

Short Communication



Diagnosis of Canine Leishmaniasis using Ear Blood PCR in Region of Brazil

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Abstract | The objective of this study was to evaluate a polymerase chain reaction (PCR) for the diagnosis of Canine leishmaniasis (CanL) using less invasive sample collection. PCR-based detection of *Leishmania infantum* using ear peripheral blood collected from dogs with symptomatic CanL and coinfection with *Ehrlichia* sp. in the city of Campo Grande (Brazil) was investigated. A total of 268 CanL seropositive dogs were sampled. Of the 268 dogs tested, 70.90% were PCR positive for CanL, 72.43% of the dogs with ear lesion/s (185) were PCR positive, whereas only 67.47% of dogs without ear lesion/s (83) were PCR positive. Additionally, 35.82% of dogs tested were positive based on parasitological diagnosis for *Ehrlichia* sp. It was concluded that ear blood PCR may be an alternative for the diagnosis of CanL. Because it is more rapid collect and less invasive sample, this makes ear-blood PCR a potentially useful supplement to traditional diagnostic methods.

Keywords | Dog, *Ehrlichia*, Peripheral blood, *Leishmania infantum*, Co-infection

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INTRODUCTION

Canine Leishmaniasis (CanL) is an anthroponosis of epidemiological importance as dogs (*Canis familiaris*) are the main hosts of *Leishmania* in urban areas and even asymptomatic dogs can transmit the parasite to the vector, thus playing an active role in the transmission of infection. Diagnosis for CanL is currently undertaken by health agencies via serological tests which are of limited use in asymptomatic cases, and for which specificity can be reduced in some cases owing to cross reactions to other infections (Dhom-Lemos *et al.*, 2019). For species identification, molecular methods are imperative as species other than *Leishmania infantum* have already been described in dogs (Souza *et al.*, 2019). The high sensitivity and specificity of polymerase chain reaction

(PCR) assays can be used to supplement the traditional testing methods. The objective of this study was thus to evaluate the conventional PCR-based *Leishmania* assay on dog ear peripheral blood for the diagnosis of CanL with coinfection with *Ehrlichia* sp.

Ear blood samples from dogs were collected for convenience by the Control Center of Zoonosis (CCZ) of Campo Grande, Mato Grosso do Sul State, Brazil, over a period of two months. The Campo Grande municipality is capital of the State and it has a territorial area of 8,000 km². It has humid tropical climate, with rainy season in summer and dry in winter. In 2016, it has estimated population of about 765,000 inhabitants with 41 confirmed cases of visceral leishmaniasis, and estimated dog population of about 162,500 with 4,231 cases of CanL. The dogs

analyzed were CanL seropositive (DPP™ Canine Visceral Leishmaniosis and ELISA) and it was sent for euthanasia following the recommendations by the Brazilian Ministry of Health. As inclusion criteria were analyzed dogs with laboratory and clinical diagnosis for CanL. Dogs without conclusive diagnosis for CanL were excluded. As for the information on sex, race and age, and clinical signs, these same animals were analyzed by [Silveira et al. \(2018\)](#). The dogs presented classic signs of CanL as alopecia, skin peeling, onychogribose, purulent ocular secretion, ear tip lesion and weight loss, and it is mostly feral dogs from the periphery of the city, with no access to treatment and with wide exposure to co-infections ([Silveira et al., 2018](#)). After the animal was anesthetized, a needle puncture was made in a blood vessel of the auricle, and peripheral blood was collected. A part was stored into an EDTA tube and stored at -4 °C until PCR was carried out. A part of ear blood was also used for direct parasitological diagnosis of *Ehrlichia*. This work was approved by the Ethics Committee for Animal Use of Universidade Federal da Grande Dourados, protocol 27/2016.

Glass slides with ear blood smears were used for direct investigation for *Ehrlichia*; the slides were stained with Giemsa and observed under optical microscopy (1000x magnification).

Peripheral blood DNA was obtained using the protocol of [Araújo et al. \(2009\)](#) with some modifications. Briefly, 300 µL of ear blood was added to 500 µL of 20% SDS (sodium dodecyl sulphate – Neon), homogenized, and incubated at 65 °C for 6 minutes. Then, 400 µL of chloroform was added and the solution was mixed, following which 300 µL of protein precipitation solution (5M potassium acetate, glacial acetic acid) was added. The solution was again mixed and centrifuged at 10,000 g for 10 minutes. The supernatant was transferred to a new tube, the volume of which was then filled made up with ice-cold absolute ethanol; the solution was mixed by gentle inversion and centrifuged at 10,000 g for 5 minutes. The supernatant was discarded and the sediment was washed twice with 1 mL of ice cold 70% ethanol with each wash followed by centrifugation at 10,000 g for 2 minutes. After discarding the final wash supernatant, the residual ethanol was evaporated off in a 65 °C digital dry bath for 5 minutes. Fifty microliters of TE buffer (10 mM Tris, 1mM EDTA, pH8.0) was added to the DNA pellet, which was then stored overnight at 4 °C and then frozen at -20 °C until use.

