Canine antibody response to *Lutzomyia longipalpis* saliva in endemic area of visceral leishmaniasis.

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Abstract

Introduction: Canine exposure to *Lutzomyia longipalpis* bites and the potential of *Leishmania infantum* transmissibility for the vector were evaluated. **Methods**: Immunoglobulin G (IgG) anti-*Lu longipalpis* saliva and -*L. infantum,* and blood parasite load were determined in dogs from endemic areas of visceral leishmaniasis. **Results**: Blood parasitism was similar between symptomatic and asymptomatic dogs. IgG anti-*L. infantum* was higher in symptomatic dogs, but IgG anti-*Lu. longipalpis* saliva was mostly observed in higher titers in asymptomatic dogs, indicating vector preference for feeding on asymptomatic dogs. **Conclusions**: Our data suggest a pivotal role of asymptomatic dogs in *L. infantum* transmission in endemic areas.

Keywords: Canine leishmaniasis. Anti-*Lutzomyia longipalpis* saliva antibody. Transmissibility.

In Brazil, visceral leishmaniasis (VL) is considered a zoonotic disease caused by the protozoan *Leishmania infantum* [syn. *Leishmania (Leishmania) chagasi*] that is mainly transmitted by the sand fly *Lutzomyia longipalpis*. Dogs (*Canis familiaris*) are considered the main domestic reservoir for VL transmission and have been the target of control actions, because they form part of the transmission cycle of *L. infantum*[1].

Previous studies concerning the potential transmission of VL from infected dogs are controversial. Whilst some authors reported that asymptomatic dogs were non-infective to vectors[2], others stated that they are indeed infective vectors, but to a lesser extension than symptomatic dogs[3]. Recently, our group demonstrated that sand fly infection can occur independently of a dog's skin parasitism, and that asymptomatic dogs were more likely than symptomatic dogs to transmit *L. infantum* to the sand fly, thus promoting a higher infection rate of sand flies feeding on asymptomatic dogs[4].

In accordance with this life cycle, during the blood meal the female sand fly injects its saliva into the hosts' skin which contains proteins that have anti-hemostatic, immunomodulatory, and antigenic properties[5]. The saliva induces antibody production in repeatedly bitten hosts that are highly specific to the sand fly species *Lu. longipalpis*, and the antibody level reflects the intensity of vector exposure[6]. Therefore, antibodies against sand fly saliva have been used in epidemiological studies as an exposure marker to the vector bite, as well as to estimate the likely risk of *Leishmania* transmission[7].

The phlebotomine sand fly could be found in the peridomiciliary zone in urban and rural areas, since the transmission of the infection is associated to precarious living conditions and environmental modifications, modulated by the intense urbanization process, which favoured the adaptation of the vector to the urban environment.[3] Usually, the sand fly feeding in the crepuscular period and dogs who lives in the street or houses backyard are exposed. Apparently, the ears skin represent the most frequently exposed areas, likely due to its lower hair density and cerumen attractiveness, leading to increased exposure to the insect vector's bite[8].

Thus, the main objective of this study was to evaluate the level of immunoglobulin G (IgG) anti-*Lu. longipalpis* in dogs living in urban endemic areas for VL to determine the vector exposure of symptomatic and asymptomatic dogs, verify whether clinical condition affects female attraction for hematophagy, and correlate the data with anti-*L. infantum* antibody and parasite burden in canine whole blood, the main source of feeding for sand flies.

