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# Canine inflammatory myopathy associated with *Leishmania Infantum* infection

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ABSTRACT

Inflammatory myopathy associated with several infectious diseases occurs in dogs including those caused by *Toxoplasma gondii*, *Neospora caninum*, *Ehrlichia canis* and *Hepatozoon canis*. However, muscle disease due to *Leishmania* infection has been poorly documented. The aim of this study was to examine the distribution and types of cellular infiltrates and expression of MHC class I and II in muscle biopsies obtained from 15 male beagle dogs from a breeder group with an established diagnosis of leishmaniasis. Myopathic features were characterized by necrosis, regeneration, fibrosis and infiltration of mononuclear inflammatory cells consisting of lymphocytes, plasma cells and histiocytes. The predominant leukocyte populations were CD3+, CD8+ and CD45RA+ with lesser numbers of CD4+ cells. Many muscle fibers had MHC class I and II positivity on the sarcolemma. There was a direct correlation between the severity of pathological changes, clinical signs, and the numbers of *Leishmania* amastigotes. Our studies provided evidence that: 1) *Leishmania* should be considered as a cause of IM in dogs; 2) *Leishmania* is not present within muscle fibers but in macrophages, and that 3) the muscle damage might be related to immunological alterations associated with *Leishmania* infection. *Leishmania* spp. should also be considered as a possible cause in the pathogenesis of human myositis.



## 1. Introduction

The most common inflammatory myopathies (IMs) in humans are immune-mediated and fall into three major subsets: polymyositis (PM), dermatomyositis (DM) [1,2], and inclusion body myositis (IBM) [3]. Other, less common forms include focal myositis, infectious myositis, macrophaghic myofasciitis (MMF) [4] and IM with abundant macrophages (IMAM) [5]. Similar to human IMs, canine IMs are a heterogeneous group of disorders characterized by infiltration of inflammatory cells into muscle [6]. The most common immune-mediated IMs in dogs include the highly specialized masticatory muscle myositis (MMM); polymyositis that resembles PM in humans; and extraocular myositis. Dermatomyositis occurs in certain breeds and is associated with characteristic skin, muscle, and vascular lesions, as in humans [7]. A canine equivalent of human IBM has not yet been found.

In dogs, IMs can also be associated with infectious diseases caused by *Toxoplasma gondii*, *Neospora caninum*, *Ehrlichia canis* or *Hepatozoon canis* [7]; however, there are few reports documenting an association of IMs with *Leishmania* infection [8]. Zoonotic visceral leishmaniasis, caused by the protozoan parasite *Leishmania infantum* (syn: *L. chagasi*), is a sand fly-borne disease found in the Mediterranean area, Asia, and Latin America [9]. In most of this range, the domestic dog is the main reservoir host. Dogs may suffer from a severe disease characterized by chronic evolution of viscero-cutaneous signs, which occurs in approximately 50% of infected animals, as shown by cross-sectional as well as longitudinal studies [10]. Canine leishmaniasis is a major veterinary and public health problem in traditional areas of endemicity, but also in areas where the disease is not endemic such as in the United States and Canada [11,12] and northern Europe [13] with only occasional reports of outbreaks. To evaluate the possibility of *Leishmania* involvement in canine IMs, we studied muscle biopsies from dogs with an established diagnosis of leishmaniasis and clinically evident muscle disease.

## 2. Materials and Methods

### 2.1. Animals and samples

Fifteen male 2 year old beagle dogs showing clinical signs of muscle weakness and atrophy, and living in a breeder group in an endemic area for *Leishmania* in southern Italy, were selected

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for the study (Table 1). All dogs had an established diagnosis of naturally-occurring leishmaniasis by serological and parasitological methods [14]. Clinical signs and laboratory findings characteristic of systemic leishmaniasis infection were also found in all dogs, such as weight loss, lymphadenopathy, exfoliative dermatitis and hypergammaglobulinemia. For comparison, 3 control dogs from the same breeder group without clinical or laboratory evidence of leishmaniasis, nor signs of muscle disease, were included. These dogs were serologically and parasitologically negative for *L. infantum* (Table 1).

