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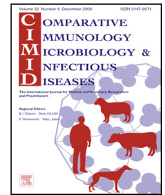

# A minimally invasive approach to spleen histopathology in dogs: A new method for follow-up studies of spleen changes in the course of *Leishmania infantum* infection

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ABSTRACT

Severe forms of zoonotic visceral leishmaniosis (ZVL) are associated with disruption of the spleen structure. However, the study of spleen histology requires splenectomy or necropsy. In this work, we present a minimally invasive cell-block technique for studying spleen tissue histology in dogs with ZVL. We examined 13 dogs with and seven dogs without *Leishmania infantum* infection. The dogs with *Leishmania* infection had a lower frequency of lymphoid follicles (2/13, Fisher's test, $P < 0.02$) and a higher density of plasma cells (score 3, Fisher's test, $P < 0.02$) than uninfected dogs (5/7 exhibiting lymphoid follicles and a plasma cell score of 1). The dogs with *Leishmania* infection also presented with granulomas (8/13) and infected macrophages (5/13). These differences in the histological presentations of spleen tissue from infected and uninfected dogs corresponded to changes observed in conventional histology. Hence, the cell-block technique described here may be used in the follow-up care and study of dogs with ZVL and other diseases in both clinical practice and research.

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## 1. Introduction

The spleen is the largest secondary lymphoid organ in mammals and is responsible for surveillance against blood-borne antigens [1]. This organ is the site of the final differentiation and homing of many leukocyte populations, including monocyte populations and memory B cells responsive to both T-dependent and T-independent type 1 and type 2 antigens [2,3]. The spleen also plays an important role in the course of neoplastic and infectious diseases [4,5]. For instance, absence of the spleen is associated with an increased risk of disseminated infections, and spleen infiltration is an important parameter for lymphoma staging in both humans and dogs [5,6].

The spleen plays a central role in the course of zoonotic visceral leishmaniosis (ZVL) in humans and in dogs and is involved throughout the course of the disease [7]. Spleen enlargement is an important clinical sign of chronic infection, so a spleen regression index is used as a marker of the therapeutic response in human ZVL [8]. Furthermore, the spleen may contribute to the progression of ZVL by (1) providing a permissive environment, thereby maintaining the parasite burden; (2) promoting anemia and thrombocytopenia through hypersplenism; and (3) modulating the immune response to favor parasite survival [9–11]. Recently, we and other groups have shown that disruption of splenic lymphoid tissue is associated with severe forms of ZVL in dogs [12–15]. Similar changes in spleen structure have also been reported in humans who died of the disease [16].

Unfortunately, most of the morphological changes in the spleen that are potentially relevant to the course of infectious or neoplastic disease can be studied only in specimens obtained by necropsy or splenectomy. A few years ago, we developed a technique for fine-needle aspiration spleen biopsy that proved to be safe for sensitive and specific diagnosis of *Leishmania* infection and allowed for the study of spleen cytology in dogs [17–19]. In that study, we observed that small and sparse fragments of spleen tissue remained in the midst of single-cell suspensions that were prepared by

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cytocentrifugation [19]. In the present work, we show that these spleen tissue fragments obtained by fine-needle biopsy can be used as a source of information about spleen morphology in dogs with ZVL. This information can be acquired using a cell-block technique for processing the fine-needle spleen aspirates.

In addition to the information obtained via conventional cytology, this cell-block technique allows spleen compartments to be studied and pathological structures, such as granulomas and blood vessel hyalinosis, to be identified. Because fine-needle aspiration biopsy is a well-tolerated and safe procedure in dogs under sedation [17], its combination with this cell-block technique may allow for sequential studies on the development, reversibility, and impact of spleen changes during the course of *L. infantum* infection and ZVL.

## 2. Materials and methods

### 2.1. Ethical considerations

All procedures were conducted in accordance with the Oswaldo Cruz Foundation guidelines for research in animals (http://sistemas.cpqam.fiocruz.br/ceua/hiceuaw000.aspx) and with the manual for the surveillance and control of visceral leishmaniosis. All the tests were performed in the animals after authorization by the Control of Visceral Services of Camacari (Bahia State, Brazil, an area endemic for ZVL) (1 animal) or by their owners (all the other infected and control animals). This study was approved by the ethics committee for the use of animals in research (CPqGM-FIOCRUZ, Ceua, license N.040/2005).

