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Case Report

Unusual Small Intestine Inflammatory Lesions in a Dog with Visceral Leishmaniasis

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Abstract

The aim of this study is to describe a case of an asymptomatic dog naturally infected with *L. infantum chagasi* with a surprising number of parasites in the duodenum. A mixed breed dog of unknown age was referred to the Center for Zoonoses Control of the Municipality of Ribeirão das Neves, Belo Horizonte Metropolitan area, Minas Gerais State, Brazil. The dog was diagnosed for *Leishmania* using an enzyme-linked immunosorbent assay (ELISA), direct parasitological examination of bone marrow aspiration, and immunohistochemistry of ear biopsy. After euthanasia samples of spleen, liver, lung, kidney, heart, cervical and mesenteric lymph nodes; ear, snout, abdominal skin and GIT segments (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum) were evaluated histologically and immunohistochemically for the presence of parasite amastigotes. Gross and microscopic examination of necropsy samples showed no severe alterations of the mucosa in any gastrointestinal segment. A conspicuous parasite load was observed in the lamina propria of the duodenum, jejunum, ileum, cecum, colon, and rectum. Parasite distribution in the small intestine was diffuse through the lamina propria, whereas in the large intestine it was concentrated close to the muscularis mucosa and distant from the intestinal lumen. The parasite load in the duodenum, mainly in the subepithelial region, was higher than in the other segments ($p = 0.0008$). This unusual case of localization in the small intestine and the distribution of the parasites in the intestinal mucosa may suggest the existence of different regulatory mechanisms in these segments.

Key Words: Canine visceral leishmaniasis, gastrointestinal tract, histopathology, immunohistochemistry, *Leishmania (Leishmania) infantum chagasi*.

Introduction

In Brazil, canine visceral leishmaniasis (CVL) is a severe zoonotic disease caused by an intracellular protozoan *Leishmania* (*Leishmania*) *infantum chagasi* (16) transmitted by the bite of blood-sucking sandflies (*Lutzomyia* sp.). Dogs are the domestic reservoir for human visceral leishmaniasis (HVL), and show clinical and pathologic profiles similar to those observed in humans (4).

The primary histological alterations observed in dogs with visceral leishmaniasis are hyperplasia of cells of the mononuclear phagocyte system mainly in spleen, lymph nodes, liver, and bone marrow; a chronic inflammation in the skin; granulomatous inflammatory reactions in liver and spleen and glomerulonephritis (19). Gastrointestinal tract (GIT) disorders occur in HVL and in naturally and experimentally infected dogs (8, 9, 23). One study in Europe described a chronic inflammatory exudate composed of macrophages, plasma cells, and lymphocytes throughout the mucosa (lamina propria) of the small and large intestine in two dogs naturally infected with *L. infantum* (5). Some authors have identified amastigotes inside macrophages of the lamina propria of the GIT (7,14). Parasites were present in all intestinal segments and layers of the intestinal wall (mucosa, muscularis mucosa, and submucosa) irrespective of clinical status.

However, the parasite load was significantly higher in the cecum and colon than in other segments of the GIT (7,14). Other studies have also revealed differences in parasite load among GIT segments in clinically affected dogs (5,8,9). Infected dogs often exhibit an increased number of macrophages (infected or not), plasma cells and lymphocytes (focal or diffuse), in GIT lamina propria, muscularis mucosae, and submucosa (14).

The aim of this study is to describe a case of an asymptomatic dog naturally infected with *L. infantum chagasi* with a surprising number of parasites in the duodenum.

Case report

Dog

A mixed breed dog approximately 2-year-old was identified during an epidemiologic survey of CVL carried out by the Center for Zoonoses Control of the Municipality of Ribeirão das Neves, Belo Horizonte Metropolitan area, Minas Gerais State, Brazil. Dogs were diagnosed with *Leishmania (Leishmania) infantum chagasi* infection using ELISA (optical density >100; 1:400 dilutions) and direct examination of parasites from bone marrow aspiration, and immunohistochemistry of ear sections. Physical examination was performed for clinical classification of the patient. The dog had good body condition and normal vital signs. The palpated peripheral lymph nodes (submandibular, cervical and popliteal) the skin and other systems evaluated were unremarkable. The dog was categorized as asymptomatic dog.