Upon clinical examination, whole blood was collected from the jugular or cephalic vein, and the serum was stored at -20°C until its use for determination of IgG anti-*Lu. longipalpis* saliva by enzyme linked immunosorbent assay (ELISA) according to the methodology described by Rohousova et al., 2005[9]. *Lu. longipalpis* captured in Cametá municipality, Pará state, Brasil were used as a source of antigens for ELISA. Its salivary glands were collected in cold phosphate buffered saline (PBS) from 5- to 7-day-old F1 laboratory-reared females. Just before use, salivary gland lysate (SGL) was prepared by disruption of glands with 3 freeze-thaw cycles. Proteins were measured by the Bradford reaction[10]. Briefly, 96-well microtiter plates were coated with SGL (60ng of protein per well) in 0.01M carbonate-bicarbonate buffer (pH 9.5) overnight at 4°C. After automated PBS washing for three times, 10% skimmed milk powder in PBS for 45 min at 37°C was used to block unspecific binding sites. After another washing, the canine serum was diluted 1:400 times in PBS containing 0.05% Tween 20 and incubated in duplicates for 90 min at 37°C, followed by another incubation with alkaline-phosphatase anti-canine IgG (Bethyl, USA) at 1/20,000 dilution for 45 min at 37°C. Para-nitrophenyl phosphate - pNPP (Sigma-Aldrich, EUA) was used as a substrate solution for 30 min at 25°C and 2N sodium hydroxide (50μL per well) was used to block the reaction. The absorbance was measured using the Multiskan reader (Labsystems, USA) at 405nm. The titers of IgG were expressed as ELISA units (EU). Another ELISA using crude *L. infantum* antigen (MHOM/BR/72/strain46) and anti-canine IgG (A40-123AP) conjugated to alkaline phosphatase (Bethyl, USA) was performed according to Laurenti et al., 2014[11] to determine the level of IgG anti-*L. infantum*.

Samples of whole blood were also used for parasite-load determination by real time polymerase chain reaction (PCR). This assay was performed using primers targeting a 120-bp sequence found in kinetoplast DNA (kDNA)[12]. The amplification was performed in a final volume of 15μL that consisted of 5μL of total DNA diluted in deionized water to 10ng/μL, 7.5μL of Kapa SYBR Fast Universal 2× qPCR Master Mix (Kapa Biosystems, USA), 0.5μL of each primer at a final concentration of 300nM, and 1.5μL of deionized water. A standard curve was generated using serial dilutions of *L. infantum* DNA from 10^6 to 10^{-3} parasites/μL. The standard curve was considered acceptable when the slopes ranged between -3.1 and -3.4 with correlation coefficients ≥0.98. The amplifications were performed in duplicate for each sample, each concentration of parasites, and negative control. The parasite load was obtained by plotting the cycle threshold (Ct) values against the standardized parasite concentrations. Prism 5.00 for Windows (GraphPad software Inc., USA) was used to perform all statistical analysis and graphics. Differences among the clinical groups were analyzed by nonparametric Kruskal-Wallis test and p value < 0.05 was considered significant.

In the current study, the IgG anti-*Lu. longipalpis* salivary gland lysate was found in many of the dogs in the endemic areas. From a total of 185 sera evaluated, 134 (72%) were positive. The highest proportion of dogs with detectable anti-saliva antibodies were found in the asymptomatic group (64/73; 88%), followed by the uninfected controls from the endemic

area (15/20; 75%) and symptomatic dogs (55/92; 60%). Levels of anti-saliva antibodies (median, interquartile range 25% to 75%) were significantly higher in asymptomatic dogs (13.7, -4.53 to 63.53 EU) in comparison with the symptomatic ones (-3.76, -10.99 to 25.07 EU) (p ≤ 0.05) (**Figure 1**).

Figure 1 shows that the levels of anti-saliva IgG were higher in asymptomatic dogs, which suggests that the state of being asymptomatic influences the attraction of the female sand fly and influences their preference for feeding. These findings are consistent with a previous study carried out on experimental conditions of xenodiagnoses, when we showed a larger number of engorged females after blood meal conducted in asymptomatic dogs compared to symptomatic dogs[4]. As early as 1931, Adler and Theodor warned that ulcerated skin lesion infected by bacteria and fungus of symptomatic animals would be less attractive to the sand fly vector, which could explain our findings[13]. Skin involvement is one of the main clinical findings in dogs with VL that usually present with alopecia, exfoliative dermatitis, and nodular and ulcerated lesions[14]. In the present study 63% of symptomatic dogs had the skin lesions aforementioned. It is important to consider that the anemic status of symptomatic dogs is not a limiting factor for vector feeding in the host[4], as the symptomatic dogs with (35%) and without anemia (65%) did not differ in the number of positive cases for anti-*Lu. longipalpis* saliva IgG as well as in their titer.

