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

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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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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 (Degrave et al., 1994). The reaction mixture consisted of 1× 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× (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× (Fermentas, USA), 1 mM LIN19, and 20.25 μL of MilliQ to a total volume of the first solution amplified.

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