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## Cardiac and pulmonary alterations in symptomatic and asymptomatic dogs infected naturally with *Leishmania* (*Leishmania*) *chagasi*

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# Cardiac and pulmonary alterations in symptomatic and asymptomatic dogs infected naturally with *Leishmania (Leishmania) chagasi*

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## Abstract

Fifteen symptomatic and seven asymptomatic dogs infected naturally with *Leishmania chagasi* were examined in order to identify the presence of parasites and changes in heart and lung. Histopathological, cytological, and immunohistochemical analyses were performed on samples of heart and lung tissues. An inflammatory reaction characterized by inflammatory mononuclear, perivascular and intermuscular infiltrates was observed in both symptomatic and asymptomatic animals on histopathological analysis of the heart. In the lung, there was thickening of the alveolar septa due to congestion, edema, inflammatory infiltrate, and fibroblast proliferation. A focal reaction was observed although a diffuse reaction was present in both groups. On cytological examination, heart and lung imprints revealed amastigotes in two symptomatic animals and heart imprints were found in 1 asymptomatic dog. Immunoperoxidase staining showed amastigotes in the lung and heart of only 1 of 6 symptomatic animals examined. Within the ethical principles and limits of this research, it can be inferred that the study of heart and lung alterations in canine visceral leishmaniasis is increasingly important for understanding the problem related to humans. Dogs with visceral leishmaniasis were a good experimental model, since infection was caused by the same agent and the animals developed clinical, pathological and immunological alterations similar to those observed in humans.

Key words: Leishmaniasis; Dog; Lungs; Heart

## Introduction

In the Americas, visceral leishmaniasis is caused by the protozoan *Leishmania (Leishmania) chagasi* (1) and transmitted by the bite of the *Lutzomyia longipalpis* sandfly (2). The dog is considered to be the primary domestic reservoir of the disease (1,3). However, further investigation is warranted to determine the precise role of the dog in the cycle of visceral leishmaniasis infection in endemic regions. In addition to dogs and animals of the synanthropic fauna (4), humans also seem to be reservoirs of this parasite in Brazil (5,6).

In order to evaluate the role of the dog in the cycle of canine visceral leishmaniasis (CVL) infection, all manifestations of the disease must be known, ranging from clinical and pathological features to the extension and progression

of lesions in various compromised organs. Dog infection with *L. chagasi* is clinically similar to human infection, although skin lesions are frequently found in dogs developing clinical manifestations, as well as visceral damage (7). CVL occurs as a chronic, fatal and systemic disease. The major clinical signs consist of cachexia, hypergammaglobulinemia, hepatosplenomegaly, anemia, and lymphadenopathy (7-12).

The main alterations are in organs rich in cells belonging to the mononuclear phagocyte system, such as the liver, spleen, lymph nodes, bone marrow, and gastrointestinal tract (13). However, parasite dissemination also occurs in other organs that do not belong to this system, e.g., the heart and lungs (14).

The clinical and pathological alterations due to cardiac

and pulmonary involvement in CVL remain largely unknown. In the heart, alterations in cardiac rhythm, necrotizing polyarteritis, granulomas, and non-suppurative myocarditis have been reported (15-18). Respiratory alterations include dyspnea, rhinitis, mucocatarhal nasal discharge, epistaxis, stertorous breath sounds, and chronic interstitial pneumonitis (16,19-21). Secondary bacterial or fungal inflammatory processes can be observed in immunosuppressive states, and pneumonia acquires a suppurative aspect (22).

The present study was conducted to assess the cardiac and pulmonary alterations in symptomatic and asymptomatic dogs naturally infected with *L. chagasi*, as well as to evaluate the nature and extension of injury to these organs, associated with the infection and the clinical form of the disease.

## Material and Methods

Twenty-two privately owned or stray dogs from a Brazilian endemic area in Teresina (State of Piauí) and Timon (State of Maranhão) were included in this study. Male and female adult dogs of unknown ages and breeds were randomly tested for leishmaniasis (serology is mandatory in areas endemic for leishmaniasis) in an epidemiological survey performed by the Center for Zoonosis Control. Visceral leishmaniasis (VL) was diagnosed by positive anti-*Leishmania* serology combined with parasite detection. An indirect immunofluorescence assay or enzyme-linked immunosorbent assay was used for the detection of serum anti-*Leishmania* antibodies. For *Leishmania* detection, direct examination of Giemsa-stained smears of the skin, spleen and popliteal lymph nodes or culture of biopsy material collected from the sternal bone marrow, spleen, and/or popliteal lymph nodes (or all) in NNN medium (Sigma-Aldrich, USA) was performed. The animals were divided into two groups: a) 15 dogs infected naturally with *L. (L.) chagasi* with clinical signs of the disease and b) 7 dogs infected naturally with *L. (L.) chagasi* with no clinical signs of VL.

