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Parasitic load and histological aspects in different regions of the spleen of dogs with visceral leishmaniasis

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ABSTRACT

Leishmania infantum causes from subclinical infection to severe disease in humans and dogs. The spleen is one of the organs most affected by the infection. Although evidence exists that the parasitic load distribution and histological alterations may not be homogeneous in the affected organs of naturally infected individuals, it has not been formally demonstrated using the current techniques used for studying the disease.

In six dogs naturally infected with *Leishmania*, parasitic load and histological changes were compared in samples collected from the lower, middle and upper third of the spleen.

Parasitic load in the spleen of the group of dogs was variable, revealing a difference of 61 times between animals with the lowest and the highest parasitism. The set of parasitic load values of each dog showed a cluster trend, when compared to the other animals. Nevertheless, the parasitic load values of each dog showed a variation ranging from 3.2 to 34.7 times between lowest and highest value. Histological changes showed recognizable variation in frequency (granulomas) or intensity (perisplenitis) in the spleen of 2 out of the 6 dogs. The agreement of histological findings between samples collected from the different thirds of the spleen was good (kappa coeficient, 0.61–0.80) very good (0.81–0.99) or perfect (1.00), for most of the parameters analyzed.

Variability of parasitic load and, to a lesser extent, histological changes in spleen of dogs with visceral leishmaniasis is observed. Such variability may be taken in account in the design of studies on pathogenesis, vaccine and therapeutic drug development.

1. Introduction

The zoonotic form of visceral leishmaniasis (VL) is found mainly in Brazil, in the Mediterranean Basin and in the Middle East [1]. The disease affects predominantly human beings and the domestic dog, being the latter the main reservoir of *Leishmania infantum* (syn. *L. chagasi*) [2,3], the causal agent. In endemic areas, the infection rate may exceed 60% of the canine population [4–7]. Unfortunately, a vaccine demonstrated to be effective to control canine VL is still unavailable [8]. In addition, dogs treated with the currently available drugs tend to develop only transient clinical cure, without parasitological clearance, and, after treatment, relapses are frequent even in the absence of re-infection [9,10]. For the reasons mentioned above, canine VL poses a major public health and veterinary medicine concern.

After dermal inoculation by the phlebotomine vector, *L. infantum* spread to organs of the mononuclear phagocytic system [11], especially the spleen, bone marrow, liver and lymph nodes. In these organs, the innate and adaptive immune system cells will determine the extent of parasitic survival and multiplication, development of host tissue damage, and fate of the infection [12].

In the infected individuals, the spleen tends to be parasitized during the whole period of the infection [11–14], and very often develops functional and architectural changes [15–18]. These changes are associated to a state of immunosuppression that compromises the host capacity to control the protozoan infection and infections caused by other microorganisms [19]. For these reasons, splenic evaluation is used to

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detect *Leishmania* infection and determine parasitic load as well as host immunological state, and response to treatment in dogs and humans with VL [16,17,20,21]. Perisplenitis, granuloma and white pulp disorganization has been associated with susceptibility and with severe forms of the disease and the spleen size regression is used as clinical evidence treatment response [16,22].

There is evidence that parasitic distribution and histological changes in organs of individuals (e.g. humans and dogs) naturally infected with *Leishmania* may be heterogeneous [16]. Yet, there are no reports formally demonstrating a heterogeneous distribution of these findings in the affected organs.

Further knowledge on distribution of *Leishmania* load and histological changes in different organs may contribute to improve diagnosis of infection/disease and understanding of compartmentalization of host response to the parasite. This will allow for designing more rational studies on pathogenesis and evaluation of outcome of immunization or treatment protocols.

In the present work, we systematically studied distribution of parasitic load and morphological changes in samples collected from the upper, middle and lower third of the spleen of dogs with VL. The parasitic load was assessed by real-time PCR using TaqMan system and morphological changes were assessed by conventional histology.