The PCR primers used for detection of *L. infantum* were FLC2 (5'-GTC AGT GTC GGA AAC TAA TCC GC-3') and RLC2 (5'-GGG AAA TTG GCC TCC CTG AG-3'), which flank a 230-bp fragment of the kDNA minicircle ([Gualda et al., 2015](#)) present in high numbers per parasite (from 10,000 to 20,000 copies). The PCR

mixture (25 µL) was composed of 0.2 µM of each primer (Sigma), 2 mM MgCl₂, 0.2 mM dNTP (Invitrogen), 1.5 U Taq DNA polymerase (Phoneutria), and 2 µL DNA. DNA amplification was performed on a Thermocycler (BIORAD, T100 Thermal Cycler) at 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds, and finally 72 °C for 10 minutes. The amplified product was stored at 4 °C until analysis. Positive controls (1 pg of *L. infantum* DNA) and negative controls (water) were used. Eight microliters of the amplified product were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, at 10-15 V/cm. The PCR products were visualized on a transilluminator (Loccus Biotecnologia).

To detect potential PCR inhibition, *L. infantum* PCR negative samples were also submitted to PCR amplification of the canine β-actin gene using the primers Forward (5'-CTT CTA CAA CGA GCT GCG CG-3') and Reverse (5'-TCA TGA GGT AGT CGG TCA GG-3') as described by [Silveira et al. \(2018\)](#).

The proportions were analyzed using a Mid-p exact test OpenEpi version 3.01, with a confidence interval of 95% (95% CI).

A total of 268 dogs were analyzed. Ear lesions were described in 69.03% (185) (95% CI; 63.26–74.26) of the dogs. Of the samples, 70.90% (190) (95% CI; 65.19–76.01) were PCR positive for *L. infantum* and 96 (35.82%) (95% CI; 30.32–41.72) were positive for *Ehrlichia* based on parasitological diagnosis; 35.90% (28/78) (95% CI; 26.15–46.97) of the *L. infantum* PCR-negative dogs were positive for *Ehrlichia*. In *L. infantum* monoinfected animals, 70.76% (121/171) (95% CI; 63.55–77.06) were *L. infantum* PCR-positive, while in co-infected animals 71.13% (69/97) (95% CI; 61.45–79.21) were PCR-positive. In terms of size, 70.34% (95% CI; 61.56–77.83) of small dogs (83/118), 72.64% (77/106) (95% CI; 63.48–80.22) of medium dogs and 69.23% (27/39) (95% CI; 53.58–81.43) of large dogs were *L. infantum* PCR-positive; and in terms of sex, 75.27% (70/93) (95% CI; 65.62–82.92) of male dogs and 67.50% (108/160) (95% CI; 59.91–74.27) of female dogs were *L. infantum* PCR-positive. In dogs with (185) and without ear lesions (83), 72.43% (95% CI; 65.59–78.37) and 67.47% (95% CI; 56.81–76.58), respectively, were *L. infantum* PCR-positive.

The high number of CanL symptomatic dogs observed in Campo Grande emphasizes the need for studies to help control CanL endemicity in the region. In Brazil, euthanasia alone has not been enough to reduce the number of cases of CanL, necessitating additional measures to control the vector population, including a treatment program, which was authorized in Brazil in 2016. The high canine and

human density and poor basic sanitation infrastructure are factors that favor CanL transmission and endemicity (Teles et al., 2015). The vector control measures in Campo Grande reflect what is happening throughout Brazil, demonstrating the precariousness of the health agencies, and the lack of investment in development and testing of new insecticides, their mode of application, and effective use strategies in priority areas.

The classic clinical signs of CanL have been described by Brazuna et al. (2012) and Silveira et al. (2018). Ear lesions may occur due to increased exposure to the insect vector bite and subsequent tissue infection. Furthermore, the formation of immunocomplexes is also responsible for lesions in the ears and ophthalmic, elbow, and plantar cushions, this being a classic pathophysiological mechanism of CanL (Brito et al., 2010).

