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*Case Report*

# Failure of Miltefosine Treatment in Two Dogs with Natural *Leishmania infantum* Infection

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Two dogs, with naturally acquired canine leishmaniasis, were treated orally with miltefosine (2 mg/kg q 24 hr) and allopurinol (10 mg/kg q 12 hr) for 28 days. Both dogs showed good initial response to therapy, with reduction in clinical signs and improvement of clinicopathological changes. However, in both dogs, clinical and clinicopathological abnormalities recurred 150 days after initial treatment and a second course of miltefosine and allopurinol was administered. One dog failed to respond to the 2nd cycle of miltefosine treatment and the other dog responded initially but suffered an early relapse. Treatment with meglumine antimoniate (100 mg/kg q 24 hr for a minimum of 4 weeks) was then started in both dogs. Both dogs showed rapid clinical and clinicopathological improvement and to date they have not received further treatment for 420 and 270 days, respectively. In view of the low number of antileishmanial drugs available and the fact that some of these are used in human as well as veterinary medicine, it is of paramount importance that drug resistance is monitored and documented.

## 1. Introduction

Canine leishmaniasis (CanL) caused by the protozoan *Leishmania infantum* is a life threatening zoonotic disease transmitted by insect vectors, sand flies (*Phlebotomus* spp.). CanL has a wide distribution in temperate and subtropical countries with a very wide prevalence covering both the old and new worlds. The dog is the main reservoir for human visceral leishmaniasis (VL) caused by *Leishmania infantum* [1] which is listed among the most important neglected tropical diseases by the WHO (http://www.who.int/neglected_disease/diseases; access April 2014).

The clinical features of CanL vary widely as a consequence of the numerous pathogenic mechanisms involved in the disease, the different organs affected, and the diverse nature of the immune responses mounted by individual hosts [2]. The main clinical findings include skin lesions (such as exfoliative dermatitis, papules, nodules, ulcerations, and alopecia), generalized lymphadenomegaly, splenomegaly, progressive weight loss, muscular atrophy, polyuria and polydipsia, ocular lesions, epistaxis, and onychogryphosis. Laboratory findings include nonregenerative anemia, serum hyperproteinemia, polyclonal beta and gamma hyperglobulinemia, hypoalbuminemia, decreased albumin/globulin ratio, renal azotemia, and persistent renal proteinuria [3].

The antileishmanial drugs currently used in dogs were originally developed to treat leishmaniasis in people, and most therapeutic protocols were developed through human clinical studies with subsequent adaption for use in dogs [4]. Many drugs (including amphotericin B, pentamidine, metronidazole, spiramycin, enrofloxacin, and ketoconazole) have been used, either alone or in combination, with variable results [4–7]. Currently, the first line treatment against CanL is meglumine antimoniate (MA), usually in combination with allopurinol [7]. This treatment protocol usually induces clinical remission, although it does not prevent relapses and in most cases does not completely eliminate parasites from the infected animal [8].

Recently, miltefosine (MLF) (in combination with allopurinol) has been suggested as an alternative to meglumine

Table 1: Case number 1: clinical score, therapy, and hematological and biochemical analysis of the first dog affected by canine leishmaniasis.