2. Methods

2.1. Animals

Blood samples and splenic aspirates were obtained from six mongrel dogs from Camaçari, Brazil, an area endemic for VL [23]. Spleen aspirate punctures were carried out by the method previously described by Barrouin-Melo et al. [24], followed by culturing the aspirate in NNN biphasic medium supplemented with 20% fetal bovine serum (FBS) [24]. All six animals showed: a) *Leishmania*-specific antibodies, detected by indirect immunofluorescence assay (IFAT, carried out with a BioManguinhos leishmaniasis detection kit, Rio de Janeiro, Brazil), b) *Leishmania* promastigote growth in cultures of splenic aspirate, and c) clinical signs compatible with VL (described in Table 1). Four months after the initial clinical evaluation, the dogs were clinically re-examined and then euthanized, in accordance with Brazilian Ministry of Health guidelines, under deep anesthesia (1 mg/kg of xylazine, 15 mg/kg ketamine and 25 mg/kg thiopental) by intravenous injection of saturated potassium chloride solution.

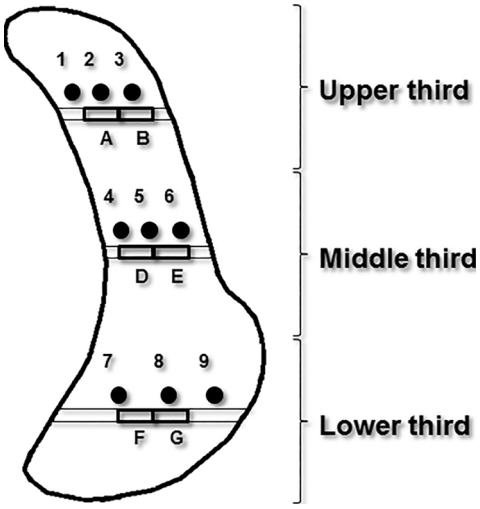

Fig 1. Schematic drawing of spleen showing sampling areas.
Schematic drawing of canine spleen indicating the spots (numbered 1–9) where separate aspirate punctures were carried out for determination of parasitic burden and splenic fragments were taken out (labelled A to G) for histological analysis. The brackets show each third section of the organ.

2.2. Collection of splenic aspirates for real-time PCR and histological study

Following euthanasia procedures, each animal's spleen was removed from its abdominal cavity and subjected to needle puncture aspiration at nine different sites (three in each third of the organ: upper, middle and lower; Fig. 1). Sample collection was performed using a separate 42 × 1.2 mm needle attached to a 20 mL syringe. Each splenic aspirate was then transferred to previously weighed Eppendorf tubes, immediately placed under dry ice and stored at −70 °C until use. Later, the weight of each sample was determined. In addition, cross sections of the spleens were performed in each third (Fig. 1) and two fragments approximately 4 mm in thickness from each third were collected and fixed in phosphate-buffered 10% formalin. Splenic sampling was carried out uniformly without representing anatomical segments of the organ.

2.3. Real-time PCR

DNA was extracted from the splenic aspirate samples using a DNAeasy Blood & Tissue kit (Qiagen, Hilden, Germany) in accordance with manufacturer recommendations, and concentrations were

Table 1
Clinical characterization of dogs with visceral leishmaniasis.

Dog #	Sex[a]	Age[b] (years)	Body[c] weight (kg)	Clinical evaluation[d]	
				First	Second
320	M	5–7	9.4	Hyperkeratosis on the nose and popliteal lymphadenopathy	Hyperkeratosis on the nose and submandibular lymphadenopathy
325	M	5–7	11	Hyperkeratosis on the nose and submadibular lymphodenopathy	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy, alopecia, periocular dermatitis, conjunctivitis and onychogryphosis
332	F	3–4	19	Hyperkeratosis on the nose, periocular dermatitis and onychogryphosis	Hyperkeratosis on the nose, periocular dermatitis, onychogryphosis, alopecia, popliteal and submandibular lymphadenopathy
335	F	3–4	11.2	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy	Hyperkeratosis on the nose, popliteal and submandibular lymphadenopathy, alopecia and onychogryphosis
343	F	5–7	25	Popliteal lymphadenopathy	Alopecia and onychogryphosis
353	F	3–4	23	Hyperkeratosis on the nose, alopecia, and popliteal lymphadenopathy	Dermatitis, conjunctivitis and submandibular lymphadenopathy

[a] Sex: male (M), female (F).
[b] Age estimated by physical examination.
[c] Body weight measured on the day of euthanasia.
[d] The two clinical evaluations were carried out 4 months apart.