Sample collection

The dog was euthanized with 1.0 mL/kg of 2.5% intravenous thiopental (Thiopentax®) and 0.3 mL/kg T61® (MSD, Brazil). Samples of spleen, liver, lung, kidney, heart (ventricles), cervical and mesenteric lymph nodes; ear, snout, abdominal skin and GIT segments (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, and rectum) were fixed in 10% neutral buffered formalin, trimmed and embedded in paraffin, sectioned 3µm tick and stained with hematoxylin and eosin (HE). A single sample was removed from each GIT segment, cut into three pieces, and processed for histology. Blind examination of slides was carried out by at least two pathologists. Immunohistochemistry to identify *Leishmania* amastigotes was performed using the streptavidin peroxidase reaction performed as previously described (20).

Ethical committee approval

In Brazil euthanasia of dogs with leishmaniasis is indicated as a measure of public health control (Decreto-Lei n. ° 314/2003 December 17, 2003). This study received the approval of the CETEA/UFMG (Comitê de Ética em Experimentação Animal/Universidade Federal de Minas Gerais), protocol 257/2008. All procedures involving animals were conducted according to the guidelines of the Brazilian College of Animal Experimentation (COBEA).

Analysis

For semiquantitative analysis, samples were scored from one (no inflammation) to four (severe inflammation), based on mononuclear cell (mainly macrophages) infiltration in all segments as previously described (6).

For the histomorphometric study, twenty five randomly chosen images (micro QColor 3 imaging system, Olympus, Pennsylvania, USA) from immunohistochemical slides of GIT tissue fragments were used to assess the area of immunolabeled amastigotes. Using a sequence of algorithms of the program KS400 (Carl Zeiss, Oberkochen, Germany), the area of immunohistochemical staining (µm²) on each image was calculated by selecting those pixels with shades of brown and the subsequent creation of a binary image (3). The statistical analysis of the inflammation score and the score for the number of immunolabeled amastigotes were compared by Kruskal–Wallis test. The accepted level of significance corresponded to $p < 0.05$.

Histopathology and immunohistochemical Analysis

Leishmania amastigotes distribution in infected organs

Liver

Grossly, the hepatic lobe was preserved. However, in microscopic analysis a chronic inflammatory reaction characterized by intralobular granulomas was observed. These granulomas were of variable size and comprised macrophages, some of which contained amastigotes, epithelioid cells, small numbers of lymphocytes, and plasma cells. We also observed large numbers of eosinophils. Hemosiderosis was found, but it was not extensive. Hypertrophy and hyperplasia of Küpffer cells loaded with amastigotes, a moderate and focal congestion of the sinusoid vessels, and moderate mononuclear inflammatory cells in the portal space were apparent.

Spleen

A discrete and diffuse chronic inflammatory reaction was observed in the capsule, subcapsular region, and red pulp. The capsule and trabecular vessels were dilated and congested. The red pulp showed intense fibroplasia of the trabecula. Epithelioid macrophages were found, but without typical granuloma formation as previously described in CVL. Focal hemosiderosis was observed. The white pulp showed lymphoid nodules without typical germinal centers. In addition, the periarteriolar lymphatic sheath was atrophic formed by reduced numbers of lymphocytes around the central arteriole. A discrete infection was observed within the

capsule, subcapsular, and perifollicular regions.

Lungs

The main lesion observed was chronic and diffuse interstitial pneumonia. The cellular infiltrate was predominantly characterized by mononuclear cells mainly composed by macrophages, plasma cells and lymphocytes with rare eosinophils and neutrophils. Hyperplasia of smooth muscle was seen in all intralobular septal walls. The endothelial and medial vessel layers were severely damaged and congested. Inflammatory foci in the subpleural region were characterized by focal accumulations of macrophages. No *Leishmania* amastigotes were found with either histological or immunohistochemical methods.