Dogs infected by *L. infantum* have detectable levels of anti-*Leishmania* antibody in the serum, which increases with the disease severity. Sixty percent (n = 99) of infected dogs from the endemic area showed detectable titers of anti-*Leishmania* IgG: 37/73 (51%) of asymptomatic and 62/92 (60%) of symptomatic dogs (**Figure 2**). Moreover, the highest titers were observed
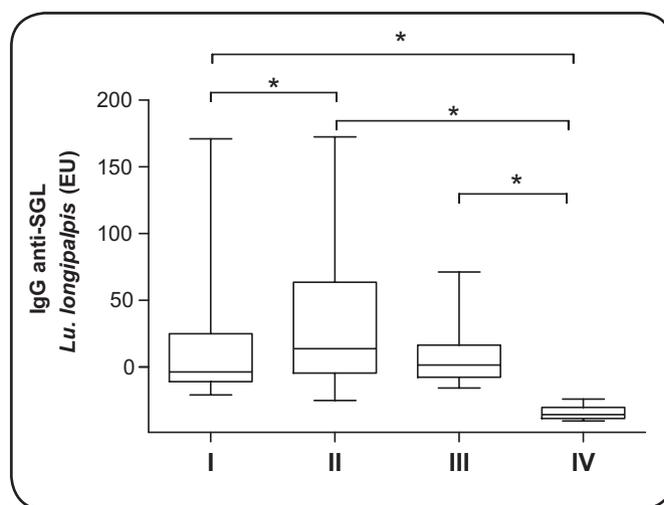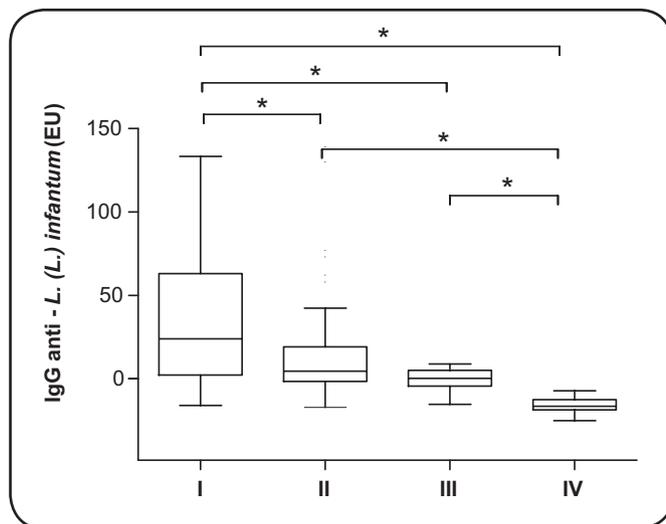

**FIGURE 1 - Boxplot showing median and interquartile range of IgG anti-*Lu. longipalpis* salivary gland lysate in EU in the sera of dogs from endemic and non-endemic area for visceral leishmaniasis. I. Symptomatic dogs. II. Asymptomatic dogs. III. Control dogs from endemic area. IV. Control dogs from non-endemic area. IgG: immunoglobulin G; anti-SGL: anti-salivary gland lysate; *Lu*: *Lutzomyia*; EU: ELISA Units; ELISA: enzyme linked immunosorbent assay. *p < 0.05.
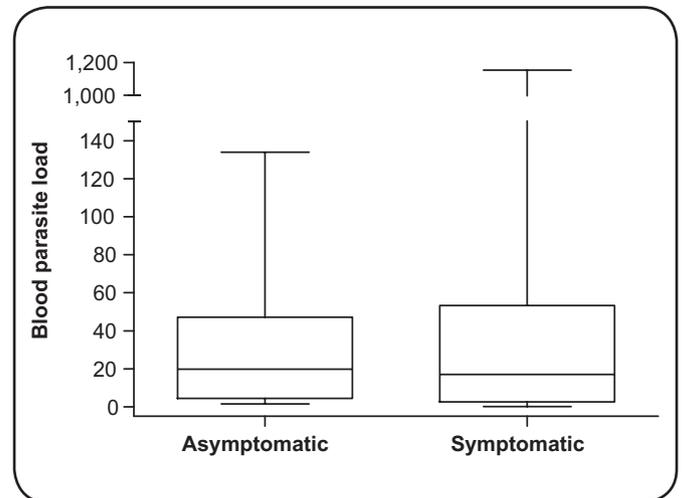
in symptomatic dogs showing that they have a high immune competency to produce antibodies against foreign antigens.