Biopsy specimens from the biceps femoris muscle were collected, frozen in isopentane pre-cooled in liquid nitrogen and stored at −80 °C until further processed. Sections (8 μm) were stained by the following histological and histochemical techniques: haematoxylin and eosin (HE), Engel trichrome [15], periodic acid-Schiff (PAS), cytochrome C oxidase (COX), succinate dehydrogenase (SDH), reduced nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), and the myofibrillar ATPase reactions at pH 9.4 and 4.3. Scoring of the major morphological alterations observed in the biopsy was performed and summarized in Table 1. The distribution of the inflammatory cells within muscle biopsies was classified as scattered, focal, multifocal and diffuse. The number of necrotic fibers in the biopsy was classified as 1+ (1 to 5); 2+ (5–10); 3+ (>10). The amount of fibrosis observed in the endomysium was reported as slight, moderate, or prominent.

### 2.2. Immunohistochemical examination

Frozen muscle specimens were sectioned (8 μm), fixed in acetone at 4 °C for 5 min, then blocked for endogenous peroxidase in 0.3% $H_2O_2$ in methanol for 20 min. Muscle sections were then incubated with carefully selected and well-characterized mouse monoclonal antibodies against canine leukocyte antigens [16, diluted 1:50] as described in Table 2. Additional incubations were performed with monoclonal antibodies against canine MHC class I (1:100, clone H58A, VMRD, Inc., USA) [17] and canine MHC class II (1:50, clone H34A, VMRD, Inc., USA) [16], and with a specific rabbit antiserum raised against *L. infantum* (gift from Dr. Mercedes Domínguez, Servicio Immunologia, Instituto de Salud Carlos III, Madrid, Spain) diluted 1:100, for 2 h at room temperature. Slides were washed three times with PBS, then incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies and labeled with streptavidin biotin (LSAB kit; DakoCytomation, Denmark) for 30 min, followed by incubation with streptavidin conjugated to horseradish peroxidase (LSAB Kit; DakoCytomation, Denmark). Color development was obtained following 5–20 min of diaminobenzidine (DakoCytomation, Denmark) treatment. Sections were counterstained with Mayer's haematoxylin. In the corresponding negative control section, the primary antibody was either omitted or replaced with normal serum.

### 2.3. Immunofluorescence and confocal laser scanning microscopy

Muscle sections were air dried, blocked with normal goat serum diluted 1:10, then rinsed in 0.01 M PBS (pH 7.4) containing 0.2% Triton X-100 and 0.1% bovine serum albumin. Sections were then incubated with either the primary antibody against canine CD8 antigen for 24 h at 4 °C or the primary antibody against *Leishmania* spp., applied overnight at room temperature, both diluted 1:50 in 0.01 M PBS with 20% normal serum. Sections with the primary antibody against CD8 antigen were then washed in 0.01 M PBS followed by incubation with goat anti-mouse IgG-Fab fragment conjugated to TRITC fluorochrome (1:50, Jackson Laboratories) for 1 h at room temperature. For double immunofluorescence staining, fluorochrome conjugated Fab fragments were used (Jackson Laboratories) to avoid antibody cross-reaction using two primary mouse antibodies, according to the method previously described [17]. The sections were rinsed again in PBS and incubated with the second primary antibody against canine MHC class I (diluted 1:50 in 0.01 M PBS with 20% normal rabbit serum) for 24 h at 4 °C. After rinsing in 0.01 M PBS, the sections were treated with affinity-purified rabbit anti-mouse IgG-Fab conjugated to FITC fluorochrome (1:50; Jackson Laboratories), for 1 h at room temperature. Finally, the sections were rinsed with PBS and mounted with glycerin diluted in PBS 1:1. Sections incubated with primary antibody against *Leishmania* spp., were washed three times in PBS and then incubated with FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA) at 1:200 dilution for 2 h at 4 °C. Slides were then rinsed in PBS and incubated for 30 minutes at room temperature with 1 μg/mL propidium iodide (Sigma, Milan, Italy). They were washed again and mounted with an aqueous mounting medium (Sigma, Milan, Italy). Controls for staining specificity included omission of the primary antibody or its replacement with non-immune sera or irrelevant antibody.

The number of immunolabeled amastigotes was scored from 0 to 3 + (0 = no amastigotes detected, 1 + = small number of amastigotes [1–3 amastigotes or macrophage-containing amastigotes per higher magnification microscopic field], 2 + = moderate number of amastigotes [4–10 amastigotes or macrophage-containing amastigotes per higher magnification microscopic field], and 3 + = large number of amastigotes [>10 amastigotes or macrophage-containing amastigotes per higher magnification microscopic field]).

