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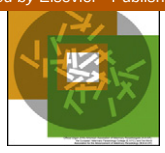

Evaluation of miltefosine for the treatment of dogs naturally infected with *L. infantum* (=*L. chagasi*) in Brazil

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ABSTRACT

Dogs naturally infected with *Leishmania Infantum* (=*L. chagasi*) were treated with miltefosine using different therapeutic regimens. The animals were evaluated for clinical evolution, biochemical parameters, parasite load (by real-time PCR), cytokine levels and humoral response. After treatment and during the following 24 months, there was progressive clinical improvement and complete recovery in 50% (7/14) of the treated animals. There was a decrease in the smear positivity of the bone marrow after treatment, and there was also a gradual and constant decrease in positive cultures at the end of the follow-up period. However, the PCR detection of parasite DNA remained positive. In general, all animals presented a significant increase in parasite load 6 months after treatment. The IFN-γ levels in all the groups tended to increase during follow-up period, regardless of the miltefosine dose administered. The IL-4 and IL-10 levels of the animals tended to decrease during follow-up, except after 300 days when only IL-10 increased. The serum antibodies identified antigens that ranged from 116 kDa to less than 29 kDa in the Western blot assay. Furthermore, 300 days after treatment, qualitative and quantitative differences in the antigen profiles were observed. Antigens of 97 and 46 kDa were the most intensely recognized. Higher levels of antigen-specific *Leishmania* IgG were detected before and 300 days after treatment in all groups. Taking together, the improvement in the clinical symptoms was not followed by parasitological clearance, suggesting that treatment with miltefosine is not recommended, especially in endemic areas like Brazil, where children are the major victims and dogs are involved in the maintenance of the parasite cycle.

1. Introduction

Leishmaniasis infects approximately 12 million individuals in 88 countries, and 350 million people are at risk of contracting the infection (http://www.who.int/en/). Depending on the species of *Leishmania* and the immune response of the host, there are several clinical manifestations of the disease: cutaneous, mucocutaneous, diffuse, and visceral. Visceral leishmaniasis (VL), a more severe disease, is considered to be an anthroponosis in India and Central Africa and a zoonosis in the Mediterranean and the Americas. The zoonotic VL caused by *L. infantum* (also known as *L. chagasi*) accounts for 20% of the global human visceral leishmaniasis (100,000 cases per year), and its incidence is increasing in urban and peri-urban areas of the

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tropics (Dye, 1996). The domestic dog is considered to be the primary reservoir and plays a central role in transmitting the parasite to humans and sand flies.

Brazil has the highest incidence of zoonotic VL in the world, with about 3000 new cases per year. Moreover, the number of areas with incidences that favor a widespread epidemic, which could affect young people or individuals with co-morbidities and cause a high number of deaths, is increasing. The mortality rate of VL has increased from 3.6% in 1994 to 6.7% in 2003, which represents an inflation of 85% (Ministério da Saúde, 2006). The control measures adopted include the diagnosis of canine and human cases, which is followed by the treatment of infected humans and euthanasia of infected dogs. The euthanasia of animals is a controversial measure, and alternatives for treatment are highly desirable. There is, however, a lack of obvious alternatives for the treatment of human and canine VL.

Miltefosine has just become available in India for the treatment of human VL. This drug is an alkyphospholipid that was originally developed as an oral antineoplastic agent (Sundar et al., 1998). Miltefosine is a simple, very stable, relatively safe, and highly efficient molecule. Currently in clinical trials, it has become the first oral drug available for the treatment of human visceral and cutaneous leishmaniasis. Miltefosine is toxic for *Leishmania* parasites, and it enhances both T cell and macrophage activation and the production of microbicidal reactive nitrogen and oxygen intermediates (Soto et al., 2006).

