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Serological evaluation of experimentally infected dogs by LicTXNPx–ELISA and amastigote-flow cytometry

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Abstract

Canine visceral leishmaniasis (CVL) is characterized by a high incidence of asymptomatic infections. Because of the high prevalence of asymptomatic dogs in the endemic areas of visceral leishmaniasis (VL), a sensitive test is required for an accurate diagnosis. In this study, we evaluated the detection of symptomatic and asymptomatic *Leishmania infantum* infection in dogs using the secreted LicTXNPx antigen (*Leishmania infantum* cytosolic tryparedoxin peroxidase) in an ELISA format and compared it to soluble *Leishmania* antigens from promastigote or amastigote forms (SPLA and SALA) and two other unrelated secreted *Leishmania* proteins (LiTXN1 and TDR1). Moreover, we evaluated the diagnostic potential using the promastigote or amastigote-flow cytometric methodologies. The assays utilized sera collected from a cohort of *L. infantum* experimentally infected dogs, in which the intravenous or intradermal parasite injection mimics a symptomatic or asymptomatic pattern of infection, respectively. Our study indicated that anti-LicTXNPx antibodies were present in both symptomatic and asymptomatic experimental infections. Among the different *Leishmania* recombinant proteins tested, LicTXNPx showed a good predictive correlation with total soluble promastigote or amastigote *Leishmania* antigens, suggesting this antigen as a good candidate for a marker in either symptomatic or asymptomatic infection. The use of flow cytometry using both forms of live parasites was also tested with the same group of dogs. Amastigotes were shown to have more advantages than promastigotes for the serological diagnostic in both symptomatic and asymptomatic dogs, since higher continuous levels of anti-amastigote antibodies were detected during the course of experimental infection. Moreover, additional studies were done using sera from non-infected dogs and clinically asymptomatic and symptomatic dogs with confirmed naturally occurring *L. infantum* infections. The sensitivities of amastigote and promastigote flow cytometry were 96% vs. 89%, respectively, while

Abbreviations: CVL, canine visceral leishmaniasis; VL, visceral leishmaniasis; DAT, direct agglutination test; i.v., intravenously; i.d., intradermally; SPLA, soluble promastigotes *Leishmania* antigens; SALA, soluble amastigotes *Leishmania* antigens; ELISA, enzyme-linked immunosorbent assays; LicTXNPx, *Leishmania infantum* cytosolic tryparedoxin peroxidase; LiTXN1, *Leishmania infantum* tryparedoxin; TDR1, *Leishmamia major* Thiol-dependent reductase.

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the specificity for both was 93.2%. Therefore, our findings showed for the first time the potential of amastigote-flow cytometry regarding their applicability to detect both symptomatic and asymptomatic VL canine infections.

Keywords: Canine leishmaniasis; Flow cytometry; ELISA; LicTXNPx; Amastigote

1. Introduction

Domestic dogs are the main reservoir hosts of *Leishmania infantum* in endemic areas. They constitute a primary source of infection by sand fly vectors, contributing to the perpetuation of the infectious cycle. An accurate evaluation of the prevalence and the incidence of canine visceral leishmaniasis (CVL) depend on the reliable identification of infected dogs. It was recently suggested that CVL control programs fail due to the underestimation of the real number of cases because of lack of sensitivity and specificity of current diagnostic tests (Solano-Gallego et al., 2001). Over the years, several techniques using different antigens were proposed for the diagnostic of CVL (Alvar et al., 2004). In spite of this, the existence of cross reactivity with antibodies present in other pathologies and the inherent subjectivity due to the necessity of a mathematical cut off to distinguish infected from non-infected dogs are still major drawbacks (Gomes et al., 2008).

An efficient field diagnostic requires specific rapid tests. Hence, some serological techniques are more appropriate for field use, since other approaches such as parasitological methods are often cumbersome and less suitable, despite their higher sensitivity and specificity. One of the major problems in field diagnostic is the unsatisfactory sensitivity associated with asymptomatic dogs (Alvar et al., 2004). This is exemplified by one of the most common field diagnostic methods, the direct agglutination test (DAT). This method uses whole stained promastigotes either in a suspension or in a freeze-dried form, which are incubated with serial dilution of serum. In a positive reaction, agglutination is visible after 18 h of incubation (Mohebali et al., 2004; Ferreira Ede et al., 2007). While the seroprevalence evaluation of CVL is relatively easy to perform in symptomatic dogs, the sensitivity of antibody detection is generally lower for early or asymptomatic infections, for which a highly sensitive test is required (Gomes et al., 2008). Therefore, the development of methods that allow the detection of asymptomatic dogs is of major importance since they are equally infectious for sandflies and represent an asymptomatic reservoir of *Leishmania*. The use of recombinant *Leishmania* proteins is considered an attractive alternative to the traditional soluble promastigotes *Leishmania* antigens (SPLA) based approaches. Recombinant proteins are reproducible and are easily adsorbed on several scaffold surfaces making them desirable targets for field diagnostic, given an acceptable specificity and sensitivity. More recently, a flow cytometry methodology was proposed for the detection of anti-promastigote *L.* (*L.*) *chagasi* immunoglobulin levels in serum samples from dogs with VL (Carvalho Neta et al., 2006). This new diagnostic method was shown to be reliable, since it achieved high levels of sensitivity and specificity and it even demonstrated the potential to distinguish the serological profile between *L. chagasi* infected and Leishmune® vaccinated dogs (De Andrade et al., 2007).

We had previously demonstrated that antibodies against LicTXNPx might be a useful constituent for a defined serological diagnostic and the monitoring of the therapeutic response in human VL (Santarém et al., 2005). In order to evaluate the potential of this secreted *Leishmania* antigen in the CVL diagnostics, we measured the presence of anti-LicTXNPx antibodies in the sera of experimentally infected dogs by ELISA and compared those with specific antibodies to SPLA or soluble amastigote *Leishmania* antigens (SALA) and other unrelated secreted *Leishmania* proteins (LiTXN1 and TDR1). Further, we examined the isotype pattern of the anti-LicTXNPx antibodies in both groups of infected dogs. In parallel, we used the same cohort of experimental infected dogs to assess the potential of flow cytometry technique using live *L. infantum* amastigotes for CVL diagnostic, with live promastigotes as a reference (Rocha et al., 2002). Finally, we evaluated the potential of the flow cytometry approach using live promastigote and amastigote forms in the diagnostic of a natural infection.

2. Material and methods

2.1. Dog populations and samples characterization

2.1.1. Samples from experimental infected dogs

The experimental infections were done in beagle dogs ($n = 15$) by an intradermal ($n = 5$) or intravenous ($n = 5$) injection of 5×10^8 and 1×10^8 stationary