Kidneys

The main lesion observed was a mesangioproliferative glomerulonephritis. The glomerular tuft almost completely filled the Bowman space due to diffuse mesangial cell proliferation. An accumulation of homogeneous and eosinophilic hyaline material was observed in the mesangial area. Thickening of Bowman's capsule occurred, and some glomeruli showed tuft atrophy due to scarring. *Leishmania* amastigotes were not detected using both histological and immunohistochemical methods.

Heart

A slight focal chronic inflammation was present in the ventricular myocardium. *Leishmania* amastigotes were not found with either histological or immunohistochemical methods.

Lymph nodes (mesenteric and cervical)

The most frequent lesion observed in lymph nodes was hypertrophy of cortical and medullar regions. Amastigotes were primarily observed inside medullar macrophages. The capsule of the cervical lymph node was thickened and colonized by a chronic inflammatory mononuclear cell exudate associated with collagen deposits. Macrophages were the main inflammatory cells in the subcapsular sinus. The cortical region showed follicular hyperplasia. A diffuse paracortical hyperplasia was observed. In the medullar region, hyperplasia of medullary cords and sinus cells occurred. The medullary areas were closely packed with differentiated plasma cells, medium-sized and large lymphocytes (lymphoblasts), and macrophages. Macrophages were morphologically differentiated as epithelioid cells due to the presence of large vesicular nuclei, branched chromatin, and abundant cytoplasm. Hemosiderosis was observed. Edema was occasionally seen only in the mesenteric lymph nodes. Mesenteric lymph nodes showed intense inflammation of the capsule, with the presence of polymorphonuclear cells in the subcapsular region. In cervical lymph nodes, we observed intense infection with numerous amastigotes within the cytoplasm of macrophages.

Bone marrow (smear)

In the bone marrow, hypoplasia, mainly of white cells, was the principal pathological alteration. Red cells predominated in all tissue sections examined. Amastigotes were observed inside monocytes.

Skin (ear, nose, and abdomen)

Skin samples from ears, nose, and abdomen were analyzed. Ear skin samples showed a chronic inflammatory reaction characterized by a cellular infiltrate mainly composed by mononuclear cells (plasma cells, macrophages and lymphocytes). In general, the cellular inflammatory infiltrate was diffuse in the upper dermis and around vessels, glands and hair follicles in the deep dermis. A presence of many epithelioid cells was noticed within the mononuclear cell exudate, parasitized or not, but granuloma formation was not observed. No amastigotes of *Leishmania* were found in the abdomen skin samples. Some amastigotes were seen in macrophages of the dermis of the nose skin samples.

Gastrointestinal tract

Macroscopic observation revealed no severe lesions in the GIT mucosa. Microscopic evaluation revealed an increased number of plasma cells, lymphocytes and macrophages and rare neutrophils or eosinophils in all GIT layers (lamina propria, muscularis mucosa, and submucosa). A chronic cellular infiltrate was observed in all samples, composed predominately of macrophages, plasma cells, and lymphocytes, with rare polymorphonuclear neutrophils or eosinophils. No macrophages or epithelioid macrophages infected with *Leishmania* amastigotes were found in the lamina propria of the mucosae. The stomach showed inflammatory foci in the submucosa, whereas, in the ileum, invasion of inflammatory cells into the lamina propria of the submucosa was observed. Both the colon and rectum exhibited marked inflammatory reaction throughout the wall. In spite of the infection, no tissue erosion or ulcers were observed in the epithelial mucosal layers or glands (Fig. 1). Immunolabeled amastigotes were evident in the duodenum, ileum, cecum, colon, and rectum, with the duodenum showing more amastigotes ($p = 0.0008$) than the other segments (Fig. 2). The topographic distribution of the parasite in the lamina propria differed in GIT. In the small intestine, the amastigote forms were distributed diffusely in the lamina propria while, in the large intestine, the amastigotes were concentrated near the muscularis mucosa at some distance from the intestinal lumen (Fig. 3). Infection was not observed in the esophagus or stomach.