After clinical examination, the dogs were sacrificed with an overdose of thiopental sodium (33%, at a dose of 5 mL/kg, *iv*). All animal procedures were performed according to the Brazilian Guide for Care and Use of Laboratory Animals (Project #3.964/97 - <http://www.planalto.gov.br>). All experimental protocols were previously approved by the Research Ethics Committee of Universidade Federal do Piauí, Brazil.

Following autopsy, lung and heart samples were obtained for touch imprint cytology. Samples were stained with Giemsa for parasite count in 50 random fields under the light microscope using oil immersion at a magnification of 100X. Fragments of heart and lung tissues were fixed in 10% neutral formalin buffered with 10 mM sodium phosphate, pH 7.4, for histopathological and immunohistochemical analysis. The fixed tissues were cut into 5- $\mu$ m sections, stained

with H-E (23) and analyzed by light microscopy for lesion classification and amastigote detection. Immunohistochemistry was performed as previously described using a mouse polyclonal anti-*L. (L.) amazonensis* antibody produced by the Laboratory of Seroepidemiology and Immunobiology of the Tropical Medicine Institute, University of São Paulo (24). Briefly, the antibody was obtained from BALB/c mice infected with *L. (L.) amazonensis* amastigotes and tested on formalin-fixed and paraffin-embedded sections of hamster liver infected with *L. (L.) chagasi* (positive control). Specificity was confirmed on the same sections using serum adsorbed with *L. (L.) amazonensis* promastigotes when the reactions were negative. Serum was diluted 1:1.600 (v/v) in PBS and incubated overnight at 4°C in a humid atmosphere. Samples were assessed using a sensitive EnVision+, peroxidase system (Dako Corporation, USA, Code K 4001) following protocols provided by the manufacturer.

Quantitative and semi-quantitative analyses were performed to measure the distribution and intensity of lesions. Lesions were classified on a scale from 0 to 5. Data were analyzed statistically by the Mann-Whitney test. The significance level was set at  $P < 0.05$ .

## Results

Our results indicated that the most frequent clinical manifestations were local or generalized lymphadenopathy (73.3%), skin lesions (73.3%), fever (46.7%), weight loss (46.7%), apathy (26.7%), alopecia (20%), onychogryphosis (13.3%), conjunctivitis (13.3%), and anemia (6.7%). Clinical examination did not reveal any manifestations of cardiac or pulmonary alterations. Table 1 shows the parameters investigated in the heart and lungs of each dog.

Microscopic examination of cardiac tissue revealed the presence of mononuclear perivascular and intermuscular infiltrates (Figure 1A) of minimum to moderate intensity in both asymptomatic and symptomatic animals. In one animal, a subepicardial inflammatory infiltrate predominantly composed of mononuclear cells and fatty changes was observed. In some cases, fragmentation, hyaline degeneration and necrosis of cardiac muscle fibers were observed.

Microscopic analysis of pulmonary tissue revealed thickening of the interalveolar septa due to congestion, inflammatory infiltrates and epithelial cell and fibroblast proliferation, in both asymptomatic and symptomatic animals (Figure 1E). The infiltrates were composed of mononuclear cells with a predominance of macrophages or lymphocytes and a smaller number of fibroblasts and polymorphonuclear cells. Congestion of the alveolar wall was also present. Peribronchial and peribronchiolar inflammatory infiltrates were observed, composed of mononuclear cells with invasion of the basal membrane and lamina propria. Bronchiolar exudate, hydropic degeneration and squamous metaplasia of the bronchial and bronchiolar epithelia were also observed (Figure 1G) with loss of cilia, in addition to

thickening and hypertrophy of the peribronchial smooth muscles and thickening of the pleura. Hemosiderin was also present inside the cytoplasm of the macrophage. In one case, a thrombus was observed in a bronchial artery branch. Lesions of mild to severe intensity were mostly focal. In a few cases, lesions were diffuse. Overall, the lung had a more expressive inflammatory infiltrate than the heart (Table 1).