### 2.4. Scanning and photography

For scanning and photography, a confocal laser scanning microscope LSM-510 (Zeiss, Gottingen, Germany) was used. FITC was irradiated at 488 nm and detected via a 505–560 nm band pass filter. TRITC was irradiated at 543 nm and detected with a 560 long pass filter. Two-channel frame-by-frame multi-tracking was used to avoid cross-reacting signals. The different frames were scanned separately, with appropriate installation of the optical path for excitation and emission of each scan according to the manufacturer's instructions.

### 2.5. Laser capture microdissection of muscle fibers

Tissue sections (4–20 μm thick) were cut and mounted directly on to glass slides for isolation by laser microdissection, or mounted on cross linked polyethylene (PEN) foil attached to a glass slide or to a carrying frame (Leica Microsystems GmbH, Wetzlar, Germany) for laser microdissection. Single muscle fibers (approximately 1500 from each case) were isolated from muscle tissue samples and microdissected with the Leica Laser Microdissector (Leica Microsystem, Milan, Italy). Only the sarcoplasm was collected and stored at −80 °C, until further processed.

### 2.6. Real time PCR

DNA extraction from tissue samples and from parasite cultures was carried out as previously described [18]. All TaqMan primers and probes were selected as previously reported and provided from Applied Biosystems. [18]. Reactions were carried out in a final volume of 25 μl of reaction mixture containing 1X TaqMan Universal Master Mix (Applied Biosystems), 100 pmol/μl of the specific primer, 10 pmol/μl of the labeled probe, and 50 ng of DNA. The thermal cycling conditions included an initial incubation for 2 min at 50 °C, followed by a 10 min denaturation at 95 °C, and 45 cycles at 95 °C for 15 s and 60 °C for 1 min each. Each standard and sample, and a negative control were analyzed in triplicate for

**Table 1**
Signalment, clinical signs, histological findings and amastigotes detected in all studied dogs.

| Dog # | Sex | Clinical signs | IFAT titles | CK (U/l) (<100) | Distribution of inflammatory cells | Myonecrosis[a] | Endomisial fibrosis | Amstigotes detected[b] |
|---|---|---|---|---|---|---|---|---|
| 1. | M | Progressive exercise intolerance, symmetric proximal muscle weakness, severe muscle atrophy | 1:2560 | 523 | Diffuse | 3+ | Moderate | 3+ |
| 2. | M | Progressive exercise intolerance, symmetric proximal muscle weakness, severe muscle atrophy | 1:5120 | 439 | Diffuse | 3+ | Prominent | 3+ |
| 3. | M | Symmetric proximal muscle weakness, moderate muscle atrophy | 1:5120 | 520 | Diffuse | 3+ | Moderate | 3+ |
| 4. | M | Symmetric proximal muscle weakness, moderate muscle atrophy | 1:5120 | 510 | Diffuse | 3+ | Slight | 3+ |
| 5. | F | Symmetric proximal muscle weakness | 1:2560 | 412 | Multifocal | 3+ | Slight | 3+ |
| 6. | M | Progressive exercise intolerance, symmetric proximal muscle weakness, moderate muscle atrophy | 1:5120 | 339 | Multifocal | 3+ | Slight | 3+ |
| 7. | F | Mild muscle weakness | 1:5120 | 240 | Multifocal | 3+ | Slight | 2+ |
| 8. | F | Mild muscle weakness | 1:2560 | 149 | Multifocal | 2+ | Slight | 2+ |
| 9. | F | Mild muscle weakness | 1:5120 | <100 | Focal | 2+ | Moderate | 2+ |
| 10. | M | Symmetric proximal muscle weakness, moderate muscle atrophy | 1:5120 | <100 | Focal | 2+ | Moderate | 2+ |
| 11. | F | Symmetric proximal muscle weakness | 1:2560 | <100 | Focal | 2+ | Slight | 2+ |
| 12. | M | Mild muscle weakness | 1:1280 | <100 | Focal | 2+ | Slight | 2+ |
| 13 | M | Mild muscle weakness | 1:640 | <100 | Scattered | 1+ | Slight | 0+ |
| 14. | F | Mild muscle weakness | 1:1280 | <100 | Scattered | 1+ | Slight | 0+ |
| 15. | F | Mild muscle weakness | 1:5120 | <100 | Scattered | 1+ | Slight | 1+ |
| 16. | F | Normal | negative | <100 | Absent | 0 | Absent | 0+ |
| 17. | M | Normal | negative | <100 | Absent | 0 | Absent | 0+ |
| 18. | M | Normal | negative | <100 | Absent | 0 | Absent | 0+ |

M, male; F, female; CK, creatine kinase; IFAT, indirect fluorescent antibody test for *Leishmania* spp. test.
[a] For method of grading, see Materials and Methods.
[b] For method of grading, see Materials and Methods.

each run. The efficiency of the amplification was close to 1. Cycle threshold (Ct) value was calculated for each sample by determining the point at which the fluorescence exceeded the threshold limit. To normalize for differences in efficiency of sample extraction, we used ß-actin as a housekeeping gene [18].