Few studies have been conducted using miltefosine for the treatment of Canine Visceral Leishmaniasis (CVL). Recently, it was shown that treatment with miltefosine alone reduced *Leishmania* replication, and the parasite was eradicated from the blood, although it was not removed from the lymph nodes (Manna et al., 2008a). This phenomenon may be a potential reason for the recurrence of the disease. Furthermore, clinical information on the efficacy and action of miltefosine for the treatment of canine leishmaniasis is limited.

The objective of the current study was to treat dogs naturally infected with *L. Infantum* in Brazil endemic area using different protocols. The animals were evaluated for clinical evolution, biochemistry, parasite load (by real-time PCR), cytokine levels and humoral response. To the authors' knowledge, this is the most complete study concerning the treatment of CVL with miltefosine in Americas.

2. Materials and methods

2.1. Animals

The study protocol was approved by the Research Ethics committee of the Federal University of Piauí.

Fourteen mongrel animals (*Canis familiaris*) of unknown ages were diagnosed by seroepidemiology for CVL, which was performed during the control program by the Zoonoses Control Center of the municipality of Teresina, Piauí, Brazil. The animals were diagnosed with an indirect immunofluorescence assay (IFA). The seropositive dogs were donated to the kennel of the Federal University of Piauí to be used in the experiments.

The diagnosis of *Leishmania* infection was confirmed by serological tests (IFA—cut off 1:80), conventional PCR and the detection of amastigotes in smears of bone marrow. Before beginning the treatment schedule, the dogs were treated for parasites (endo and exo) and clinically inspected for other possible co-infections (Babesiosis, Erlichiosis and dermatosis). They were kept in a kennel protected by an anti-sand fly screen with water and food "ad libitum" during the treatment and throughout the follow-up period.

Three different miltefosine treatment schedules were administered as described below:

Group 1 ($n = 5$): 100 mg miltefosine/animal, administered daily over a period of 28 days.
Group 2 ($n = 5$): 200 mg miltefosine/animal, administered daily for 28 days.
Group 3 ($n = 4$): 100 mg miltefosine/animal, administered daily for 45 days.

2.2. Clinical evaluation

Before, immediately after, and at 3-month intervals over a period of 2 years after treatment, the animals were evaluated for the presence of clinical symptoms of VL, such as dermatitis, skin ulceration, alopecia, ocular lesions, lymphadenopathy, weight loss, epistaxis, diarrhoea, and onychogryphosis. For each assessment, blood samples were collected for laboratory analysis. At the end of these analyses, all animals were sacrificed according to resolution no. 714 of the Federal Council of Veterinary Medicine (CFMV) and submitted to necropsy. A spleen fragment was collected for *Leishmania* isolation, and the parasite load was determined by q-PCR (quantitative PCR using real time-PCR).

2.3. Biochemical evaluation

The alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), total protein, albumin, globulin, direct/indirect bilirubin, urea, and creatinine levels were determined in blood samples collected from each experimental group at the intervals mentioned above. An automated biochemical analyzer (Ciba® Express 550) and the compatible, commercial reagents (Labtest®) were employed. The samples were processed by the kinetic enzymatic method according to the manufacturer's recommendations. All of the tests were performed in the Clinical Pathology Laboratory of the Veterinary Hospital of the Federal University of Piauí.

2.4. Parasitological evaluation

The presence of *Leishmania* was investigated in bone marrow samples obtained by sternal puncture from animals under sedation. Giemsa smears were obtained from these samples and evaluated by microscopy. An aliquot of the bone marrow aspirate was transferred to the NNN-LIT culture medium, which was maintained at 25 °C and examined weekly for 30 days.

The parasite loads in the bone marrow and spleen samples were evaluated by q-PCR. The DNA in the samples was

extracted with phenol–chloroform, as described by Silva et al. (2010). To minimize errors in the quantification of the parasite load due to differences in the concentration of total DNA, all of the samples were normalized to the lowest concentration of DNA obtained from a smear, which was 20 ng/ml, while the spleen samples were adjusted to 200 ng/mL.