On cytologic examination, the presence of amastigotes in touch imprints of both the heart (Figure 1B) and lungs was an important finding (Figure 1F). Two of 15 symptomatic animals presented amastigotes in heart and lung imprints.

Amastigotes were found free in heart tissue. In the lungs, amastigotes were observed free and inside the cytoplasm of mononuclear cells of the interalveolar septa. None of the 7 asymptomatic animals showed amastigotes in lung imprints, but 1 animal exhibited leishmanias in heart tissue (Figure 1C).

The immunoperoxidase technique was performed only in 6 asymptomatic and 6 symptomatic dogs among the 22 dogs examined. Immunohistochemical analysis revealed amastigotes in the heart of only 1 of the 6 symptomatic animals examined. Only 1 was negative, and the remaining animals had minimum to moderate antigen levels (Figure

**Table 1.** Clinical and pathological parameters of the heart and lung of 22 seropositive dogs infected naturally with *Leishmania (L.) chagasi* and testing positive for the parasite.

Animals	Parameters								
	Clinical signs	Serology (IFI)	Parasitology	Cytology		IMH		Inflammatory infiltrate	
				Heart	Lung	Heart	Lung	Heart	Lung
1	Asymptomatic	1:160	Positive	0	0	ND	ND	++	++
2	Asymptomatic	1:320	Positive	0	0	+	-	++	++
3	Asymptomatic	1:160	Positive	0	0	-	+++	N	++++
4	Asymptomatic	1:40	Positive	0	0	-	++	+	++
5	Asymptomatic	1:80	Positive	0	0	+	++	+	+++
6	Asymptomatic	Negative	Positive	0	0	++	+	+	+++
7	Asymptomatic	1:80	Positive	1	0	+++	++	+	++++
8	Symptomatic	1:320	Positive	1	3	++	+++	++	++++
9	Symptomatic	1:80	Positive	0	0	+	-	++	++++
10	Symptomatic	1:320	Positive	0	0	+	++	+	+++
11	Symptomatic	Negative	Positive	0	0	-	++	+	+++
12	Symptomatic	Negative	Positive	0	0	+++	++	++	+++
13	Symptomatic	Negative	Positive	0	0	++	++	+	++++
14	Symptomatic	1:80	Positive	0	0	ND	ND	+	+++
15	Symptomatic	1:160	Positive	0	0	ND	ND	+	++++
16	Symptomatic	1:160	Positive	0	0	ND	ND	N	+++
17	vymptomatic	Negative	Positive	0	0	ND	ND	N	++
18	Symptomatic	Negative	Positive	0	0	ND	ND	+	++
19	Symptomatic	1:80	Positive	1	1	ND	ND	+	+++
20	Symptomatic	1:40	Positive	0	0	ND	ND	N	++++
21	Symptomatic	Negative	Positive	0	0	ND	ND	+	++
22	Symptomatic	ND	Positive	0	0	ND	ND	++	++

Serology (IFI) = indirect immunofluorescence. Positive parasitology was determined by the presence of leishmania amastigotes in smears of bone marrow and/or lymph node. Cytology scale was the number of amastigotes in 50 random fields under a light microscope using oil immersion at a magnification of 100X. IMH = immunohistochemistry. Scale used for IMH and inflammatory infiltrate: N = normal; + = minimum or dubious; ++ = mild; +++ = moderate; ++++ = severe-moderate; +++++ = severe; ND = not determined.

1D). Furthermore, there was evidence of antigens in the vessel endothelium. In asymptomatic animals, 2 tested negative and 4 showed minimum to moderate antigen levels. In the lung, amastigotes were detected in only 1 animal, the same with positive cytology among 6 symptomatic animals examined. Five of these animals had mild to moderate antigen levels (Figure 1H) and 1 animal tested negative. Nine animals were not tested. Among the asymptomatic animals, 5 had antigens varying from minimum to moderate in intensity and 1 animal was normal. The antigen was present in the heart myocytes and in phagocytes of the inflammatory infiltrate in the pulmonary interalveolar septa. Antigen was also present as particulate matter and found free in the interstitial space of these organs.