| Followup | Clinical score | Therapy | RBC $\times 10^3/\mu$L | Hb g/dL | PCV% | PLT /uL | TP g/dL | ALB% | $\gamma$G% | A/G | IFAT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D0 | 8/86 | MLT + A 28/d | 5220 | 12.6 | 34.8 | 37.000 | 9.3 | 31.7 | 42.7 | 0.46 | 1 : 1280 |
| D30 | 3/86 | A | 5840 | 12.6 | 34.5 | 15.000 | 7.8 | 35.2 | 37.6 | 0.54 | 1 : 640 |
| D60 | 2/86 | A | 6440 | 14.3 | 38.7 | 65.000 | 7.5 | 42.9 | 25.6 | 0.75 | 1 : 320 |
| D90 | 0/86 | A | 6110 | 13.9 | 37.1 | 149.000 | 7.4 | 42.1 | 21.4 | 0.75 | 1 : 320 |
| D150 | 11/86 | MLT + A 28/d | 6350 | 14.2 | 40.5 | 232.000 | 8.3 | 41 | 31.7 | 0.6 | 1 : 640 |
| D 210 | 0/86 | A | 6220 | 13.9 | 43.3 | 138.000 | 7.6 | 49.3 | 11.4 | 0.97 | 1 : 160 |
| D240 | 2/86 | MA 28gg | 6160 | 15 | 42.5 | 68.000 | 6.8 | 38.6 | 20.3 | 0.63 | 1 : 320 |
| D270 | 0/86 | A | 5750 | 13.2 | 35.2 | 151.000 | 5.8 | 46.7 | 10.4 | 0.88 | 1 : 160 |
| D330 | 0/86 | A | 6420 | 15.7 | 38.9 | 116.000 | 6.7 | 46.1 | 10 | 0.86 | 1 : 160 |
| D390 | 0/86 | A | 6560 | 14 | 40.2 | 174.000 | 7.1 | 47.5 | 8.9 | 0.9 | 1 : 160 |
| D660 | 1/86 | — | 6100 | 13.8 | 36.8 | 215.000 | 6.4 | 46.2 | 9.8 | 0.86 | 1 : 160 |

antimoniate for the treatment of CanL [9–13]. MLF is an alkyphospholipid originally developed as a topical and oral antineoplastic agent [8]. Multiple *in vivo* and *in vitro* trials have demonstrated the leishmanial killing activity of miltefosine [12] through disruption of both signaling pathways and cell membrane synthesis, which induces an apoptosis-like cell death. In people, MLF is an effective oral drug for the treatment of leishmaniasis although, in common with other antileishmanial drugs, some reports suggest possible development of resistance [14–16]. Some studies in dogs have reported the short-term efficacy of MLF therapy in association with allopurinol and suggest that this combination is a safe, convenient, and effective alternative treatment option for canine leishmaniasis which has only mild (and self-limiting) side effects [9–12]. Recent studies have reported cases where relapse of clinical CanL occurs between 3 and 6 months after cessation of treatment, in dogs treated with a combination of MLF and allopurinol, but no data has been provided on the outcome of further treatments in these cases [9, 11].

In this paper, we describe two dogs with naturally occurring CanL that, after an initially successful treatment with two cycles of MLF and allopurinol, relapsed, but subsequently responded to further treatment with meglumine antimoniate. These cases demonstrate that a failure of therapeutic response to MLT therapy, as has been reported in human patients, may also occur in dogs. Resistance surveillance is particularly important because the same drugs are used in dogs and human patients although, in Europe, miltefosine is not typically used to treat human visceral leishmaniasis.

## 2. Case Reports

*Case Number 1.* A 1-year-old, 10.7 kg, neutered female mixed-breed dog, adopted 4 months previously from a kennel in Sicily (a region in which canine leishmaniasis is endemic), fully vaccinated against canine distemper virus (CDV), canine parvovirus (CPV), leptospirosis, and infectious canine hepatitis (ICH), but not treated against endo- and ectoparasites. The dog was referred to the Internal Medicine Service of the Department of Health, Animal Science and Food Safety of the University of Milan, with a 30-day history of erythema and exfoliative dermatitis that had not responded to antibiotic therapy (cephalexin, ICF vet 20 mg/kg q 12 hr for 15 days).

Physical examination revealed a generalized lymphadenopathy, dry, nonpruritic dermatitis with generalized scaling and alopecia of the auricular pinna, eyelids, axilla, and groin. A provisional diagnosis of canine leishmaniasis was made. A blood count revealed a mild normochromic, anemia, and thrombocytopenia. Biochemical analysis showed hyperproteinemia with hypoalbuminemia and hypergammaglobulinemia. Serum protein electrophoresis showed a polyclonal gammopathy and a decreased albumin-globulin ratio (A/G) ratio (Table 1).