L. infantum promastigotes culture in monophasic medium was used to construct standard curves of DNA mass vs. number of parasites. The cultured promastigotes were counted 10 times in a Neubauer hemocytometer and used to calculate the mean count, and the culture was serially diluted to produce samples that contained 1, 10, 100, 1000, 10,000 and 100,000 parasites. The DNA in each of these samples was then extracted using the Wizard kit (Promega, Madison, WI) according to the manufacturer's instructions.

TaqMan (Applied Biosystems) assays were performed using the ABI PRISM 7500 system (Applied Biosystems) and primers based on leishmanial minicircle kinetoplast DNA (Applied Biosystems) with threshold cycle numbers (Ct) that were determined using the RQ Study software package (Applied Biosystems). Reactions were performed in triplicate, and the mean Ct values were used in the final analysis. The following protocol was programmed into the thermocycler: 2 min at 50 °C, 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C.

The forward and reverse primers – 5′-GGT TAG CCG ATG GTG GTC TT-3′ and 5′-GCT ATA TCA TAT GTC CAA GCA CTT ACCT-3′, respectively – were used with TaqMan (Applied Biosystems) and an internal probe (5-ACC ACC TAA GGT CAA CCC-3) (Rolão et al., 2004). For each assay, 20-μl reaction mixture contained 2 μl of DNA sample, 10 μl of TaqMan Universal PCR master mix (Applied Biosystems), 1 μl of a 200-nM solution of each unlabeled primer, and TaqMan MGB probe (FAMTM dye-labelled) mix (Applied Biosystems). The standard curve and negative control samples were also submitted to these procedures. The point at which fluorescence exceeded the threshold limit indicated the Ct value.

2.5. Leishmania antigens

The antigens used for the Western Blot analyses and in vitro stimulation of peripheral blood mononuclear cells to assess the production of cytokines were prepared by culturing *L. infantum* promastigotes (MHOM/BR/1967/BH46) in NNN-LIT for 7 days at 23 °C (Andrade et al., 1999).

2.6. Assessment of cytokines

Peripheral blood mononuclear cells were harvested in a Histopaque gradient (Sigma Chemical Co., USA) in sterile, conical bottom, polystyrene tubes (Falcon-Corning, USA). After centrifugation at 400 × *g* for 45 min at 18 °C, the pellet of mononuclear cells was recovered. The cells were washed three times with RPMI-1640 medium and centrifuged at 200 × *g* and 4 °C for 10 min. The cell concentration was adjusted to 1.5×10^6 cells/mL in RPMI-1640 medium supplemented with 5% fetal bovine serum, 2-mercaptoethanol, HEPES, and 10 IU of penicillin/streptomycin/mL. One milliliter of cell suspension was added to two wells of a 24-well polystyrene plate (Coast, USA). One well was filled with 50 μL of *L. infantum* whole antigen extract (1 mg/mL), while another was reserved for the negative control (without *Leishmania* antigens). This 24-well plate was incubated for 72 h at 37 °C in an atmosphere containing 95% relative humidity and 5% CO_2. The supernatant was collected and stored at −70 °C until assay. The levels of IFN-γ, IL-4 and IL-10 cytokines were evaluated with double sandwich Enzyme Linked Immunosorbent Assay (ELISA) using antibodies and reagents purchased from R&D Systems.

2.7. Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed in a vertical gel apparatus (Bio-Rad). The antigen was boiled for 5 min in sample buffer and immediately subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% SDS as described by Laemmli (1970). Three hundred micrograms of protein was used for electrophoresis. The gels were run at 15 mA for the stacking gel and 30 mA for the resolution gel until the bromophenol blue dye migrated to 1 cm from the bottom of the gel in Tris–glycine–SDS buffer (pH 8.3).