Discussion

Classic histological lesions were found chiefly in organs rich in cells of the mononuclear-phagocytic system.

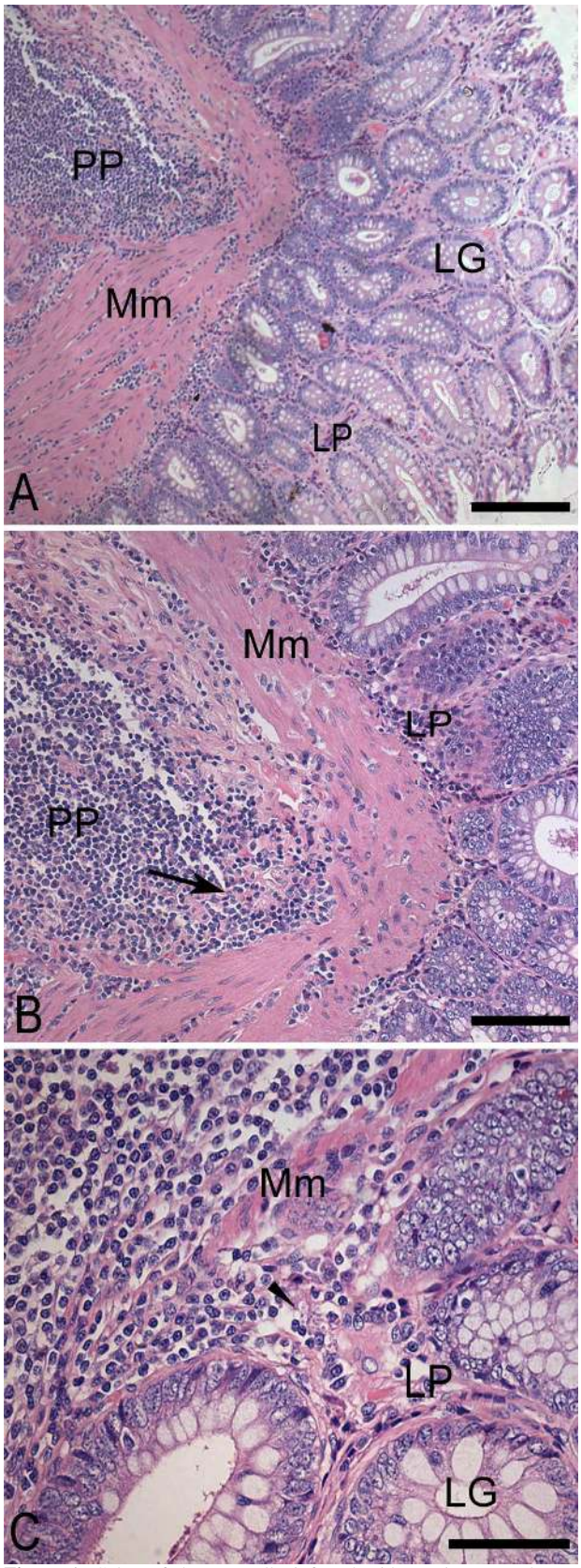

Figure 1. Colon of dog naturally infected with *Leishmania infantum chagasi*. (A) submucosal layer, muscularis mucosa, and lamina propria (HE staining, Bar = 64µm). (B) Peyer's patch hyperplasia (arrow) and inflammatory infiltrate (HE staining, Bar = 32µm). (C) higher magnification of Figure 1B showing extensive presence of amastigotes (head arrow) distributed in muscularis mucosa and lamina propria (HE staining, Bar = 16 µm). LG: *Lieberkühn's* gland; LP: Lamina propria; Mm: Muscularis mucosa; PP: Peyer's patches.

As formerly described by many authors, an intense chronic inflammatory reaction consisting of mononuclear cells (macrophages, plasma cells, and lymphocytes) infiltrated the liver and spleen (2,18,23), skin (4,19, 22) bone-marrow (18), lymph nodes (10,12), gastrointestinal tract (1, 7, 14, 21), and kidneys (17).