### 2.7. Statistical analysis

Data were analyzed using Microsoft Excel for Windows. Owing to the small sample size, the Fischer test was used to test for significance.

## 3. Results

### 3.1. Normal control dogs

The muscle specimens from healthy control beagles, serologically and parasitologically negative for *L. infantum*, were histologically normal without obvious cellular infiltrations. MHC class I and class II expression was restricted to endothelial cells of arterioles, venules, and capillaries. As expected, no leishmanial DNA was detected in muscle tissue.

### 3.2. L. infantum-infected dogs

Pathological changes in muscle from all affected dogs were similar, although they varied in severity (Table 1). H&E and Engel Trichrome staining of muscle from affected dogs showed variable numbers of mononuclear cells, represented by lymphocytes and some macrophages, with an endomysial and perimysial distribution (Figs. 1A and 2B). A perivascular pattern of cellular infiltration was evident in the perimysium. In the endomysium, where cellular infiltration was marked, it was difficult to discern whether the infiltration accumulated around blood vessels. Various stages of myonecrosis and phagocytosis were observed in all studied cases and invasion of non-necrotic muscle fibers by inflammatory cells was also observed. Marked variation in fiber size including atrophic and hypertrophic fibers, and fibers with a basophilic cytoplasm suggestive of muscle regeneration, were also found. With the ATPase reaction at pH 4.3, a few type 2C fibers were detected, indicating muscle regeneration. Furthermore, we observed mild to prominent proliferation of connective tissue consistent with fibrosis. In H&E and Engel Trichrome stains, it was very difficult to identify *Leishmania* amastigotes due to the poor morphologic details using frozen sections.

**Table 2**
Monoclonal antibodies used for leukocyte classification.

| Canine antigen | Leukocyte expression |
|---|---|
| CD3 | T lymphocytes (from P. Moore, University of California, Davis) |
| CD4 | MHC class II-restricted cells, T, tissue macrophages, granulocytes (from P. Moore, University of California, Davis) |
| CD45 | Leukocyte common antigen (from P. Moore, University of California, Davis) |
| CD45RA | Restricted isoform of CD45 (naive CD4 T-cells, resting activated CD4 T-cells, CD8 T-cells, B-cells, mast cells, NK cells) (from P. Moore, University of California, Davis) |
| CD18 | All blood leukocytes (from P. Moore, University of California, Davis) |
| CD8α, CD8β | MHC class I – restricted cells; cytotoxic T lymphocytes (from P. Moore, University of California, Davis) |
| MHC I | MHC class I clone H58A. from VMRD, Inc., USA |
| MHC II | MHC class II clone H34A. from VMRD, Inc., USA |

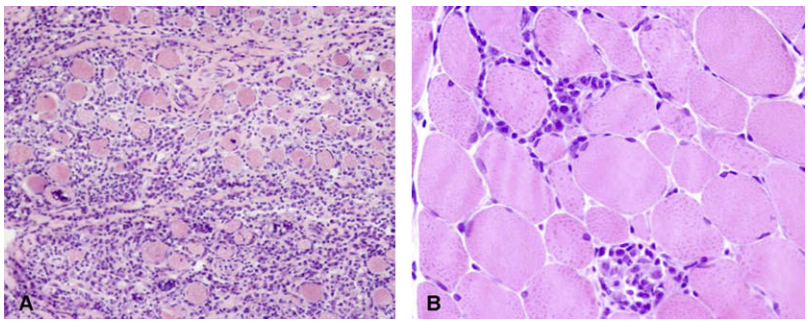


**Fig. 1.** Histological features of canine myositis associated with *Leishmania infantum* infection. A. Severe and diffuse infiltration of mixed mononuclear cells, with only few remaining histologically normal fibers. (Dog 1). B. Different histological pattern of canine myositis associated with *Leishmania infantum* infection with scattered clusters of inflammatory cells that appear to outline the circular remnants of myofibers that have undergone necrosis. (Dog 9) (H&E stain, Original magnification X10 for A and X20 for B).

