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Value of the oral swab for the molecular diagnosis of dogs in different stages of infection with *Leishmania infantum*

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ABSTRACT

This study was based on the need to employ a sensitive and specific method with samples that could be easily collected for diagnosing dogs infected with *Leishmania infantum*. To this end, we used real time-PCR (qPCR) to assess the value of the oral swab (OS) in detecting infected sick dogs (SD; n = 62), including, for the first time, the analysis of apparently healthy infected dogs (AD; n = 30), both from endemic areas for visceral leishmaniasis (VL). For comparison, we also evaluated the performance of the conjunctival swab (CS), blood (BL), lymph node (LN) and serology. We detected the presence of *Leishmania* DNA in the oral cavity in 62 out of the 92 dogs studied. The OS positivity (67.4%) was equivalent to the CS (68.5%) ($p > 0.05$), higher than BL (52.2%) ($p \leq 0.05$), and lower than LN (84.8%) ($p \leq 0.05$). OS and CS performed well in SD dogs (82.3% and 83.9%, respectively) but not in AD dogs (36.7% for both samples). BL showed the lowest positivity (52.2%) and provided equivalent results between AD (60.0%) and SD (48.4%) dogs ($p > 0.05$). LN yielded the highest positivity (84.8%), and it was also higher in the SD population (93.5%) compared to the AD population (66.7%) ($p \leq 0.05$). Parasite load was high in LN, moderate in OS and CS, and low in BL, showing the relationship between the levels of parasitism and the positivity rates found in these samples. Serology was positive in 82.2% of the SD group and in 70% of the AD dogs ($p > 0.05$). Among the 20 seronegative dogs, seven (35%) were positive in either OS or CS, and 12 (60%) were positive when both noninvasive samples were jointly considered. The OS/CS combination resulted in a significant increase of positivity ($p \leq 0.05$) for the AD dogs (from 36.7% to 63.4%), as well as OS/serology (80%) and OS/CS/serology (83.4%). For the SD population, positivity reached up to 95.2% with the same combinations, showing that combination of samples and/or tests is required for the identification of dogs infected with *L. infantum* and that the OS and CS combination based on qPCR notably improves the detection of both AD and SD dogs.

In conclusion, OS proved to be a suitable sample for the molecular diagnosis of infected dogs with clinical signs of VL, but not for dogs with inapparent infection. For these, we recommend the combination of OS results with CS and/or serology in order to reach relevant positivity for *L. infantum*. Finally, another advantage of using OS or both noninvasive samples is the increased likelihood of diagnosing seronegative dogs.

1. Introduction

Visceral leishmaniasis (VL) is a neglected vector-borne tropical disease that is endemic in 65 countries, including Brazil. The zoonotic disease is caused by the intracellular protozoan parasite *Leishmania infantum* (syn *Leishmania chagasi*) that is mainly transmitted by *Lutzomyia longipalpis* in the New World (WHO, 2011; Alvar et al., 2012).

VL can affect humans, as well as both wild and domestic animals. Dogs (*Canis familiaris*) are the main reservoir in urban areas, and have been responsible for dispersing the disease in endemic regions (Michalsky et al., 2007; Laurenti et al., 2013). From the per-

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spective of human risk, canine visceral leishmaniasis (CVL) must be taken into account, given that canine disease usually precedes the emergence of human cases, and a clear correlation between canine and human infection rates has been demonstrated (Werneck et al., 2007; Falqueto et al., 2009).

Brazil accounts for up to 90% of all cases in Latin America, recording 3500 cases per year (Alvar et al., 2012), and canine infection can be as high as 57% in some endemic areas (Falqueto et al., 2009), which shows the public health problem VL represents in Brazil. Measures such as the early diagnosis and treatment of human cases, control of vectors, health education, and the culling of seropositive dogs are advocated to control the spread of VL (Romero and Boelaert, 2010)

The infection of dogs can be inapparent or present with a wide range of clinical signs that can overlap with other canine diseases, making the diagnosis of CVL challenging (Gomes et al., 2008). The canine diagnosis is based on clinical manifestations as well as on laboratorial tests to confirm the disease in dogs suspected to be ill or to investigate infection in apparently healthy dogs (Miró et al., 2008). Serology is by far the most widely used for this purpose, especially in surveys carried out in endemic areas, but it does not fully discriminate dogs infected with *L. infantum* from those with other diseases (do Rosário et al., 2005; Zanette et al., 2014; Laranjeira et al., 2014; Laurenti et al., 2014), as it fails to detect a portion of animals with subclinical infection (Porrozzi et al., 2007), and dogs vaccinated against leishmaniasis from those naturally infected (Marcondes et al., 2013). The insufficient accuracy of serological assays has caused the euthanasia of false-positive animals and, conversely, the maintenance of false-negative dogs in transmission areas, increasing risk to humans by the transmission route dog-sand fly-human (Bevilacqua and Alves, 2004; Laurenti et al., 2013).