In CVL, a notable parasite load in the lamina propria of GIT segments has been previously described, mainly in the cecum and colon (14). However, in the dog of this investigation parasites where found in all GIT segments except in the esophagus and stomach. Distribution (topography) and number of parasites in the duodenum differed from those reported in previous studies. In the duodenum, a notable diffuse parasite load was observed within the lamina propria, with a distinct distribution of parasites in the subepithelial region. This distribution was also observed in the jejunum and ileum. In contrast, in the large intestine, parasites were concentrated near the muscularis mucosa distant from the intestinal lumen. Platt et al. 2008 (15) studying murine macrophages and regulation of the immune response in the intestinal mucosa found that the microbiota present in the intestinal lumen varied significantly with the intestinal area. Resident commensal microbiota of the large intestinal lumen consisted of 10^{21} microorganisms more than in the small intestine. Thus, they could assume that there is a relationship between the intestinal microbiota and the immune system. Despite of this data not been related to CVL, these results could indicate that intestinal microbiota may regulate the immune response and directly affect the distribution of parasites in the lamina propria. Our group has characterized intestinal macrophages in dogs with CVL, obtained from two distinct segments of canine GIT, the jejunum and colon, and considered the regulatory role of microflora in the GIT immune response. The microflora of naturally infected dogs expressed TLR2 and TLR9. We investigated the macrophage phenotypes $CD11b^+$, $CD11c^+$, and $CD14^+$, which demonstrated a higher frequency of TLR2 in colon than in jejunum. In contrast, a higher frequency of TLR9 was evident in jejunum (unpublished observations).

Clinical abnormalities of the GIT in dogs, both naturally (5) and experimentally (8) infected, have been described. Some studies reported diarrhea to be the first clinical manifestation of infection in dogs experimentally infected with *Leishmania (Leishmania) infantum chagasi* (8). In our study, the clinical status of the animal with respect to vomiting or diarrhea was unknown. During necropsy, we did not find gross GIT alterations such as hyperemic mucosae with ulcers, submucosal edema, or punctiform bleeding areas as described by Toplu and

