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Research paper

Xenodiagnosis on dogs with visceral leishmaniasis: Canine and sand fly aspects related to the parasite transmission

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ABSTRACT

One of the main limitations for the effective control of canine leishmaniasis in endemic areas is the difficulty in identifying infectious dogs. The objective of this study was to determine factors, related to dogs and to parasite detection in sand flies, which are associated with the positive xenodiagnosis of *Leishmania infantum* using the vector *Lutzomyia longipalpis*. The xenodiagnosis was performed in 50 owned dogs residing in endemic areas, which were divided into three different groups: G1—26 dogs proved to be infected and classified by severity of VL clinical signs on physical examination; G2—15 dogs proved to be infected and classified by severity of clinical signs and intensity of laboratory abnormalities; G3—nine dogs that were seropositive for anti-*Leishmania* IgG in ELISA tests. Parasite search in the sand flies after having fed on dogs in the xenodiagnosis was performed by both methodologies, PCR and dissection followed by microscopy. In G1, 58% (15/26) of dogs were able to transmit *Leishmania* to the vector, when parasite detection in sand flies were performed by PCR technique, 5 days after blood meal, whereas in G2, 53% (8/15) transmitted the parasite to the vector, however, confirmation was performed by direct observation of parasite through optical miscroscopy held 10 days after blood meal. Rate of infectiousness of dogs to sand flies was positively associated to severity of disease ($p = 0.042$ and $p = 0.040$), regardless the method used for clinical classification or for parasite detection in sand flies after xenodiagnosis. In G1 30% (3/10) of dogs with subclinical infection were infectious to the vector, while 80% (12/16) of dogs with clinical disease were also infectious. Even more, 17% (1/6) of dogs that had moderate disease were infectious to the sand flies, while 78% (7/9) of dogs with severe disease were infectious in G2. Still in G2, the proportion of sand flies infected (grade of infectiousness) was significantly lower ($p = 0.0098$) when they fed on dogs with moderate disease (1%) in comparison with dogs with severe disease (38%). The dogs from G3 presented a rate of infectiousness of 11% (1/9), demonstrating that the indirect ELISA is not a good indicator of infectiousness and, therefore, should not be used as a confirmatory test for the euthanasia of dogs, as it is currently done in Brazil.

1. Introduction

The dog is considered an important reservoir of zoonotic visceral leishmaniasis (VL), which is caused by *Leishmania infantum* in Brazil, and represents an important worldwide public health issue (Mauricio et al., 2000; Quinnell and Courtenay, 2009). The disease affects mainly children and immunocompromised adults, occurring endemically in the Americas (Campos-Ponce et al., 2005; Lainson and Rangel, 2005), Europe and Mediterranean (Ballart et al., 2012) and in Asia (Stauch et al., 2011). In the Americas, the sand fly *Lutzomyia longipalpis* (Lutz and Neiva, 1912) is considered to be the primary vector for *L. infantum* infection (Lainson and Rangel, 2005).

In the last decades, expansion of VL has been following a strong urbanization tendency. The increasing incidence of human and canine cases in endemic areas (Lopes et al., 2010) has evidenced

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the ineffectiveness of current control measures and the need for broadening scientific knowledge (Romero and Boelaert, 2010). In Brazil, the control of VL has been focused on vector control by using environmental chemical pesticides, early diagnosis and treatment of human cases, health education and elimination of seropositive dogs (Brasil, 2014).

The control of VL in Brazil is mainly based upon the elimination of seropositive (above the cut-off value) dogs for anti-*Leishmania* antibody in ELISA tests. The dog culling program has been rejected by society and criticized by scholars in the field, whether for the ethical and social aspects involved or the low efficiency (Courtenay et al., 2002; Costa-Nery, 2011; Costa et al., 2013). It has been shown that once the seropositive dogs are removed, they are immediately replaced by puppies that are susceptible to developing high parasitic loads due to their immunological immaturity (Andrade et al., 2007; Nunes et al., 2008). VL has a complex pathophysiology that involves the immunological competence of the dog (Pinelli et al., 1994; Amorim et al., 2011), and culminates with the development of extremely variable and individual clinical manifestations (Solano-Gallego et al., 2011). Studies on dog infectiousness have shown that the mere positive result in serological tests is not enough to indicate that a given dog is actually capable to transmit the pathogen to the vector. The lack of a sensitive method to distinguish the dogs that actually transmit the parasite from all seropositive dogs is an important aspect associated with the low efficiency of control measures for VL (Courtenay et al., 2002). A well standardized and sensitive method would rather allow greater focus on the control actions of infectious dogs and, consequently, a better control program effectiveness, also considering the high heterogeneity that exists in the infections by *L. infantum*, which implies in different levels of infectiousness of the dogs (Courtenay et al., 2013).