The skin parasitism found in both symptomatic and asymptomatic animals has been implicated as an important source of infection for sand flies[3]. However, our previous report showed that vector infection can occur independently of dogs' skin parasitism[4]. Thus, parasite loads in the blood, the main source of feeding for *Lu. longipalpis* females, was also investigated in the present study. The parasite load was measured in the buffy coat by quantitative real-time PCR and no significant difference ($p = 0.55$) was observed in the blood parasitism between symptomatic and asymptomatic dogs (**Figure 3**). Although peripheral blood is not the favorite tissue for the proliferation and maintenance of *L. infantum*; and a fluctuation of parasitemia may occur during the course of infection[15], the lack of significance in parasite load between clinical groups suggests asymptomatic and symptomatic dogs likely have similar potential of transmissibility.

In summary, besides asymptomatic dogs being competent for infecting phlebotomine sand flies[3][4], our present study showed that asymptomatic dogs have blood parasitism comparable to symptomatic dogs and were preferably bitten by *Lu. longipalpis*. The higher attraction of female sand flies to asymptomatic dogs may signify their contribution in parasitic transmission for vectors. Together, these findings suggest that asymptomatic dogs play a pivotal role in the maintenance of the *L. infantum* cycle and spread of parasite in endemic areas of VL. Moreover, to the best of our knowledge, this is the first study to show the relationship between the *Lu. longipalpis* anti-sand fly saliva antibodies, and blood parasite load, main source of vector feeding, and clinical status of dogs naturally infected by *L. infantum*.

FIGURE 3 - Box plot showing median and interquartile range of parasitic load in the blood of symptomatic and asymptomatic dogs from endemic areas of visceral leishmaniasis.

ETHICAL CONSIDERATIONS

In the present study, 185 breed dogs (Rottweiler n = 50, Labrador Retriever n = 72 and Shepherd dogs n = 63) domiciled or resident for at least 18 months in high endemic areas for VL were used. According to the clinical signs, biochemical laboratory features, and infection status (presence/absence of *Leishmania* deoxyribonucleic acid (DNA) in lymph nodes), dogs were grouped into symptomatic (n = 92), asymptomatic (n = 73), and control (n = 20) from endemic area of São Paulo state, Brazil. Blood serum of uninfected dogs (n = 20) from non-endemic area was used as the negative control. This study was approved by the Ethics Committee for the use of animals in the Veterinary School of the University of São Paulo, under protocol 2391/2011. All procedures were performed in accordance with the guidelines of Brazilian College of Animal Experimentation (COBEA), under the owners' signed consent.

Conflict of interest

The authors declare that there is no conflict of interest.

FIGURE 2 - Boxplot showing median and interquartile range of IgG anti-*L. infantum* in EU in the sera of dogs from endemic and non-endemic area for visceral leishmaniasis. I. Symptomatic dogs. II. Asymptomatic dogs. III. Control dogs from endemic area. IV. Control dogs from non-endemic area. IgG: immunoglobulin G; **anti-*L. infantum***: anti-*Leishmania infantum*; **EU**: ELISA units; **ELISA**: enzyme linked immunosorbent assay. *p < 0.05.

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