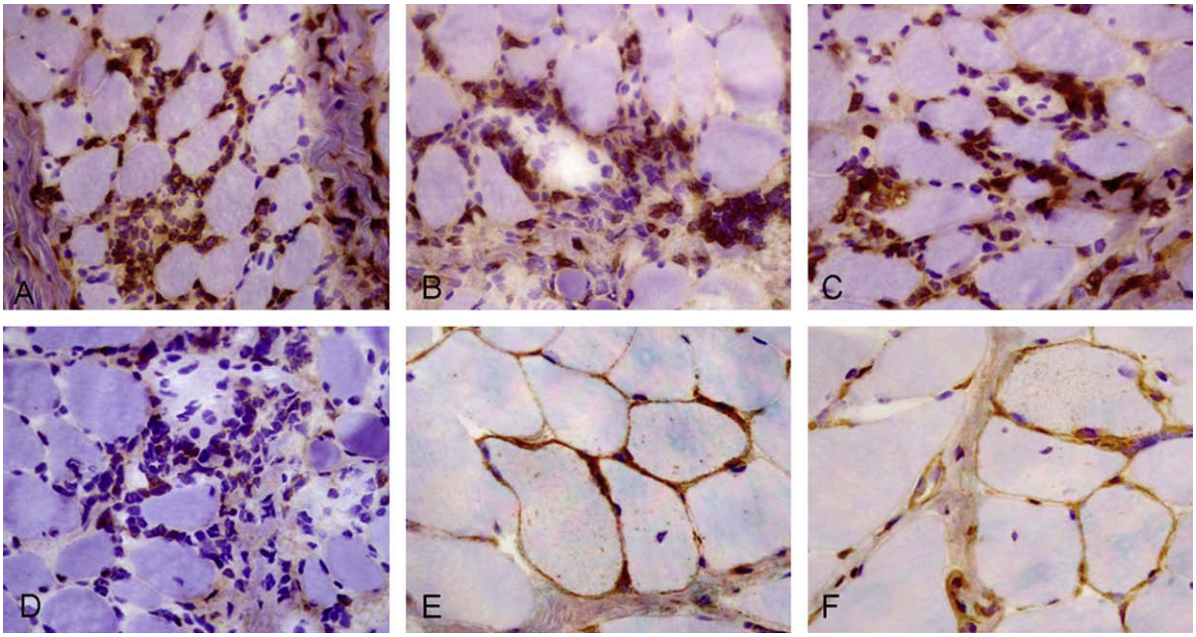


**Fig. 2.** Immunohistochemical staining of muscle for phenotypes of infiltrating cells and MHC identification. A. T-cells (CD3+) were the most frequently encountered cells with an endomysial distribution. B. CD8+ cells located around a blood vessel. C. CD45 positive cells and D. CD45RA positive cells. Expression of both MHC class I (E) and MHC class II (F) was present on muscle fibers in the presence or absence of cellular infiltration. (Avidin-biotin-peroxidase complex method with Mayer's haematoxylin counterstain, Original magnification X20).

*3.2.1. Immunohistochemistry and confocal laser scanning microscopy*

Lineages of inflammatory cells were identified in the biopsies based on staining patterns of monoclonal antibodies against cell-surface proteins. All leukocytes were identified with the antibody against CD45 (leukocyte common antigen). In all cases, the predominant cell populations were CD3+, CD8+, CD4+, and CD45RA+ positive with an endomysial, perimysial, and in some cases perivascular distribution (Fig. 2A–D). CD8+ T-cells were present in greater numbers than CD4+ cells. Only a few cells were CD18+ and were localized mainly in the perivascular areas.

Vascular adventitia, endothelial cells, and cellular infiltrates within the perimysium and endomysium stained intensely for MHC class I and MHC class II antigens. In addition, many muscle fibers had MHC class I and class II positivity on the sarcolemma (Fig. 2E, F). CD8+ T lymphocytes invaded histologically healthy muscle fibers expressing MHC class I antigens (Fig. 3 demonstrates the CD8/MHC-I complex [19]).