Parasitological diagnosis is definitive and can be accomplished by direct observation of *Leishmania* amastigotes in lymphoid tissues or, less frequently, by immunohistochemistry (IHC) or parasite isolation (Grimaldi and Tesh, 1993; Xavier et al., 2006; Moreira et al., 2007). All these methods are carried out on invasive samples, are time-consuming and impracticable to be performed on a large scale. On the other hand, molecular tools have become increasingly prominent in detecting *Leishmania* infection due to their notable sensitivity, specificity, and flexibility in choice of samples (Srividya et al., 2012). Several studies have confirmed PCR's superior performance when compared with direct exam, IHC and serology (Moreira et al., 2007; Quaresma et al., 2009; Santos et al., 2014). Others point out the advantage of using the real-time PCR (qPCR) format, especially when based on multicopy targets, such as the *Leishmania* kinetoplast DNA (kDNA) (de Paiva Cavalcanti et al., 2009; Reis et al., 2013).

One of the limiting factors of using a diagnostic technique on a large scale is the collection of the clinical material, which ideally should be quick, simple, and painless. Thus, the association of PCR with noninvasive samples could represent an important contribution to the diagnosis of CVL.

Results are encouraging with the use of the conjunctival swab in the molecular diagnosis of infected dogs (Francino et al., 2006; Leite et al., 2010; de Almeida Ferreira et al., 2012; Di Muccio et al., 2012; Lombardo et al., 2012; Leite et al., 2015). Nevertheless, many animals reject the conjunctival scraping, and the cornea could be damaged during the procedure.

The oral swab is a practical solution for sample collection and does not cause any harm to the patient. Recently, Lombardo et al. (2012) demonstrated *Leishmania* DNA in canine oral mucosa for the first time, and de Ferreira et al. (2013) showed its potential use in sick dogs. Following in this direction, the aim of the present study was to evaluate the oral swab as an alternative sample for the molecular diagnosis of *L. infantum* infection, including, for the first time, apparently healthy infected dogs that represent the most prevalent population in VL-endemic areas, for which serological and parasitological diagnoses may fail. In the current study, we used a potent diagnostic tool, qPCR targeting *Leishmania* kDNA, and compared the oral swab performance with that of the conjunctival swab, lymph node, blood and serology, considering clinical groups (dogs with inapparent infection and sick dogs), parasite load, and combined results of the oral swab with the other samples and serology.

2. Material and methods

2.1. Ethics statement

This study was approved by the Ethics Committee for the use of Animals in Research of the Medical School- University of São Paulo, under the protocol no. 375/12. All procedures were conducted in accordance with the guidelines of the Brazilian College of Animal Experimentation (COBEA) and with the Brazilian Federal Law 11.794/08.

2.2. Population and areas of the study

We investigated adult mongrel dogs with different ages and gender referred to the Centers for Zoonosis Control of Araçatuba (21°12′32″S, 50°25′58″W), Andradina (20°53′45″S, 51°22′44″W), and Embu das Artes (23°38′56″, 46°51′7″W), municipalities of the São Paulo State (Brazil) where CVL is endemic. In those places, we selected dogs presenting clinical signs compatible with CVL. Apparently healthy dogs were obtained in private clinics from the same regions above mentioned. Those with proven infection (n = 92 dogs), i.e, with positive molecular diagnosis at least in one of the samples studied (section 2.2) were enrolled in the study and assorted into two groups, one composed of 30 apparently healthy infected dogs (AD), and another with 62 sick dogs (SD). The signs found in SD group were lymphadenomegaly and/or splenomegaly (79.0%, 49/62), dry exfoliative dermatitis and/or ulcers, (69%, 43/62), ocular signs (58.1%, 36/62), onychogryphosis (40.3%, 25/62), diffuse alopecia (33.9%, 21/62), and epistaxis (4.8%, 3/62).

2.3. Clinical samples

Peripheral blood (BL) was collected from venous punctures for qPCR, and serological tests. All samples were stored at −20 °C until use. Exfoliative cells from oral cavity and conjunctiva were collected with rayon sterile swabs (Inlab, Brazil). The oral swab (OS) was firmly scraped against the right and left sides of the oral cavity, including the gums, and the conjunctival swab (CS) was scraped against the lower eyelid of both eyes. The tips were broken into sterile microtubes containing 200 μL of preservation NET buffer (0.15 M NaCl, 50 mM EDTA, 0.1 M Tris-HCl, pH 7.5) and kept them at 4 °C until use. The lymph node aspirate (LN) was collected from cervical or popliteal region (the most palpable) with a 10 mL syringe and 25 × 7 mm needle. The material was transferred to sterile microtubes containing 200 μL of preservation NET buffer and stored at 4 °C until use.

2.4. Serological tests

DPP® (DPP® CVL rapid test, BioManguinhos, Rio de Janeiro, Brazil) was performed according to the manufacturer's recommendations and the results were interpreted visually (Grimaldi et al., 2012). Enzyme-immunoassay (EIE® Bio-Manguinhos, Rio de Janeiro, Brazil) was developed according to the manufacturer's recommendations. The optical densities (OD) were evaluated with the