Western blotting (WB) was carried out as described (Cuquerella et al., 1991). Proteins were electroblotted overnight onto nitrocellulose membranes (0.45-μm pore size, Hybond® GE Healthcare) and the strips probed with dog sera (1:100 in 1% bovine serum albumin–TBS) for 2 h. Secondary antibody was anti-dog IgG (1:500) horseradish peroxidase-labelled (Sigma) and after incubation, the membrane was washed three or four times with Tween 20–TBS and developed with the 3,3′-diaminobenzidine plus 4-chloro-1-naphthol system (SIGMA) for 5 min. The reaction was stopped with deionized water. Molecular weight (MW) markers (116, 97.4, 66, 45 and 29 kDa) were purchased from GE Healthcare.

2.8. Assessment of IgG levels

Plasma obtained from the blood samples collected for the cytokine evaluation was used for the IgG levels determination through ELISA performed exactly as described by McLaren et al. (1980).

2.9. Statistical analyses

Considering the nature of the variables, non-parametric tests with a significance level of 0.05 were used. The Wilcoxon test was employed for the parasite load analysis. The Kruskal–Wallis non-parametric test was used for the cytokine analysis within the different groups, and ANOVA was used for the evaluation of serum IgG levels.

3. Results

3.1. Clinical evaluation

The animals monitored here started as asymptomatic ($n = 1$), oligosymptomatic ($n = 5$) and symptomatic ($n = 8$) in agreement to Mancianti et al. (1998). The most prevalent

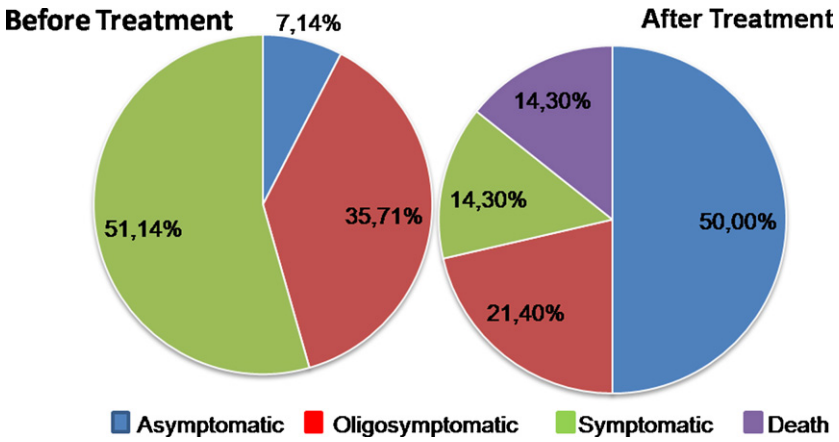

Fig. 1. Clinical evaluation before and 24 months of follow-up after treatment.

clinical signs were dermatitis (61.5%), onychogryphosis (53.8%), alopecia (46.1%), lymphadenopathy (30.7%) and conjunctivitis (23.5%). After treatment and during the 24 months of follow-up, there was progressive improvement and complete clinical recovery in 50% (7/14) of the treated animals. For the others, 21.4% (3/14) presented two or three symptoms of CVL, 14.3% (2/14) were symptomatic and two (14.3%) of the dogs died after 22 and 23 months of acute renal failure and cachexia, respectively (Fig. 1).

3.2. Biochemical evaluation

Reference values were used for all laboratory tests as reported by Kaneko et al. (1997). The hepatic function was estimated through the total protein, globulin, albumin, ALAT, ASAT and bilirubin levels. The most frequent finding was an increase in the total protein and globulin levels. Albumin levels remained within the normal limits. Direct bilirubin increased in group 3 (animals treated with 100 mg miltefosine for 45 days) after treatment.

Regarding the renal function, most animals presented an increase in the urea concentration before treatment, and the level did not return to normal after 24 months of follow-up. The full blooded values ranged from 35 to 55 mg per dL.