Ear peripheral blood was used owing to the common presence of lesions in this region, which may increase the likelihood of the presence of *Leishmania* in the peripheral blood vessels of the ear. Although the ear peripheral blood sample has lower positivity in parasitological examinations than do other samples, such as those from bone marrow and lymph node, the low invasiveness of this sample collection method makes it more accessible. Ear blood is easy to collect as a small needle punch is sufficient for the blood to exit; thus, ear blood collection may be particularly useful when testing large numbers of animals.

The sensitivity of PCR in blood is due to the low number of parasites (Quaresma et al., 2009). In addition, the existence of inhibitors of TAQ (specifically the heme group) and intrinsic factors, such as the DNA extraction method and primers selected, affect the efficiency of the PCR assay (Nunes et al., 2007). The FLC2/RLC2 primers were used owing to their high sensitivity and specificity for *L. infantum* (Gualda et al., 2015), the main etiological agent of CanL. However, infection in dogs by other *Leishmania* species has previously been described (Sanchez et al., 2016). Thus, primers that target the genus *Leishmania* rather than a single species could avoid false negative results and detect all *Leishmania* species.

Although the presence of *Leishmania* in tissue macrophages is greater than that in blood, increased proximity of the sample site to the lesions did not increase sensitivity. The sensitivity of PCR-based CanL detection is higher in lesions than in blood (Manna et al., 2004); however, collection of the lesion is more invasive, considering the already weakened state of the infected animal.

Canine monocytic ehrlichiosis is a widely distributed parasitosis, and in Brazil, *E. canis* is the main causative species. Both *E. canis* and *L. infantum* spread from the

skin to the spleen, bone marrow, and liver, activating the host immune system and inducing diverse clinical and immunopathological responses. Infection can vary from asymptomatic to chronic infection or even death (Andrade et al., 2014). The frequent coinfection of *E. canis* and *L. infantum*, demonstrated in our results, highlights the fact that, to be effective, the CanL diagnostic test must be able to identify CanL in symptomatic, asymptomatic, and non-cross-reactive dogs.

All animals in this study were symptomatic. In the presence of symptoms, the possibility of co-infection with *Erlichia* should be considered, as *E. canis* infection causes a reduction in class II histocompatibility complex receptors (Harrus et al., 2003). This may exacerbate the clinical evolution of CanL and consequently increase the parasitic load. In contrast, infection by *L. infantum* can also impair humoral and cellular immune responses and favor either subsequent *E. canis* infection or the reactivation of a pre-existing *E. canis* infection (Barbieri, 2006).

The animals studied here were diagnosed according to the Brazilian Ministry of Health (DPP™ Canine Visceral Leishmaniosis for screening and confirmatory ELISA technique). Prior to 2012, ELISA was used for screening and indirect immunofluorescence reaction was used as the confirmatory test for CanL, as part of the “Visceral Leishmaniasis Control Program.” This diagnostic change improved health services. The high sensitivity and specificity of DPP™ contributed to diagnosis, especially in symptomatic dogs. However, serological tests for CanL present limitations, as cross-reactions with *Trypanosoma* species and other hemoparasites have been reported (Dhom-Lemos et al., 2019). Thus, it is necessary to use increasingly sensitive and specific diagnostic techniques in zoonosis control programs. This study demonstrates the importance of using molecular tests to supplement serological methods for CanL diagnosis. Conventional PCR and its variants can contribute to the initial diagnosis and, when necessary, be used to supplement traditional methods. An analysis of this PCR technique in healthy animals and asymptomatic dogs is important to verify test parameters such as specificity. Also, an analysis on blood collected from other regions of the animal.

The parasitological test is rapid and inexpensive, but the sensitivity depends on the type of biological material due to the low parasitemia in blood. The technique also requires qualified personnel to read the slides, which limits its use in routine clinical practice. According to our results, the use of PCR in ear blood may be subsequent to the negative parasitological test.

It was concluded that the PCR assay using ear blood may be an alternative for the diagnosis of CanL. Because it is

more rapid collect and less invasive sample, this makes ear-blood PCR a potentially useful supplement to traditional diagnostic methods. Ear blood PCR-base testing may be a valuable addition to traditional diagnostic methods and should be evaluated with other molecular targets to increase its sensitivity.

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AUTHORS CONTRIBUTION

HCNA, MSCLJ: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - original draft, review & editing. VBDV, APSS, LSB, LVG, SOC: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft, review & editing. SBC, EF: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing - review & editing.

CONFLICT OF INTEREST

There are no conflict of interests.

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