Xenodiagnosis is the only technique that evaluates whether a particular host that is infected with a pathogen is able to transfer it by natural means to its potential vector (Guarga et al., 2000a). It is relatively sensitive, despite its high specificity, and may vary according to animal-related factors, such as its parasite load, or to variations in procedure implementation (Travi et al., 2001).

The objective of this study was to evaluate the infectivity of dogs, classified by different methods, with different clinical presentations of the infection by *L. infantum* to the vector *L. longipalpis*, evaluating the infectiousness of dogs under different procedures of xenodiagnosis and ways to classify the animals clinically. Moreover, due to the indication of culling seropositive dogs in Brazil, seropositivity in ELISA tests was a criterion to select dogs for xenodiagnostic evaluation.

2. Materials and methods

2.1. Animals and ethical aspects

The groups studied were composed by animals from different areas of the state of Bahia, northeast of Brazil, both sexes, with varying race and age. The criteria for animal inclusion in the study were the following: (1) owned dogs residing in the endemic area for VL, and (2) presenting positivity to *L. infantum* by parasitological tests (Groups 1 and 2) and indirect ELISA (Group 3). All animals underwent xenodiagnosis, biological sample collection for laboratory tests and a physical examination on one occasion.

All procedures were previously approved by the Animal Ethics Committee of the School of Veterinary Medicine and Animal Science, of the Federal University of Bahia (Protocol number 19/2011).

2.2. Experimental design

The animals were studied in three experimental groups. These groups were divided according to the period the tests were performed, being the dogs, in each group, classified according to the presentation of clinical-pathological abnormalities by different methodologies (Fig. 1). The dogs in Group 1 (G1) were evaluated between 2011 and 2013 and positive by parasitological tests of the spleen. These dogs were divided in two clinical groups according to Soares et al. (2011): infected dogs, but clinically healthy or presenting with skin focal mild and non-characteristic lesions of VL, or with other only, mild clinical alteration (here named as subclinical infection); and clinically ill dogs, with manifestation of more than one characteristic clinical signs of VL (here named as clinical disease).

The dogs of the Group 2 (G2) were evaluated between 2014 and May 2015 and were confirmed positive by spleen aspiration and culture. These animals were grouped according to clinical staging proposed by Solano-Gallego et al. (2011): Stage 1—mild disease; Stage 2—moderate disease; Stage 3—severe disease; and Stage 4—very severe disease.

A third group, denominated G3, was evaluated between 2011 and 2013. It was composed by dogs that tested positive by indirect ELISA; however, they were negative in cultures of spleen aspirates. All dogs of this group had at least one suggestive clinical manifestation of VL, according to the clinical signs described in Section 2.3, but the presence and the intensity of these abnormalities were quite varied (supplementary material).

2.3. Physical examination and biological sample collection

All dogs underwent a physical examination to search for the presence and intensity of clinical signs of VL. Their history including previous preventive measures against common infectious diseases such as vaccination, deworming and use of repellent collars or topical drugs against blood-sucking ectoparasites have been carefully taken during anamneses. The dogs were thoroughly inspected to search for skin lesions, epistaxis, abnormal growth of nails, ocular lesions, signs of weight loss, presence of lymphadenomegaly and/or splenomegaly.

The dogs were sedated with acepromazine for splenic fine needle aspiration biopsy, following the method described by Barrouin-Melo et al. (2006). While sedated, 10 mL of blood was sampled by puncturing the cephalic or jugular vein for hematological analysis and serology for anti-IgG against *L. infantum* by indirect ELISA.

Hemograms were performed to search for evidences of inflammation, anemia and infection by other blood borne pathogens. Smears of fresh blood were prepared and after being dry at room temperature (23° C), they were stained (panoptic staining kit, Instan Prov®, Brazil). The slides were examined under optical microscopy (100X) for manual cell counting and morphology, and to search for the presence of parasitic inclusions within cells.

Only for animals of G2 were performed serum biochemistry, that included measurements of urea by UV enzimatic (Labtest®), creatinine by modified Jaffé reaction, (Labtest®) total proteins by BIURETO (Labtest®) and albumin by bromocresol green with citrate buffer (Labtest®), globulin fractions (arithmetic calculation), and enzymes alanine aminotransferase (ALT) by kinect UV (Labtest®) and alkaline phosphatase (AP) by modified Bowers and McComb (Labtest®), to evaluate inflammatory response, and hepatic and renal function. Urine samples were obtained by cystocentesis in female dogs and use urethral catheter in male dogs to perform urinalysis and UPC.