3.3. Parasitological evaluation

Parasites were identified in bone marrow samples through microscopic examination of smears after Giemsa staining, cultures, and qPCR. All of the samples were analyzed before and 3, 6 and 24 months after treatment. At the beginning of the study, amastigotes were identified in bone marrow slides from all of the animals, and the parasite was isolated in NNN-LIT culture from 75% of subjects. There was a decrease in the number of positive smears at 3 months post-treatment (14.2%), which was lower at 6 months (7.1%), and returning a 14.2% after two years. There was also a gradual and constant decrease in the number of positive cultures at the end of the monitoring period. However, the detection of the parasite DNA by PCR remained positive. These data are listed in Table 1.

It was not possible to assess the parasite loads in the bone marrow smears in 5 of the 14 animals (35.7%). Two progression patterns were observed. One group presented a significant reduction in parasite load ($p = 0.0277$) after 3 months of treatment, which included 66% of the animals studied. In the second pattern, the animals showed a progressive increase in parasite load after treatment (Fig. 2). These patterns have not any relationship with treatment schedule, and thus, we are presenting the results according to these patterns, and not according to the treatment group. In general, all of the animals presented a significant increase in parasite load 6 months after treatment relative to the first pattern ($p = 0.04664$).

Also, qPCR was used to assess the parasite load in the spleen 24 months after treatment. The reaction was positive in 13/14 animals and ranged from 3250 to 66,493. 920 parasites/200 ng DNA (data not shown).

3.4. Cytokines production

IFN-γ was produced in all of the groups, and the levels of this cytokine showed a tendency to increase during follow up treatment (Fig. 3) and to decrease at the end of follow up in all of the groups (data not shown), regardless the miltefosine dose. There was no significant difference between the different time intervals analyzed for each group ($p > 0.05$), however the p values were 0.68, 0.06 and 0.21 to the groups 1, 2 and 3, respectively, showing an increase tendency, mainly in group 2 whose animals were treated with the highest miltefosin dosage.

Table 1
Leishmania detection in dog bone marrow before and after miltefosine treatment. The results are expressed as the percentage of positivity.

Method	Before treatment (%)	After treatment		
		3 months (%)	6 months (%)	24 months (%)
Smear	100	14.2	7.1	14.2
Culture	75	65	53.8	30.7
qPCR	100	100	100	100

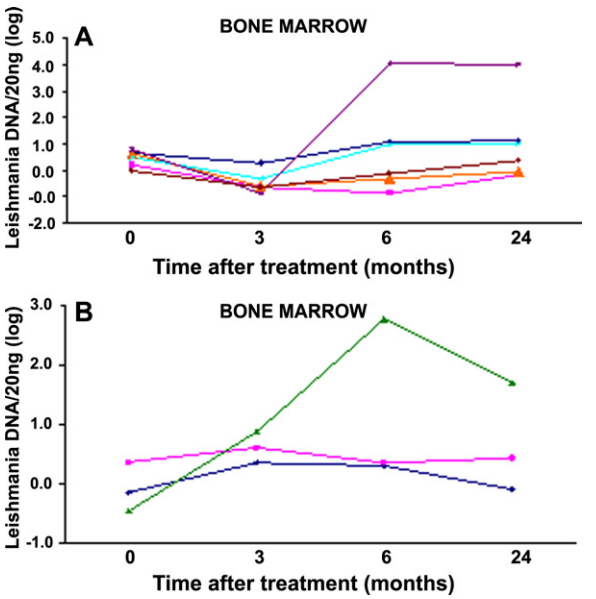

Fig. 2. *L. infantum* loads determined by real-time PCR in bone marrow. Load ranges are expressed as parasite DNA/20 ng on a log scale.

Contrary to the effect on the levels of IFN-γ, the IL-4 (Fig. 4) and IL-10 (Fig. 5) levels tended to decrease 180 days after treatment, however, 300 days after treatment IL-4 remains low while IL-10 increases. The IL-4 decrease was significant ($p < 0.05$) in groups 1 and 3. However, the differences in IL-10 levels during the time intervals the p values for the groups were 0.15, 0.17 and 0.09, respectively.