The serum indirect immunofluorescence antibody test (IFAT) for *Leishmania infantum* specific antibodies yielded a high positive titer of 1 : 1280 (reference range, <1 : 80) and conventional polymerase chain reaction (PCR) analysis of blood was positive for *L. infantum*. Indirect immunofluorescence assay (IFAT) for *Ehrlichia canis* was negative.

Diagnosis of CanL was made and the severity of clinical signs attributable to *Leishmania* infection was scored on a scale from 0 to 3 for a total of 86/86 (Table 2). The clinical score of the case 1 was 8/86 at diagnosis. Treatment was started with 2 mg/kg q 24 hr of MLF *per os* in combination with allopurinol at 10 mg/kg q 12 hr for 28 days. After the combined therapy, allopurinol was continued at the same dosage until the last follow-up (D390).

Complete clinical and blood examination was performed at 30, 60, 90, and 150 days from the start of treatment with MLF. Results of follow-up clinical scores, blood examinations, and biochemical analysis are shown in Table 2.

After the first cycle of therapy, the clinical score showed a gradual and constant decline. The anemia and the thrombocytopenia resolved during the first 90 days of followup and gamma globulin declined. At D150, the dog was presented

Table 2: Score for clinical parameters (on a scale from 0 to 86) used in dogs affected by canine leishmaniasis.

| Clinical sign | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| Appetite | Normal | Slight decrease | Moderate decrease | Anorexia |
| Mentation | Normal | Slight depression | Depression | Prostration |
| Lethargy | No | Slight | Moderate | Refusal to move |
| Weight loss | No | Slight | Moderate | Severe |
| Polyuria | No | Slight | Moderate | Severe |
| Polydipsia | No | Slight | Moderate | Severe |
| Localized muscular atrophy (temporal muscles) | No | Slight | Moderate | Severe |
| Generalized muscular atrophy | No | Slight | Moderate | Severe |
| Lymphadenomegaly | No | 1-2 nodes | 2 > 4 nodes | Generalized |
| Splenomegaly | No | | Yes | |
| Conjunctivitis and/or blepharitis | No | Unilateral and slight | Bilateral or unilateral severe | Bilateral and severe |
| Uveitis and/or keratitis | No | Unilateral and slight | Bilateral or unilateral severe | Bilateral and severe |
| Pale mucous membranes | No | Slight | Moderate | Severe |
| Epistaxis | Never presented | Sporadic | Frequent | Incoercible |
| Mouth ulcers or nodules | No | 1 or 2 small ulcers or nodules | >2 small ulcers or nodules | >1/4 or oral mouth cover by ulcers or nodules |
| Vomiting | No | Sporadic | Frequent | Frequent with blood |
| Diarrhea | No | Sporadic | Frequent | Constant |
| Lameness | No | Sporadic | Frequent | Constant |
| Itching | No | Sporadic | Frequent | Constant |
| Erythema | No | <10% body surface or slight generalized erythema | 10–25% body surface or moderate generalized erythema | >25% body surface |
| Dry exfoliative dermatitis | No | <10% body surface or slight generalized erythema | 10–25% body surface or moderate generalized erythema | >25% body surface |
| Ulcerative dermatitis | No | 1-2 ulcers | 3–5 ulcers | >5 ulcers |
| Nodular dermatitis | No | 1-2 nodules | 3–5 nodules | >5 nodules |
| Sterile pustular dermatitis | No | 1-2 pustules | 3–5 pustules | >5 pustules |
| Alopecia | No | <10% body surface | 10–25% body surface erythema | >25% body surface |
| Altered pigmentation | No | Localized | Multifocal | Generalized |
| Hyperkeratosis of nasal planum and pads | No | Slight | Moderate | Severe |
| Generalized hyperkeratosis | No | Slight | Moderate | Severe |
| Onychogryphosis | No | Slight | Moderate | Severe |

with a clinical deterioration (clinical score 11/86) and worsening of hematological parameters. A relapse was diagnosed and a second 28-day cycle of MLF in combination with allopurinol at the same dose as in the first cycle was started.