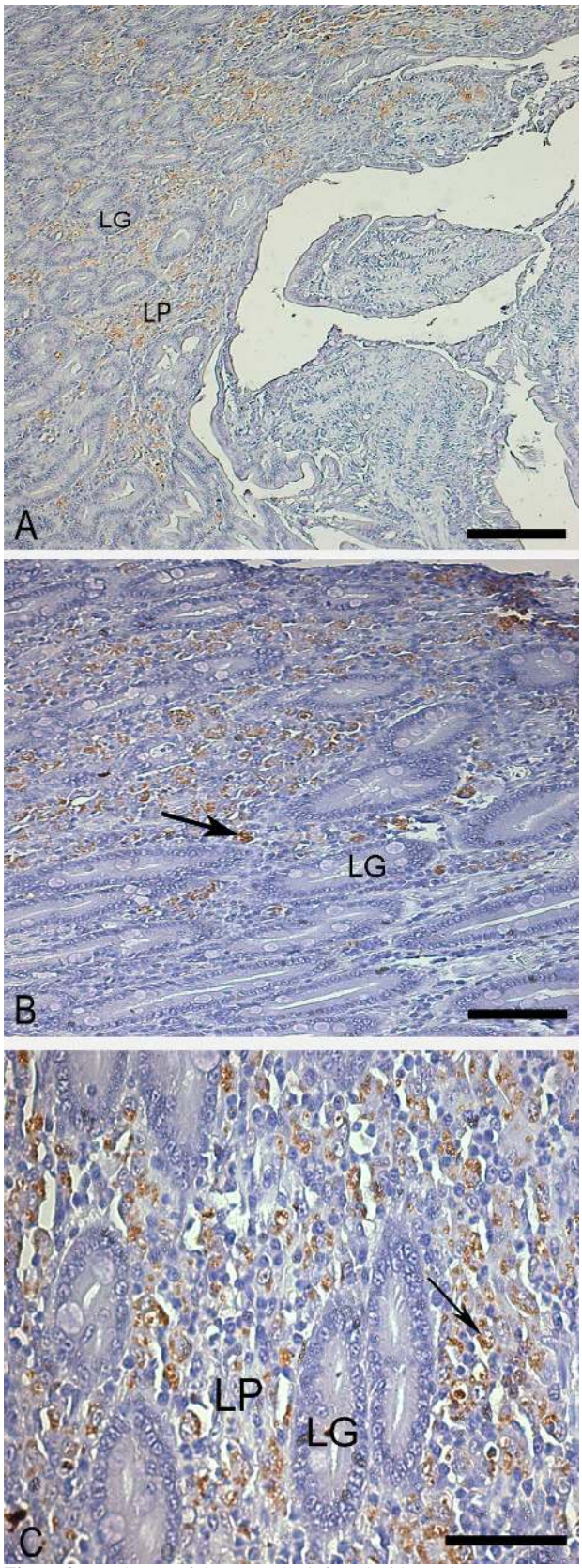

Figure 2. Duodenum of dog naturally infected with *Leishmania infantum chagasi* amastigotes visualized by immunolabeling (streptoavidin peroxidase reaction). (4) lamina propria with amastigotes distributed throughout the tissue (streptoavidin peroxidase reaction, Bar = 64µm,); (5) amastigotes diffusely distributed (arrow) (streptoavidin peroxidase reaction, Bar = 32µm); (6) intense staining of amastigotes distributed throughout the lamina propria (arrow) (streptoavidin peroxidase reaction, Bar = 16µm). LG: Lieberkühn's gland with goblet cells; LP: Lamina propria; Mm: Muscularis Mucosa; Sbm: Submucosa, PP; Peyer's patches.

Aydogan, 2011 (21). Adamama-Moraitou et al., 2007 (1) observed asymptomatic colitis, as indicated by the presence of hyperemia of the mucosa with either patchy edematous areas or small areas of mucosal erosion.

Confirming what Adamama-Moraitou et al., 2007 reported (1), we showed that colonoscopy was important in identifying disease lacking intestinal involvement but the absence of clinical signs characterizing the chronic or recurrent colitis in dogs can be explained by the fact that endoscopic abnormalities and/or histological changes do not always correlate with the severity of the clinical disease. In our group, we have observed an association between mononuclear cells of the GIT lamina propria associated with the parasite load (14). There was a predominance of plasma cells, macrophages, and lymphocytes, but polymorphonuclear cells (neutrophils and eosinophils) were rarely observed (14). Our results are in accordance with studies conducted in Europe (5) and in Brazil with naturally infected dogs (16) and also with experimentally infected dogs (8). The morphology of infected macrophages showing pale cytoplasm and intracytoplasmic vacuoles with *Leishmania* amastigotes has been also characterized in our studies. In addition, the presence of epithelioid cells (enlarged macrophages, pale cytoplasm, and vesicular nucleus with loose chromatin) and/or giant cells (fusion of macrophages) was found, usually associated with mononuclear cell infiltrates. Ferrer et al., 1991 (5) and Adamama-Moraitou et al., (2007) (1) also observed infected macrophages in the gut. The latter authors found pyogranulomas in 90% of their animals, in contrast to our study. Toplu and Aydogan, 2011 (21) observed mononuclear cell infiltrate and eosinophilic infiltrate in the mucosa of the intestine in one dog with the presence of necrotizing vasculitis. Our analysis revealed the presence of infection in the intestinal mucosa, but without large lesions and extensive erosion or ulcers

Conclusion

In conclusion, the histopathological changes in the GIT of dogs with CVL are commonly found independent of the clinical status of the host. This unusual case of parasitism in the small intestine and the distribution of the parasites in the intestinal mucosa suggest that different regulatory mechanisms exist in these segments.