3.5. Humoral response

Regardless of drug dose and therapeutic schedule of treatment, the serum antibodies from the animals identified a number of antigens with molecular weights ranging from 116 kDa to less than 29 kDa. Furthermore, qualitative and quantitative differences in the antigen profiles were

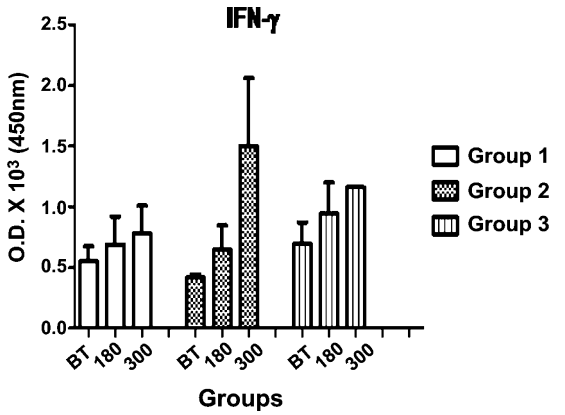

Fig. 3. IFN-γ levels in the culture supernatant obtained from mononuclear cells of dogs naturally infected with *L. infantum*, treated with miltefosine, and stimulated with *L. infantum* antigens. Numbers 1–3 refer to treatment groups. BT, before treatment. The follow-up periods are shown at 180 and 300 days. O.D., optical density at 450 nm. The vertical line represents the standard deviation.

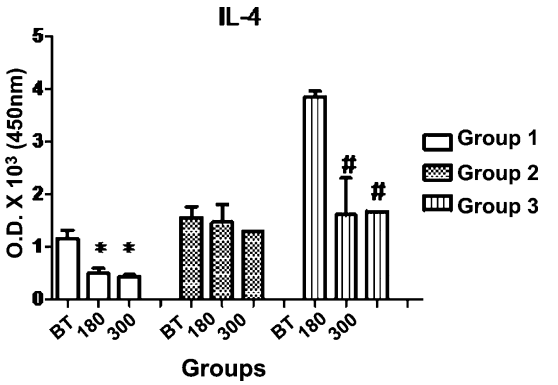

Fig. 4. IL-4 levels in the supernatant obtained from mononuclear cells of dogs naturally infected with *L. infantum*, treated with miltefosine, and stimulated with *L. infantum* antigens. Numbers 1–3 refer to the treatment groups. BT, before treatment. The follow-up periods are shown at 180 and 300 days. O.D., optical density at 450 nm. The vertical line represents the standard deviation.

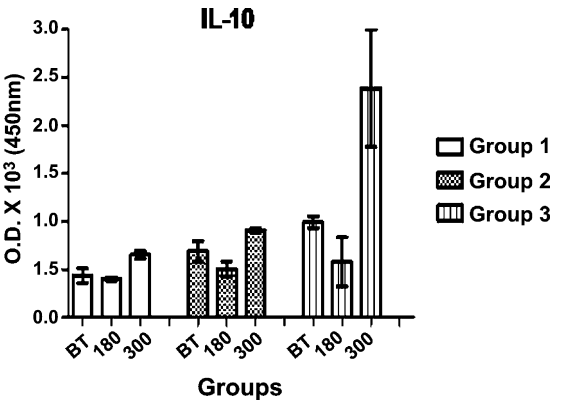

Fig. 5. IL-10 levels in the supernatant obtained from mononuclear cells of dogs naturally infected with *L. infantum*, treated with miltefosine, and stimulated with *L. infantum* antigens. Numbers 1–3 refer to the treatment groups. BT, before treatment. The follow-up periods are shown at 180 and 300 days. O.D., optical density at 450 nm. The vertical line represents the standard deviation.

observed 60 days after treatment. In general, few antigens were identified during this period. Furthermore, 300 days after treatment, other antigens were developed, particularly those of 116, 97, 66 and 46 kDa molecular weight (Fig. 6).