Following the second treatment with MLF, clinical signs were resolved at D210 (clinical score 0/86) with improvement of clinicopathological abnormalities, but, at D240, the dog showed again clinicopathological signs (clinical score 2/86). Following the classification by Oliva et al. (2010) [4], the dog was classified as "early relapse" and treatment with an alternative drug was initiated.

Therapy with meglumine antimoniate (100 mg/kg/sc) in combination with allopurinol (10 mg/kg/q 12 hr *per os*) for at least 4 weeks was started.

At D270, after 4 weeks of therapy, the dog had a clinical score of 0/86, biochemical analysis showed low total protein,

Table 3: Case number 2: clinical score, therapy, and hematological and biochemical analysis of the second dog affected by canine leishmaniasis.

| Follow-up | Clinical score | Therapy | RBC $\times 10^3/\mu L$ | Hb g/dL | PCV% | PLT /uL | TP g/dL | ALB% | $\gamma$G% | A/G | IFAT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D0 | 11/86 | MLT + A 28/d | 5100 | 11.9 | 32 | 288.000 | 7.8 | 35.5 | 30.1 | 0.5 | 1 : 640 |
| D30 | 7/86 | A | 5360 | 12.7 | 32.5 | 272.000 | 8.3 | 36.1 | 25.5 | 0.6 | 1 : 640 |
| D90 | 3/86 | A | 6550 | 15.4 | 40.4 | 186.000 | 6.4 | 41.2 | 17.6 | 0.7 | 1 : 320 |
| D150 | 7/86 | MLT + A 28/d | 5970 | 13.9 | 37.3 | 172.000 | 7.2 | 33.7 | 24.7 | 0.5 | 1 : 320 |
| D180 | 11/86 | MA 28/d | 4280 | 8.9 | 26.3 | 295.000 | 8 | 37.6 | 26.4 | 0.6 | 1 : 320 |
| D210 | 5/86 | A | 6470 | 15.3 | 41.2 | 197.000 | 5.9 | 32 | 19.8 | 0.5 | 1 : 320 |
| D270 | 2/86 | A | 6700 | 14.6 | 40.2 | 307.000 | 7.4 | 38.6 | 14.7 | 0.63 | 1 : 160 |
| D450 | 0/86 | A | 6760 | 14.5 | 42.7 | 230.000 | 6.9 | 46.3 | 13.9 | 0.7 | 1 : 160 |

RBC: red cells $\times 10/\mu L$ (reference range $5700–8800 \times 10/\mu L$).
Hemoglobin: Hb (reference range: 12.9–18.4 g/dL).
Haematocrit: PCV (reference range: 37.1–57%).
Platelet: PLT (reference range: 143300–400000/uL).
Total protein: TP (reference range: 6–8 g/dL).
Albumine: ALB (reference range: 46.3–58.5%).
Gamma globuline: $\gamma$G (reference range: 5.3–9.9%).
Albumine/globuline: A/G (reference rang: 0.8–1.7).
IFAT: immunofluorescence antibody test (reference range: <1 : 80).
Miltefosine: MLT.
Allopurinol: A.
Meglumine antimoniate: MA.
Days: d.

gamma globulin values were close to normal range, and the IFAT titer decreased at 1 : 160.

At examinations performed at D330, D390, and D660 (15 months after completing antimonial therapy), the dog was asymptomatic and no abnormalities were present on complete blood examination and urinalysis, whilst the IFAT titer was stable at 1 : 160. Allopurinol was discontinued 6 months after the end of antimonial therapy.