Competing interests

Research was supported by grants Conselho Nacional de Desenvolvimento da Pesquisa Tecnológica e Científica (CNPq/ 473601/2009-5) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG/CBB 00219/09-80 and APQ-01355-09), Brazil. Students fellowship financed by Conselho Nacional de Desenvolvimento da Pesquisa Tecnológica e Científica (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. We do not have any kind of commercial interesting or competition with other researches. It is only an academic manuscript.

Authors' contributions

*Contributed equally (AJWP and MMF).

AJWP and MMF did all the clinical exams, necropsie, histology and morphometrical and statistical analysis. WLT, MVC and RAF advisors, revise the manuscript.

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All authors read and approved the final manuscript.

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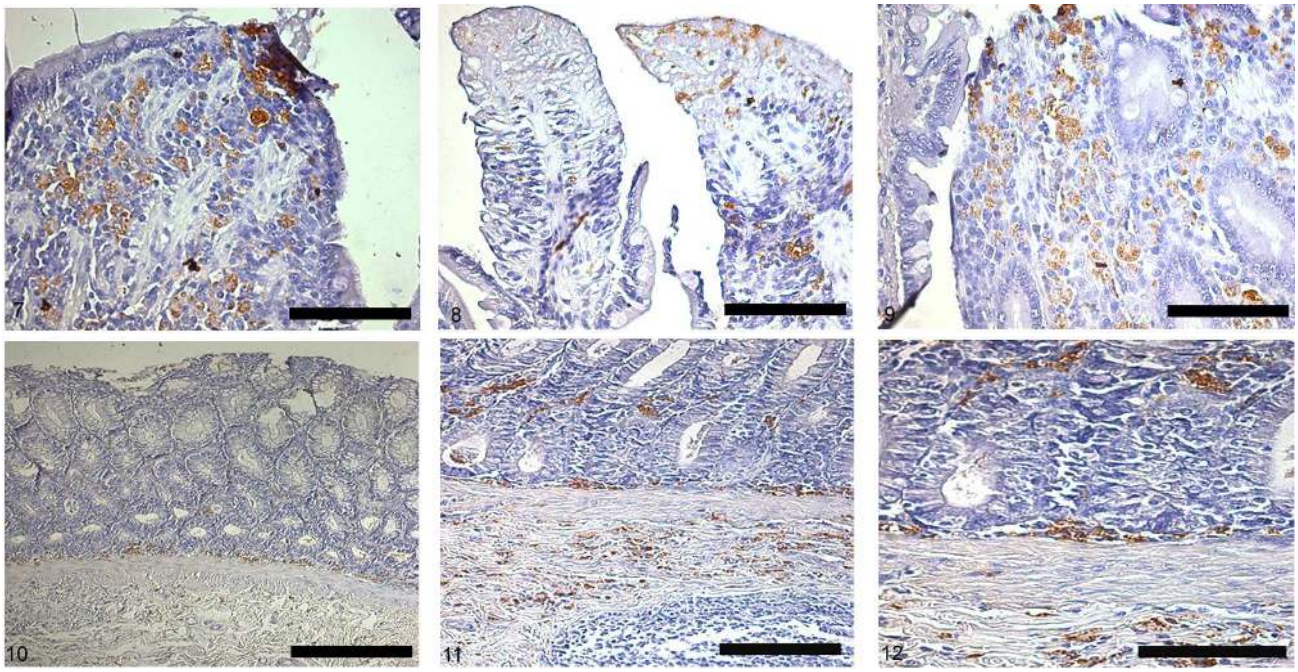

Figure 3. Duodenum and colon of dog naturally infected with *Leishmania infantum chagasi* (streptoavidin peroxidase reaction). (7-9) lamina propria of duodenum; note amastigotes throughout the tissue(streptoavidin peroxidase reaction, Bar = 32µm). (10-12) lamina propria of colon; note amastigotes concentrated near the muscularis mucosa deep below the lumen (streptoavidin peroxidase reaction, Bar = 64µm, 32µm and 16µm).

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