on ocular parasitism and tissue tropism (Geisweid et al., 2013) highlights the importance of studies in canine conjunctiva for DNA research by *Leishmania* spp.

All animals had more than five clinical signals related to the development of the disease. In this study most animals had ocular signs (64.93%). Purulent eye discharge followed by the periocular injury was the most frequent finding of the study. Ocular signs, including keratoconjunctivitis, uveitis, and blepharoconjunctivitis, have been detected in dogs with *Leishmania* sp. (Ciaramella et al. 1997; Brito et al., 2010; Baneth et al., 2008). This indicating the possibility of parasites in this tissue.

The absence between the age and gender with the prevalence of LeishCan evidenced in our results corroborates with other results (Azevedo et al., 2008). However, the greater positivity in medium-sized animals in our results differs from the scientific literature that reports the greater susceptibility of large and giant breeds due to the greater body surface favoring the bite of sandflies (Gálvez et al., 2010).

PCR positivity was not high in this study (45.45%). We used primers for the *Leishmania* genus to detect any species, although cases of LeishCan caused by another species than *L. infantum* are rare. PCR is less sensitive than serological tests such as ELISA and Indirect Immunofluorescence Reaction (IIF) (Duthie et al., 2018), due the abundance of anti-*Leishmania* antibodies is greater than *Leishmania* genetic material. In addition, factors related to the method of collection, conservation and processing of samples can influence the positivity of PCR. In serology inconclusive cases (Santarém et al., 2020), molecular tests can assist in the diagnosis of LeishCan, complementing serological tests.

In our study, PCR was positive in 42.0% of dogs had ocular signals. One study showed positive conjunctival cytology for *Leishmania* even with negative direct parasitological examination at on lymph node and marrow samples (Santos et al., 2017). Studies have reported high positivity in the conjunctiva PCR in symptomatic animals (Ferreira et al., 2012; Pereira et al., 2016). Conjunctiva cytological investigations for amastigote research on *Leishmania* also demonstrated good positivity (Barbosa et al., 2012). The significant positivity in dogs without periocular injury can be explained by recent infection and the immune response. Ferreira et al. (2013) found DNA *L. infantum* in dogs that did not present conjunctival lesions, emphasizing the practicality and potential for diagnosis. These data indicate a significant relevance of the presence of the parasite in conjunctiva, even without tissue damage.

Using a part of the same dogs, PCR positivity was higher in blood (84.75%) (Silveira et al., 2018) and ear blood (70.90%) (Neitzke-Abreu et al., 2020) than the conjunctiva swab (45.45%), but this, in addition to being a less invasive collection allows us to obtain purer DNA and less discomfort for the animal. Other authors have shown

that PCR is superior to blood (Lombardo et al., 2012; Pereira et al., 2016). The comparison in nasal and conjunctival swabs showed no difference in terms of parasitic load and was close to detection in skin biopsy, but lower than bone marrow samples (Ferreira et al., 2013). This maybe is due to a positive synergy between the DNA extraction technique and the primers used.

PCR was negative in 58.0% (29/50) dogs had ocular signals, and in 48.15 % (13/27) dogs had no ocular signals. This can indicate decreased parasitic load but show the limitation in using the conjunctiva for the diagnosis of LeishCan. Adequate cellular immune response in dogs is considered to control the spread of the pathogen in tissues such as mucous membranes and skin (Solano-Gallego et al., 2001). Ocular changes can occur due to the immune response, without the local presence of parasites. This shows the need for further studies to improve the diagnosis and understanding of ocular changes in dogs produced by *Leishmania*.

Although some authors affirm the significant existence of parasitic load in the ocular conjunctiva, our results did not show a good result of PCR in conjunctiva swab. The importance of exploring a less invasive, quick, and painless collection technique, in contrast to the methods for collecting samples from bone marrow and lymph nodes, helps reduce the suffering of symptomatic dogs in a weak and extremely fragile state. Conjunctive PCR is indicated in to complement the diagnosis, especially in dogs without periocular injury, independent of the presence of purulent eye discharge.

Acknowledgements

The authors would like to thank the and Universidade Federal da Grande Dourados (UFGD) for the English review and financial support.

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