*Case Number 2.* A 10-year-old male Yorkshire terrier, 3.6 kg, was referred to the Internal Medicine Service of the Department of Health, Animal Science and Food Safety of the University of Milan with an 8-month history of weight loss, generalized scaling, and alopecia not responding to shampoo therapy. The dog had previously visited the south of Italy (Sicily Island), a region where canine leishmaniasis is endemic. Prophylaxis had been given for ectoparasites (fipronil and s-methoprene) but not for sandfly vectors of CanL. Physical examination revealed a poor body condition, depression, generalized lymphadenopathy, exfoliative, dry, nonpruritic dermatitis with alopecia and scales on the entire head, back and limbs, and onychogryphosis (clinical score 11/86). The presence of dermatophytosis or demodicosis was excluded by both negative hair culture and negative deep skin scrapings followed by antiparasitic treatment. A blood count revealed mild normocytic hypochromic anemia. Biochemical analysis showed polyclonal hypergammaglobulinemia and hypoalbuminemia (Table 3).

The serum indirect immunofluorescence antibody test (IFAT) for *L. infantum*-specific antibodies yielded a high positive titer of 1 : 640 (reference range, <1 : 80) and conventional polymerase chain reaction (PCR) analysis of blood was positive for *L. infantum*. Indirect immunofluorescence assays (IFAT) for *Ehrlichia canis* were negative.

Diagnosis of CanL was made and treatment with oral administration of 2 mg/kg q 24 hr of MLF in combination with allopurinol at 10 mg/kg q 12 hr for 28 days was started and follow-up examinations were performed at days 30, 60, 90, and 150. Results of follow-up clinical scores and blood biochemical examinations are shown in Table 3.

Following the initial treatment with MLF at D30, the dog showed weight gain and resolution of lymphadenopathy, although the alopecia, dry exfoliative dermatitis, and onychogryphosis persisted (clinical score 7/86). At D90, a general clinical improvement was seen (clinical score 3/86) and the only clinicopathological abnormality was hypergammaglobulinemia and an increase of A/G. At D150, the dog presented with recurrence of clinical signs (clinical score 7/86): extreme lethargy, dry and exfoliative dermatitis, and increased hypergammaglobulinemia. A relapse of CanL was diagnosed and a second cycle of MLF in combination with allopurinol was started.

Following the second treatment, with MLF, there was no clinical improvement by D180. The dog suffered further weight loss and showed lymphadenopathy, diffuse hair loss and crusting lesions (clinical score 11/86), and hematological abnormalities (Table 3). On the basis of the lack of improvement in both clinical score and laboratory tests, the dog was classified as "unresponsive" [3] and meglumine antimoniate

therapy was started at a dose of 100 mg/kg/q 24 hr sc in combination with allopurinol at a dose of 10 mg/kg q 12 hr for at least 4 weeks.

At D210, following meglumine antimoniate treatment, the clinical status of dog was greatly improved with resolution of the dry, exfoliative dermatitis and of the alopecia (clinical score 5/86) and improvements in the clinicopathological abnormalities.

At D270 (from the start of therapy with MA), hair regrowth was almost complete and at D450 the dog was asymptomatic and the only clinicopathological abnormality was hypergammaglobulinemia and IFAT title at 1 : 80.

## 3. Discussion

We report the failure of therapeutic response in two dogs with CanL, following a second cycle of treatment with MFT in combination with allopurinol, which both responded promptly to a third therapeutic cycle using another leishmanicidal drug.

These reports draw attention to the need for close monitoring of the pharmacological activity of new molecules, such as MLT, against CanL in order to identify the best treatment protocol and monitor development of resistance in dogs.

It is important to emphasize that, although both dogs had travelled to areas where leishmaniasis is endemic, after diagnosis they remained in nonendemic areas and were treated using deltamethrin collars to prevent sandflies from feeding. It is therefore extremely unlikely that reinfection could have occurred between therapeutic cycles.

Several factors may have contributed to the failure of therapeutic response to MLF in the two cases described: these could be related to the parasite, the drug, or the host. It is known that differences in exposure to antigens, drug pharmacokinetics, doses, frequency of administrations of the therapy, and immune response of the host may affect outcome [17].