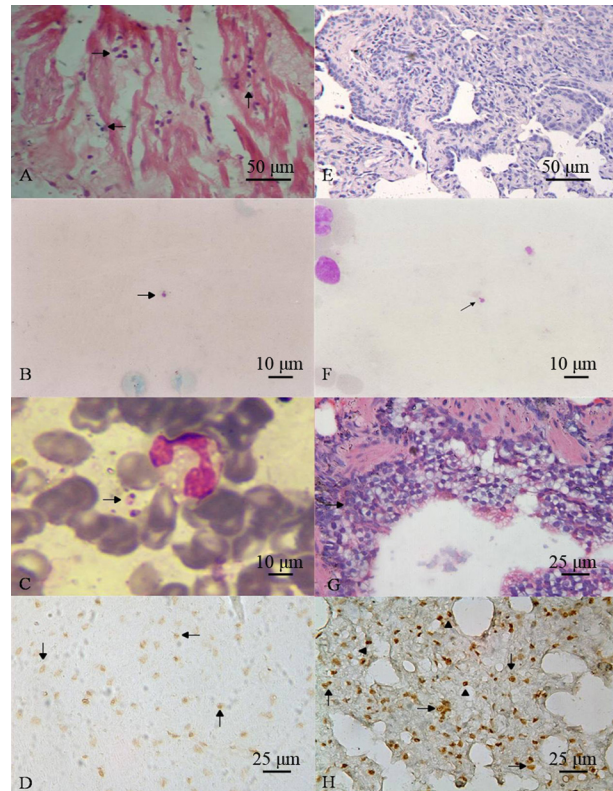
## Discussion

It is important to be aware of the changes in body organs caused by VL, especially organs that do not belong to the mononuclear phagocyte system, in order to understand the development of the disease. Canine VL runs a chronic course in most cases and affects several vital organs, such as the heart and lungs (16). Within the ethical considerations and limits of this type of research, it can be inferred that the study of heart and lung alterations in CVL is critical for understanding the problem in relation to humans. The dog serves as an excellent model for the study of VL in humans (25) since the disease is caused by the same agent that elicits similar alterations. Injury in dogs, therefore, can be considered a parameter to be used to evaluate the disease in humans. When the disease is diagnosed early, correct treatment may be instituted.

In the present study, the animals were probably in the chronic phase of the disease. Clinical manifestations ranged from infected animals without signs of the disease to animals with full-blown disease. However, it was not possible to determine the time-course of the disease, since the animals had been infected naturally by *L. (L.) chagasi*.

In VL, cardiac involvement seems to be a rare event and the clinical manifestations due to cardiac dysfunction are not always detected. Macroscopic lesions are not always evident, requiring histopathological analysis to reveal a severe myocarditis associated with vasculitis and infarction in infected animals (17). In our investigation, despite low-grade degeneration and myocardial necrosis, the low parasite load and the discreet inflammatory response affecting a small portion of the heart tissue were probably insufficient to produce macroscopic changes and clinical manifestations that could alter myocardial function. These results differ from the intense cardiac involvement reported by López-Peña et al. (18).

In VL, tissue parasite load appears to be important in the pathogenesis of the inflammatory reaction. The parasite stimulates cellular infiltrates that determine the intensity of local injury (26). Studies have shown that amastigotes



**Figure 1.** Histological sections from dogs infected naturally with *Leishmania (Leishmania) chagasi*. Heart tissue: A, Symptomatic dog. Mononuclear perivascular and intermuscular infiltrate (arrows). H-E staining (bar = 50  $\mu$ m); B, Symptomatic dog. Amastigotes (arrow). Giemsa staining (bar = 10  $\mu$ m); C, Asymptomatic dog. Amastigotes (arrow). Giemsa staining (bar = 10  $\mu$ m); D, Symptomatic dog. *Leishmania* antigen (arrows). Immunoperoxidase staining (bar = 25  $\mu$ m). Lung tissue (symptomatic dogs): E, Thickening of the inter-alveolar septa due to inflammatory infiltrate, epithelial cells and fibroblast proliferation. H-E staining (bar = 50  $\mu$ m); F, Amastigotes (arrow). Giemsa staining (bar = 10  $\mu$ m); G, Hydropic degeneration and squamous metaplasia of the bronchiolar epithelium (arrow). H-E staining (bar = 25  $\mu$ m); H, *Leishmania* antigen (arrows) and amastigotes (arrowheads). Immunoperoxidase staining (bar = 25  $\mu$ m).

may be involved in the degradation of the extracellular matrix component of the skin (27). This probably occurs in other tissues, such as the heart muscle, contributing to the development of tissue changes.