### 2.2. Animals

This was a convenience sample of 20 dogs of different breeds, genders, and estimated ages that were collected either by the Surveillance and Control of Visceral Services of Camacari (Bahia State, Brazil) or from the Veterinary School Hospital (Federal University of Bahia). Eight dogs from the hospital and five from Camacari had clinical signs of ZVL or positive laboratory tests for *Leishmania* infection. One animal from the hospital was healthy, having been admitted for castration, and was enrolled in the control group. Six other healthy animals were from a non-governmental animal shelter located in Salvador (Bahia State, Brazil, an area non-endemic for ZVL). All the animals were subjected to a clinical exam, serological testing for anti-*Leishmania* antibodies and culture of spleen aspirates for *Leishmania* isolation. These tests are further detailed below.

### 2.3. Clinical data

All animals were subjected to a clinical exam using a standardized form and were considered symptomatic if they had any of the following clinical signs, which are considered to be indicative of canine visceral leishmaniosis: alopecia, dermatitis, skin erosion, ulcerations, anemia (pale, light pink mucous membranes), conjunctivitis, onychogryphosis, weight loss, and lymph nodes larger than expected for the size of the animal.

### 2.4. Anti-Leishmania antibody activity in the serum

The presence of anti-*Leishmania* antibodies in the serum of all studied dogs was investigated using a classical indirect ELISA using soluble *L. infantum* antigens, as previously described [17]. Briefly, 96-well plates were coated with the antigen overnight at 4 °C (incubation with 100 mL per well of 5 mg of protein per mL of 0.1 M carbonate-bicarbonate solution, pH 9.6). Non-specific antibody binding was blocked with 5% skimmed milk (w/v) in 0.15 M phosphate-buffered saline (PBS) solution, pH 7.4, containing 0.05% Tween 20 (PBS-T) for 1 h, at 37 °C. Control and test serum samples were added at a 1:400 dilution in 1% skimmed milk in PBS-T, with 100 mL/well, and incubated for 1 h at 37 °C. A peroxidase-conjugated rabbit anti-dog IgG (Sigma-Aldrich Chemical Co., St. Louis, Missouri, EUA) was added at a dilution of 1:5000 in 1% skimmed milk in PBS-T, with 100 mL/well, and incubated for 1 h at 37 °C. The enzymatic reaction was developed with o-phenylenediamine (Sigma Chemical Co.) and stopped after 15 min with 25 mL/well of 4 N $H_2SO_4$. Absorbance values were read at 490 nm. The cut-off value for IgG antibodies was determined based on the receiver operating characteristic (ROC) curve using corrected absorbance values obtained for sera from 30 *L. infantum*-infected dogs positive in serological and parasitological tests and from 71 healthy dogs living in non-endemic areas. All determinations were carried out in duplicate or triplicate, and mean values above the cut-off for IgG were considered to be positive results.

### 2.5. Fine-needle spleen biopsy

Fine-needle aspiration spleen biopsies were performed according to the technique previously described by Barrouin-Melo et al. (2006a). Briefly, dogs were sedated through intravenous injection of 0.5 mg/kg body weight acepromazine in the right lateral decubitus. Asepsis of the area that would be punctured was ensured using 2% iodized alcohol. A splenic puncture was made in the left flank of each animal near the caudal border of the last rib using a 40 × 12-mm needle coupled to a 20-mL syringe [17]. Part of each splenic aspirate was cultivated in a biphasic culture medium for parasitological diagnosis, and part was dispensed into a 15-mL non-adhering (propylene) tube containing 10% formalin and was used for the cell-block technique, as described below.

### 2.6. Culture for parasitological diagnosis of Leishmania

Samples obtained by spleen aspiration (approximately 100–200 μL) were cultured in a biphasic culture medium containing 1.5 mL of solid medium (blood agar) and 2 mL of Schneider's medium supplemented with 20% fetal bovine serum. The samples were kept in culture at 23 °C and examined weekly by optical microscopy for four weeks or until they became positive [20].

### 2.7. Cell-block preparation and analysis

Spleen aspirates (approximately 100–200 μL) were placed in non-adherent polypropylene tubes containing 14 mL of 10% formalin and fixed under agitation for 12–24 h at room temperature. The preparations were then centrifuged for 10 min at 1000g. The supernatant was removed, and the sediment was carefully placed in a small parcel made of filter paper wetted with formalin. The specimens were then dehydrated with progressive concentrations of ethanol and embedded in paraffin. Sections of 3-μm thickness were obtained for conventional microscopy and immunohistochemistry. For conventional histology, cell-block sections were stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), and periodic acid-silver methenamine (PAS-M). Serial sections of the cell-block preparations were analyzed without knowledge of the parasitological or serological test results according to the following criteria:

### 2.8. Splenic tissue features

The number of spleen fragments; the sizes of smaller and larger spleen fragments; spleen compartments (red pulp, white pulp, periarteriolar lymphoid sheaths, lymphoid follicles, and marginal zone); trabeculae; blood vessels, including sheathed capillaries; and pathological structures (granulomas and *Leishmania*-infected

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