Antigen-specific *Leishmania* IgGs were detected in higher levels before treatment and 300 days after follow up in all three groups. The average levels after 180 days of treatment, were significantly different ($p < 0.05$) (Fig. 7). The IgG levels observed in the other two groups presented the same profile as that discussed for group 1 (data not shown).

4. Discussion

In the present study, miltefosine was employed for the first time in Brazil to treat dogs naturally infected by *L. infantum*. Significant clinical improvement that progressed to full recovery was observed in most animals. The main side effect observed was transient, self-limited, and

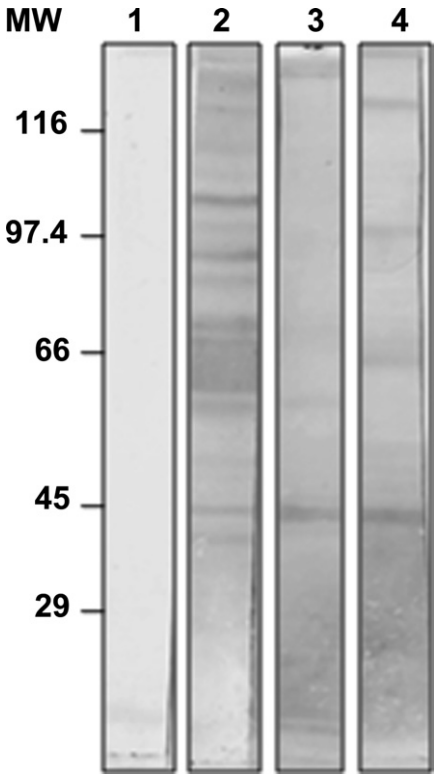

Fig. 6. Western blot profile. Line 1 = negative control, line 2 = before treatment (BT), lines 3 and 4, 60 and 300 days after treatment, respectively.

reversible vomiting, similar to that described by Woerly et al. (2009).

The anti-*Leishmania* activity of phospholipid analogues has been demonstrated previously (Croft et al., 2003). In other countries, most of the clinical trials based on the therapeutic efficacy of different active compounds on dogs naturally infected with *L. infantum* presented good clinical improvement and, in some cases, the total remission of clinical symptoms (Valladares et al., 2001; Pasa et al., 2005; Oliva et al., 2006; Ikeda-García et al., 2007), especially when miltefosine was used alone or in combination with other drugs (Manna et al., 2008a,b,c; Mateo et al., 2009; Woerly et al., 2009).

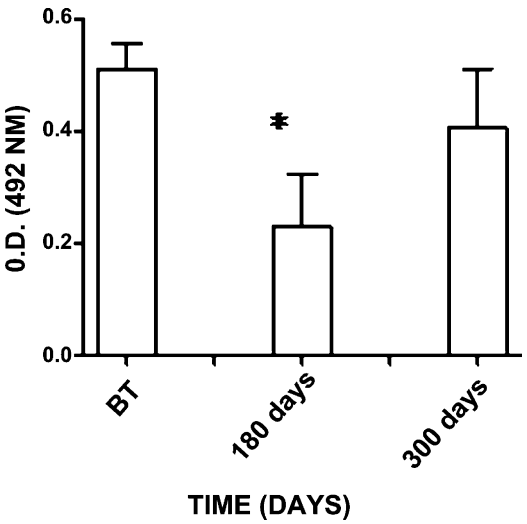

Fig. 7. Levels of immunoglobulin G anti-Leishmania antigens in the plasma of dogs treated with miltefosine (group 1). BT, before treatment. O.D., optical density at 492 nm. The vertical line represents the standard deviation.

The main biochemical change observed was an increase in the globulin level, which has been thoroughly described in the literature as the most common clinical and laboratory finding for CVL. The parasites presence in the spleen promotes an intense polyclonal activation of B cells and a large increase in the antibodies production, which reflects the increase in the globulin level (Amusategui et al., 2003).