Following oral administration of MLF, there may have been a lack of, or incomplete, drug intake by the dogs or the incomplete absorption of the molecule from the intestine. Underdosing, due to poor owner compliance, is also a possibility and this is less likely to occur with a parenterally administered drug (such as salts of antimony) used for the third therapeutic cycle [4].

Dorlo et al. [18] established the first evidence for a drug exposure-effect relationship in human patients. When treating VL, it is essential to achieve sufficient miltefosine exposure for treatment success. In man, it has been recently reported that the cure rate of mucosal leishmaniasis is about 71% after 4 weeks of treatment with miltefosine (2.5 mg/kg/day) and the duration of therapy was increased in this study to try to increase the cure rate [19].

Development of resistance is one of the major concerns with the wide use of miltefosine [20], and one of the important factors contributing to drug resistance is the use of subtherapeutic doses and/or insufficient duration of therapy [14, 20]. Furthermore, miltefosine has a long half-life (approximately 150 hours) which makes it highly susceptible to the development of resistance [21].

After early reports of therapeutic efficacy, there have been many reports in recent years of the failure of MLT therapy and the resistance to therapy with miltefosine against both visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) on the Indian subcontinent and new world [14–17, 22, 23]. Studies of *in vitro* susceptibility of *Leishmania infantum* isolated from cases in both people and dogs [24] highlight the possibility of cross-resistance to the drugs, including MLF, used in man for the treatment of leishmaniosis.

Clinical disease occurs in patients with a poor cell-mediated immune response. It is well known that the dog is a more sensitive host for *L. infantum* infection than human patients, but the therapeutic protocol used in the dog of MLT 2 mg/Kg/for 28 days is similar to that used for human beings (2.5 mg/Kg/for 28 days) [19]. In a study of 28 dogs treated with one cycle of 28 days with MLF and allopurinol, Pandey et al. [11] report that 4 dogs had a relapse and needed a second cycle of therapy which still failed to eradicate the parasite from lymph nodes.

In dogs, there is virtually no treatment that will completely eliminate parasites from the host and, even if temporary clinical remission is achieved, a relapse is to be expected in weeks to years after drug withdrawal [5, 8]. In this species, successful treatment is thought to depend, at least in part, on alterations in the host immune response to the parasite. This makes it difficult to distinguish whether a lack of treatment efficacy is attributable to the lack of immune surveillance that allows reactivation of the parasite or a true failure to respond to therapy. In animal models, T-cell-dependent immune mechanisms are not essential for miltefosine to be effective, suggesting that this agent may be useful in patients with depressed parasite-specific mediated immunity, such as sick dogs [12, 23].

This clinical report is limited by the fact that it was not possible to demonstrate the presence of a resistance to the drug, because we were not able to select the strain of *Leishmania* present in the two cases before and after treatment cycles. However, after an initial clinical improvement following the MLT treatment, both dogs relapsed or were unresponsive to the second therapeutic cycle.

Similar to findings in human medicine [19], it can be assumed that the cycle of therapy was insufficiently long to prevent the resumption of parasite replication and the activation of parasite-specific cell-mediated immunity in the host. It is also possible that the first cycle of MLF selected a resistant strain of *Leishmania* which was sensitive to the salts of antimony. Certainly, the therapeutic response to MLT was insufficient, whilst the two subjects responded readily to another molecule remaining disease-free for 420 and 270 days, respectively.

The importance of assessing whether treatment with miltefosine in dogs can lead to the selection of drug-resistant *Leishmania* strains has already been reported [3] and this is particularly relevant because of the sharing of drugs between human and canine medicine [23]. Therefore, in view of the relatively low number of antileishmanial drugs available and the fact that some of these are used in human as well as in

veterinary medicine, vigilance of the clinical efficacy of MIL in dogs is crucial. Clinicians should be encouraged to try to isolate parasites collected from unresponsive dogs and submit these to a suitable laboratory so that the possible onset of drug resistance can be monitored.

## Conflict of Interests

None of the authors (Daniela Proverbio, Eva Spada, Giada Bagnagatti De Giorgi, and Roberta Perego) declared any conflict of interests.

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