In CVL, myocarditis is characterized by an inflammatory reaction with lymphocyte, histiocyte and plasmocyte infiltrate, followed by necrosis and degeneration of cardiac muscle fibers (28). The presence of granulomas in the heart ventricle, necrotizing systemic vasculitis clinically expressed by diffuse hemorrhage in multiple organs, and thrombosis have also been observed (29-32). In the present study, however, this degree of myocardial injury was not found. Clinical and pathological manifestations of CVL seem to

differ in endemic regions and probably depend on intrinsic factors of the vector, host, parasite, and environment, which lead to different responses concerning the extension and severity of disease.

Furthermore, lesions in organs that do not belong to the mononuclear phagocyte system do not evolve in the same manner with disease progression. However, many animals may have clinical manifestations characteristic of fully expressed disease. This fact is observed with increased parasite load and tissue damage as the disease progresses to severely compromise the immune system, predisposing the animal to secondary infections. In this study, animals that exhibited a high parasite load in organs from the mononuclear phagocyte system had a low parasite load in the heart and lung tissues.

The morphology and mechanism by which visceral leishmaniasis determines pulmonary alterations are poorly understood, particularly in cases of persistent injury. In these cases, the parasite load is very low, similar to that observed in the present study. Interstitial pneumonia or pneumonitis is the most evident lung injury in CVL, but this injury is not clinically manifested in immunocompetent dogs (22). Resistance to injuries is quite peculiar in these species. In these immunocompetent animals, pulmonary structure is characterized by a lack of secondary lobulation, a thin pleura, well-developed respiratory bronchioles, sufficiently effective collateral ventilation, and thin-walled vessels, facilitating the resolution of pulmonary lesions (33).

The major pulmonary change observed in symptomatic and asymptomatic dogs with VL is thickening of the alveolar septa due to an inflammatory response consisting of mononuclear cells, fibroblasts and congestion. Several investigators have described these well-known alterations (22,34-36).

In endemic regions for VL, relatively frequent pulmonary lesions have been observed (Costa FAL, unpublished data), particularly in dogs originating from the city of Teresina. Autopsies were performed in the Animal Pathology Laboratory of Universidade Federal do Piauí. These alterations are different from the pattern of interstitial pneumonitis, which is easier to diagnose by microscopy. A lobar pneumonia pattern is present, as observed in animals developing full-

blown disease. We were unable to conclude that this injury was due to a direct action of the parasite. The initial lesion may have evolved as the result of the immunosuppression caused by VL or another concomitant infection (23).

In interstitial pneumonitis, the inflammatory infiltrate seems to have an important effect on the pathogenesis of injury (37). Inflammation may be caused by leishmania and maintained by continuous antigen stimulation. Antigens reach the lungs and are detected by the immunoperoxidase technique employed in the present study. Moreover, the role of the macrophage seems to be essential in the development of lesions. Constant stimulation causes cell hyperactivity, leading to tissue injury. Therefore, exudate and cellular fibrosis appear to be the basic mechanisms responsible for thickening of focal or diffuse interalveolar septa in canine VL, as previously suggested (22).

Immunohistochemistry is a more specific and sensitive technique for parasite detection (38,39) in tissue samples. However, only 1 animal in the current study showed amastigotes in heart and lung tissues. Animals examined by immunohistochemistry were fewer (6/15) than those studied by cytology (15/15).

Immunohistochemical analysis was fundamental to demonstrate an association between heart/lung injuries and leishmania infection. Amastigotes and leishmania antigens related to inflammatory infiltration and tissue injury were identified by this technique. In other studies, there was also evidence for the presence and involvement of the parasite as a causative agent in these lesions (17,28,36).

Thus, we suggest that veterinarians become aware of potential cardiac and pulmonary involvement in the course of VL. In the present study, the duration of infection was unknown and the animals showed no signs of clinical disease. However, microscopy detected lesions in these organs that were insufficient to cause manifestation of the disease.

Cardiac and pulmonary changes related to the presence of the parasite and tissue antigens were observed here in CVL. However, these alterations did not reflect the degree of disease progression, since infection was chronic. The presence of amastigotes and antigens was observed in both symptomatic and asymptomatic dogs.

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