Immunoreactivity to *Leishmania* amastigotes was observed in macrophages within the inflammatory cell infiltrate and in macrophages within the connective tissue adjacent to muscle fibers (Fig. 4). The distribution of immunostaining was variable and correlated with the inflammatory changes. In fact, the highest number of amastigotes was found in the biopsies with diffuse or multifocal inflammation ($P < 0.001$). It was not possible to identify any immunoreactivity to *Leishmania* amastigotes in only two clinical cases (dogs 13 and 14). Furthermore, amastigotes were not found within single muscle fibers in any of the clinical cases or detected by immunofluorescence in any of the muscle biopsies from seronegative dogs.

*3.2.2. Leishmanial DNA detection in muscle tissue*

Leishmanial DNA was detected in muscle tissue samples from all infected dogs in variable amounts (Fig. 5). The amount of *Leishmania* DNA correlated well with the severity of inflammation in the muscle biopsies ($P < 0.05$). By comparison, no leishmanial DNA was amplified from the sarcoplasm of the isolated muscle fibers.

## 4. Discussion

Here we demonstrate that skeletal muscle from dogs with established *L. infantum* infection showed pathological features of an inflammatory myopathy, and interestingly, the parasite load

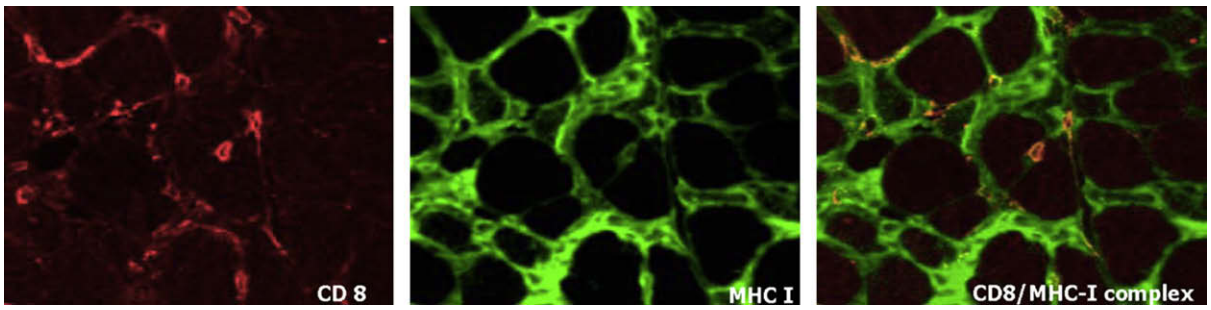


**Fig. 3.** Double immunofluorescence staining analyzed by confocal laser scanning microscopy showed co-localization of MHC class I and CD8. A. CD8+ cells within a muscle fiber (red fluorescence). (B) Localization of MHC class I to the muscle fiber membrane (green fluorescence). Overlapping images demonstrated co-localization of MHC class I and CD8+ antigen (C, yellow color) (Original magnifications X100 for all figures).

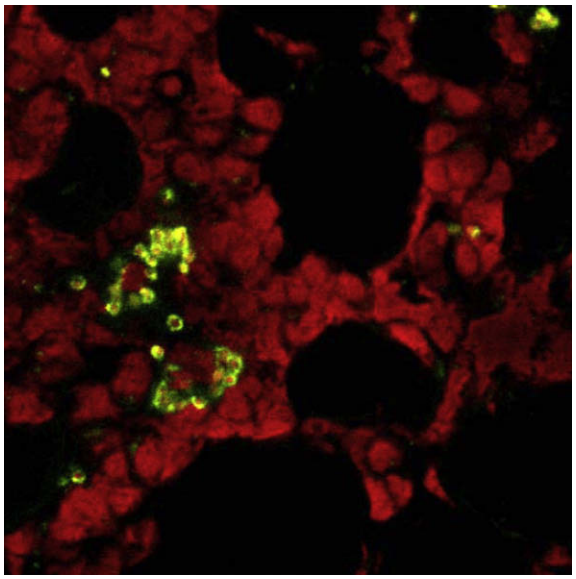

**Fig. 4.** Double immunofluorescence staining analyzed by confocal laser scanning microscopy. Numerous *Leishmania* amastigotes in the inflammatory infiltrate (green fluorescence). (Original magnifications X 400. Nuclei counterstained with propidium iodide).