Although other studies reporting absence of parasite in bone marrow of miltefosine-treated dogs during follow up (Miró et al., 2009), here was registered only a significant reduction in parasite load 3 months after treatment. The presence of *Leishmania* DNA in tissue samples is indicative of the presence of the intact parasite because the nuclear DNA and kDNA are rapidly degraded after amastigote death (Disch et al., 2004; Prina et al., 2007). Therefore, the use of miltefosine did not result in parasitological clearance of CVL, and it caused a progressive increase in the parasite load, especially after 6 months. This result has also been observed by Manna et al. (2008a). It is important to highlight that these discrepancies between the results of qPCR could be reasonably ascribed to the different sensitivity of the chosen target. It has been shown by Ovalle et al. (2007) that the minicircle kDNA target used here and in Manna et al. (2008a) is more sensitive than the ribosomal DNA target used by Miró et al. (2009). Thus, we suggest that qPCR using kDNA target indicates that early intervention is needed once recurrence is observed, which will thereby increase the chances for canine recovery and reduce vector contamination.

The profile of the main cytokines involved in the VL healing process was investigated because the success of any kind of treatment requires a good understanding of the specific immunological responses to *Leishmania* infection.

Similar to other groups, we also observed, increased levels of IFN-γ and a decrease in IL-4 during follow up (Manna et al., 2008b). Similarly, Chamizo et al. (2005) observed that the expression of IFN-γ was lower before therapy than during treatment and IL-4 was active during the initial defence against Leishmaniasis. In the present study, when the parasite load in bone marrow aspirates were decreased 90 days after treatment, the IFN-γ levels were increased.

In addition, mixed production of the IFN-γ and IL-4 cytokines, with Th2 dominance, was observed at the time of diagnosis. During treatment with miltefosine, the Th1 response became predominant. In fact, IFN-γ expression levels increased. These findings demonstrate a relationship between the detection of IFN-γ and the control of Leishmaniasis and suggest that IFN-γ may represent a good marker for predicting relapse of the disease in infected dogs and for determining the effectiveness of treatment. Furthermore, the results also demonstrated that IL-4 represents a marker for the active disease. This fact suggests that, as observed by other authors, clearance of parasites by activated macrophages during the *Leishmania* infection

is regulated by mechanisms that govern the production of gamma interferon (Kaye et al., 2004; Wilson et al., 2005).

The improvement of symptoms was, at least in part, the result of the cytokine profile that developed in the immune animals during treatment. An increase in IL-10 levels at the end of follow up was observed. IL-10 might regulate the possible damage caused by the exacerbated effects of IFN-γ as a consequence of the dominant Th1-like response. Additionally, IL-10 could act as a regulatory factor that maintains the balance between the Th1- and Th2-type cells (Kemp et al., 1999).

Apparently, the Th1 response could be prevalent at the end of follow up treatment. In fact, IFN-γ expression levels showed a tendency to increase. However, immunological and parasitological relapses, a significant reduction in IFN-γ, and an increase in IL-4 expression were observed in some dogs after 24 months.

The humoral immune response occurs during the active phase of infection, with the appearance of high titers of antibodies that decreased a few months after treatment, which represents a temporary response. This fact has made the assessment of antibody profiles a useful tool for the follow-up of dogs after therapy and for defining the prognostic period during which the veterinarians should observe the animals.

Similarly, the antigenic profiles revealed by western blot were correlated with the serological data. More complex antigenic profiles were identified before and 300 days after treatment, which may reflect increased antigen stimulation during these periods that lead to an increase in the concentration of IgG in these time points as shown by the immunoenzymatic reaction.

5. Conclusion

In conclusion, miltefosine promotes clinical improvement in animals with low parasite loads and low degrees of stimulation of humoral immunity. It also caused an increase in IFN-γ production. However, the improvement in the clinical symptoms was not accompanied by parasitological clearance, suggesting that treatment with miltefosine is not recommended, especially in endemic areas like Brazil, where children are the major victims and dogs are involved in the maintenance of the parasite cycle.

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