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PCR identification of *Leishmania* in diagnosis and control of canine leishmaniasis

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Abstract

Leishmaniases are endemic in many countries, mainly in rural areas. In Brazil, *Leishmania* infection is responsible for many cases of Leishmaniases, including recent reports in urban regions. Despite their sensitivity, traditional serological and parasitological methods for detecting Leishmaniases have proven inadequate for species discrimination. This study aimed to identify *Leishmania* species in biological samples by a fast methodology, avoiding ''in vitro'' cultivation. Knowledge of the *Leishmania* species is an important tool in regions where both New World visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) are prevalent. As these new foci appear in areas not traditionally endemic for VL, the main problem is to distinguish between true autochthonous infections and infections acquired in other well-known endemic areas. Since, domestic dogs are known to be the main VL and CL reservoir, they are regularly investigated in endemic areas to prevent, principally, severe and often fatal VL in humans. However, several infected dogs present no clinical signs or clinical signs similar to other canine diseases. Here, we evaluated the ability of PCR to diagnose VL and distinguish *L.* (*L.*) *chagasi* from other *Leishmania* species in domestic dogs. Samples from 114 dogs from 30 cities (Sao Paulo, Brazil) were divided into two groups: 44 symptomatic and 70 asymptomatic. They were assayed by parasitological methods (culture and microscopic examination) and PCR to determine *L.* (*L.*) *chagasi*, *L.* (*V.*) *braziliensis*; and in some cases, *Leishmania* spp. Parasitological tests and PCR-*L. chagasi* were concordant in 105 samples (92%). VL was confirmed in 49 dogs, while 56 had negative results. Of the 114 samples, 9 had discordant results, but were further tested by PCR-*Leishmania* spp. with positive results. VL was also confirmed in 4 dogs having negative parasitological tests and positive PCR-*L. chagasi*. Consequently, this PCR was positive for 100% (53/49) of dogs with parasites detected in parasitological tests. Also, PCR demonstrated high specificity detecting 61 dogs negative for VL. *Leishmania* infection was negative in 56 dogs, and 5 with positive culture and PCR-*Leishmania* spp. had CL since they were positive in PCR-*L. braziliensis*. This study shows the importance of including PCR in diagnosis of Leishmaniases by differential diagnosis contributing to the surveillance and control of VL programs.
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1. Introduction

The genus *Leishmania* causes a wide spectrum of human diseases, ranging from self-limited cutaneous to the more severe diffuse cutaneous and visceral forms, as a consequence of the complex host immunological response (Grimaldi and Tesh, 1993; Lainson and Shaw, 1998).

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Leishmania species in Latin-America belong to two different taxonomic groups. The first is the sub-genus *Leishmania*, composed of *L. mexicana* and *L. amazonensis*, responsible for localized or diffuse cutaneous; and *L. chagasi*, the causative of the New World viscerotropic leishmaniasis. *L. braziliensis*, *L. panamensis*, *L. guyanesis* and others comprise the second sub-genus *Viannia* group and cause cutaneous or mucocutaneous lesions (Degrave et al., 1994; Lainson and Shaw, 1998).

The traditional diagnosis of Leishmaniases consists of serological tests (ELISA and Immunofluorescence), direct examination of smears after Giemsa staining, ''in vitro'' culture and histological techniques (Grimaldi and Tesh, 1993; MSB, 2006). Despite its sensitivity, the traditional diagnosis has been found inadequate for species discrimination. For this purpose immunological methodologies, such as serodeme or monoclonal antibodies, have been developed, but they present limitations such as cross-reactions among other phylogenetic similar species (Grimaldi and McMohon-Pratt, 1996).

The variability of *Leishmania* has been traditionally studied by a multilocus enzyme electrophoresis technique (MLEE, isoenzyme analyses) based on 15 enzymes. The amino acid polymorphism was responsible for change in enzyme mobility producing different phenotypes (or zymodemes) (Rioux et al., 1990). This taxonomic methodology has been regularly used in epidemiological studies (Cupolillo et al., 1994). Generally, the definitive species identification is determined within 2 or 3 months. This technique requires the isolation of the parasite and its growth in culture medium, but many promastigotes isolates do not amplify in culture medium. Additionally, the culture is rarely done in routine diagnosis. Thus, this method is time-consuming, requiring culture facilities and individual expertise (Degrave et al., 1994; Schallig and Oskam, 2002).

Knowledge of the *Leishmania* species is particularly important in regions where both New World visceral leishmaniasis (VL) and New World cutaneous leishmaniasis (CL) are prevalent (Schallig and Oskam, 2002). Recent findings showed *L. chagasi* dispersion in endemic areas for *L. braziliensis*, the causative agent of CL in Sao Paulo State, Brazil (Camargo-Neves, 2004; CVE, 2006). As these new foci appear in areas not traditionally endemic for VL, the main problem is to distinguish between true autochthonous infections and infections acquired in other well-known endemic areas. Since domestic dogs known to be the main VL and CL reservoir, they are regularly investigated in endemic areas to prevent, principally, severe and often fatal VL in humans (Grimaldi and Tesh, 1993). However, the canine VL surveillance is limited since most infected dogs present no clinical signs or clinical signs similar to other canine diseases. Studies analyzing sera from symptomatic and asymptomatic dogs with VL showed the difficulty in determining the proportion of infected dogs that develop a detectable specific immune response. The asymptomatic ones often remain seronegative or borderline positive (Ashford et al., 1995; Berrahal et al., 1996; Paula et al., 2003).

Despite the significant contribution of a variety of molecular methods for identifying *Leishmania* (Rodriguez et al., 1994; Fernandes et al., 1996; Schallig and Oskam, 2002; Volpini et al., 2003; Marfurt et al., 2003), these methods are time consuming and expensive for diagnosistic purposes. In contrast, a specific PCR-based method is appealing as it is rapid, sensitive, and specific, avoiding culturing of parasites, thus being suitable for Leishmaniases surveillance programs that require efficient laboratorial response for rapid and effective actions.

This study aimed to determine *Leishmania* species in biological samples by a fast and simple methodology, avoiding ''in vitro'' cultivation thus; we evaluated the efficacy of PCR for New World VL diagnosis and its capacity to distinguish *L. chagasi* from other *Leishmania* species in domestic dog samples. This PCR amplifies a fragment present in the highly reiterated minicircles of DNA, having been shown to be highly sensitive and specific for VL caused by *L. infantum* in samples from Mediterranean region (Lachaud et al., 2002). We opted for these primers on the basis of the current taxonomy of the *L. donovani* complex, which has three designated species, *L. donovani*, *L. infantum* (syn. *L. chagasi*) and *L. archibaldi* (Mauricio et al., 2006).

2. Material and methods

2.1. Strains of parasites and canine samples

VL diagnosis was performed in 114 dogs from October 2003 to November 2005. All dogs analyzed in this study were selected considering epidemiological risk factors for VL, such as proximity of other infected dogs as well as signs or symptoms of the disease. The dogs lived in different localities of Sao Paulo State, including 30 cities (small and medium size) endemic for CL, as well as having endemic conditions for VL. The dogs were divided into two groups according to symptomatology. Group I, composed of 44 dogs

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