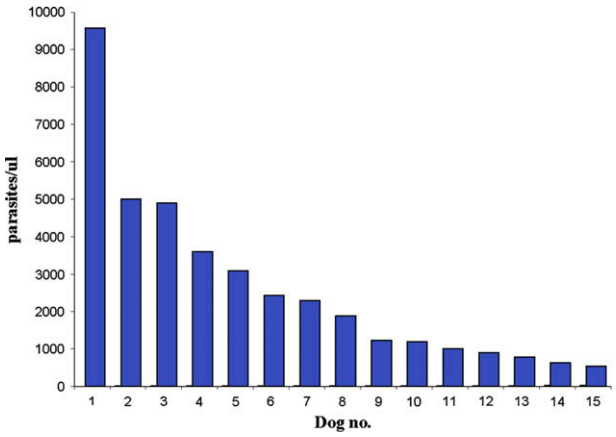


**Fig. 5.** Quantification of leishmanial DNA in muscle tissue samples from infected dogs.

in the muscle and the amount of *Leishmania* DNA correlated well with the severity of inflammation. This finding could be associated with the dissemination of *Leishmania* amastigotes within macrophages to well vascularized tissues such as muscle following an infection where they may act as a factor triggering the inflammatory response.

Pathological changes in our cases were similar to those of a previously published report [8] and included necrosis, fibrosis and infiltration of mononuclear inflammatory cells consisting of lymphocytes and histiocytes. However, *Leishmania* amastigotes were reported within macrophages and myofibers, which differ from our findings. Considering that *Leishmania* lacks the capacity to penetrate muscle cells, we studied single muscle fibers looking for leishmanial DNA in the sarcoplasm. As expected, no leishmanial DNA was amplified from the sarcoplasm of the isolated muscle fibers. Consistent with biological features of the organisms and conventional tissue microscopy, we have confirmed that *Leishmania* parasites cannot enter muscle fibers.

The predominant infiltrating cell types within the muscle biopsies were T-cells and macrophages. Macrophages were identify morphologically because they were larger, had more cytoplasm than lymphocytes, contained *Leishmania* amastigotes within the cytoplasm and were CD4 positive. The localization of CD8+ T-cells within the endomysium, and the presence of MHC class I and II antigens on muscle fibers, are also characteristic of the diagnosis of polymyositis in dogs [17]. Furthermore, the predominant localization of infiltrates in the endomysium rather than in perivascular areas and the frequent occurrence of CD8+ T-cells invading non-necrotic muscle cells, all of which express MHC class I (CD8/MHC-I complex), are also characteristic feature of PM. However, an infectious agent was demonstrated in our cases which differs from the current classification of idiopathic immune-mediated PM in humans and in dogs [20]. It is of interest that a similar pattern of cellular infiltrations and MHC upregulation is found in both infectious and idiopathic autoimmune myositis.

For the first time, our data provides a detailed antigenic characterization of infiltrating mononuclear cells in myositis associated with *Leishmania* infection. CD45RA+CD8+ T-cells are involved in production of IFN-gamma and IL-10 in response to *Leishmania* antigens [21]. It has been reported that during canine visceral leishmaniasis, there is a decrease of CD45RA+CD8+ T-cell numbers among peripheral blood mononuclear cells. An increase in the numbers of this lymphocyte subset has been used as a marker of efficacy of specific immunochemotherapy against *L. infantum* infection in dogs [22]. Our finding of CD45RA+ cells infiltrating the muscle fibers may suggest a redistribution of these cells from blood to the skeletal muscle. The CD8+ lymphocytes have been linked with protective immunity, activation of macrophages and lysis of *Leishmania* infected cells, and nitric oxide production and killing of intracellular amastigotes [23].

In a study of asymptomatic experimentally infected dogs, lymphocytes produced interferon-gamma upon parasite antigen-specific stimulation, whereas lymphocytes from symptomatic dogs did not. The T-cell lines were generated by restimulation *in vitro*

with parasite soluble antigen and irradiated autologous lymphocytes as antigen-presenting cells [24]. The study showed that lysis of infected macrophages by T-cell lines was MHC restricted. Characterization of parasite-specific cytotoxic T-cell lines revealed that the responding cells were CD8+. However, in some animals, CD4+ T-cells that lysed infected macrophages were also found. In contrast to asymptomatic dogs, lymphocytes from symptomatic dogs failed to proliferate and produce interferon-gamma after *Leishmania* antigen stimulation *in vitro* and were not capable of lysing infected macrophages [24]. This and other studies (for review see [23]) led to the hypothesis that CD8+ T-cells are involved in resistance to canine leishmaniasis. The presence of CD8+ T-cells found on our muscle biopsies may at least partially explain the relatively large number of dogs with normal serum creatine kinase activities and mild muscle weakness, even in cases with a relatively large amount of *Leishmania* DNA and immunofluorescence antibody test (IFAT) titers. Further, CD18 antigen immunoreactivity is characteristic of leishmaniasis. *Leishmania* promastigotes interact with macrophages through the association of multiple membrane surface receptors. Macrophage complement receptor CR3 (CD11b/CD18 or Mac-1) has been implicated in the interaction of serum-opsonized promastigotes with murine, human and canine macrophages [25] .

The presence of *L. infantum* in inflammatory muscle biopsies confirmed by immunofluorescence and PCR analysis, strongly suggests that this infection may be responsible for myositis in dogs. Accordingly, further experiments are needed to confirm that *Leishmania* was the only factor that induced such muscle damage in our dogs. Our preliminary experimental study in a hamster model is consistent with this hypothesis [26]. Numerous investigations have been devoted to the search for environmental factors that could trigger the onset of autoimmune diseases. There are a number of examples where the etiological role of an infectious factor has indeed been demonstrated (for review see [27]). It remains true, however, that in most cases, an infectious etiology has not been directly demonstrated even though several indirect arguments are strongly suggestive [27].

Several mechanisms can be proposed to explain the role of infectious factors as a trigger for autoimmune disease. The first hypothesis involves polyclonal B- or T-cell activation. It would imply, as far as autoreactive T-cells are concerned, that one should find no or few somatic mutations in the autoantibody gene segment corresponding to complementarity determining regions (CDR), since autoantigen-driven selection is not a primary event in this setting. This is in fact rarely the case, with the exception of some forms of systemic lupus erythematosus [28]. It is possible, however, that the major B- and T-cell activation which is observed in some diseases, notably viral and parasitic diseases, such as *Leishmania*, may explain some autoimmune states. It has been reported that *Leishmania* infection may mimic systemic lupus erythematosus [29]. B-cell responses in visceral leishmaniasis are exemplified by hypergammaglobulinemia and anti-*Leishmania* antibodies. Some of those antibodies (e.g. anti-dsDNA, ANA) are lupus erythematosus- associated autoantibodies (details in [29]).

The second mechanism is antigen mimicry. It has been noted that the protein sequence of a number of bacterial or viral proteins is homologous with autoantigen sequences [30,31]. However, such homologies often do not show definite evidence for a possible role for shared antigenic determinants between the infectious factor in question and the autoantigen. For these reasons, it is important to collect more direct evidence on the responsibility of the shared epitope in question. Such evidence has only been obtained in a very limited number of diseases [27].

The third hypothesis is the increased immunogenicity of organ autoantigens secondary to infection-mediated inflammation. A number of infectious agents induce localized inflammation of the target organ. This is notably the case for a wide spectrum of viruses [27]. This information may be at the origin of an organ-specific autoimmune response which will enhance and perpetuate the inflammation. Some experimental models support this mechanism. For example, in Theiler's disease, the infection initially provokes a virus-specific encephalomyelitis associated with T-cell reactivity to viral proteins [32]. However, within a few weeks, the virus-specific immune response is replaced by a bona fide autoimmune response, including myelin-basic protein (MBP) and proteolipid protein (PLP)-specific T-cell reactivity. It is this autoimmune response which is at the origin of the chronicity of the disease. Similarly, infection of mice with the Coxsackie B3 virus induces a long-term myocarditis which develops in two phases, the first of which is viral and the second autoimmune [33]. In these models, it is assumed that the initial virus-induced inflammation triggers overexpression of molecules participating in autoantigen recognition by T-cells. These molecules include MHC molecules (class I and II), and co-stimulatory and adhesion molecules. However, no direct demonstration of such a mechanism has been made in human autoimmune diseases, possibly due to the difficulties in identifying etiological agents.

## 5. Conclusions

Our studies provide evidence that: 1) *Leishmania* should be considered as a possible cause of canine IM; 2) *Leishmania* is not present within muscle fibers; 3) *Leishmania* amastigotes in the muscle may act as a causing factor triggering the inflammatory response; and 4) the muscle damage might be related to immune-mediated mechanisms associated with *Leishmania* infection.

We suggest that *Leishmania* spp. infection has to be considered in the differential diagnosis of IMs in dogs, and accordingly, *Leishmania* spp. should also be considered as a possible cause of human myositis. Such comparative studies would be important for understanding disease pathogenesis and may identify new therapeutic options including anti-infectious agents, chemicals, monoclonal antibodies and vaccination.

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