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Treatment of canine leishmaniasis: long term molecular and serological observations

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Summary

The aim of the present paper was to evaluate the anti-Leishmania activity of 3 different protocols of treatment (miltefosine plus allopurinol, difloxacin cloridrate plus metronidazole and meglumine antimoniate plus allopurinol) in 42 dogs naturally infected by L. infantum, during a 24-months parasitological and clinical follow-up. Our results suggest that, apart from miltefosine, the other two therapeutic regimens could be evaluated to treat animals with canL in medium-endemicity areas.

Keywords: canine leishmaniasis, treatment, IFAT, PCR

Canine leishmaniasis (CanL) in Mediterranean countries is a protozoan disease caused by *Leishmania infantum*. This disease can be caused not only by *L. infantum*. Several different Leishmania species are described (24).

CanL occurs also in South and Central America, East and North Africa, and South Asia. Epidemiological studies indicate that CanL has become an emerging problem in some dog breeds in the USA and Canada (6). Since dogs are the main reservoirs of the parasite, a decreased incidence of CanL in this animal species seems to be related to the control of infection. Dogs culling fails to reduce the transmission of the parasite and should be abandoned as a control measure, for ethical considerations, also (5). The treatment of individual pet dogs is intensively practiced in Europe and it is proven to reduce clinical disease, parasite load, and infectivity to sandflies (17). Together with the administration of anti feeding and insecticide drugs, the treatment represents a milestone of the control. Currently available therapeutic options are limited, and first choice drug is the combined treatment with meglumine antimoniate (a pentavalent antimonial), and allopurinol. Sodium stibogluconate is a further pentavalent antimonial available in anglo-saxon countries. Unfortunately, treatment does not prevent relapse of disease, so there is a search for effective and safe anti-*Leishmania* drugs. Other compounds used to treat such infection comprehend aminosidine, miltefosine, amphotericin B (1) and different quinolones (3, 19).

WHO recommends to avoid to use miltefosine, paromomycine and amphotericin B for dogs (2) given that they can induce parasite resistance (11, 16), although miltefosine has been licensed since 2007 in Europe for veterinary use and has been extensively used. The data available from literature report conflicting results about the efficacy of miltefosine in dogs (13, 15, 23). Similarly, among quinolones marbofloxacin has successfully been employed *in vitro* against *L. infantum* (8) and *in vivo* (19), while enrofloxacin was proven to be poorly effective (3).

The aim of the present paper was to evaluate the anti-*Leishmania* activity of 3 different protocols of treatment on dogs naturally infected by *L. infantum*, during a 24-months parasitological and clinical follow-up.

Material and methods

Forty-two client owned dogs diagnosed with canine leishmaniasis were included in the study. The animals were of different breed, both genders, and aged from 1 to 13 years (mean 6, median 5).

Inclusion criteria were: an indirect immunofluorescence antibody test (IFAT) titre ≥ 80, positive polymerase chain reaction (PCR) from blood, lymph-node and conjunctiva, the presence of at least one clinical sign of canine leishmaniasis (lymphadenopathy, weight loss, skin lesions, muscular atrophy, pale mucous membranes, articular pain, onychogryphosis, conjunctivitis) at the moment of diagnosis together with the fulfilling criteria for stage II or III according to Solano-Gallego et al., (2009) (20). Dogs with

severe renal failure, (uraemia ≥ 1 g/l, creatininaemia > 20 mg/l), positive serology to *Ehrlichia canis* ≥ 1 : 200, already treated for CanL and without informed consent from the owner were excluded from the trial. Thirty-two animals fulfilled the criteria for stage II, the others were rated as stage III. IFAT titres were ranging from 80 to 5120.

IFAT was carried out as described elsewhere (12) starting from 1/80 dilution until to extinction value. A PCR for *Leishmania* DNA detection was carried out on blood samples, lymph-node biopsy and conjunctival swabs. DNA was extracted from tissues with a DNA purification kit (Wizard® Genomic, Promega), according to the manufacturer's protocol. PCR for *Leishmania* sp. was performed as previously described (18).

A complete physical examination as well as complete blood count (CBC), biochemical profile and urinalysis, including protein/creatinine ratio, were performed on all the animals at day 0. A complete clinical and parasitological examination was carried out at days 90, 180, 360 and 720.

Dogs were randomly assigned to three different treatment regimen groups. Fourteen dogs were administered *per os* miltefosine (Milteforan®, Virbac) 2 mg/kg SID plus allopurinol (Zyloric® Glaxo, Wellcome) 10 mg/kg BID, for 30 days and for 1 year, respectively (group A). Further 13 animals were given *per os* difloxacin cloridrate (Dicural®, Fort Dodge Animal Health) 5 mg/kg plus metronidazole (Flagyl®, Zambon Italia) 25 mg/kg for 30 days. The other dogs (n = 15) were treated with meglumine antimoniate (Glucantime®, Merial Italia, SRL) at a dose of 100 mg/kg BID subcutaneously plus allopurinol (Zyloric® Glaxo, Wellcome) 10 mg/kg BID *per os*, for 30 days and for 1 year, respectively. A further course of each treatment was administered at day 360, to the subjects showing at least a parasitological positivity. All the enrolled subjects had a normal renal function, 9 and 10 animals showed anemia from groups A and B, respectively, as well as 7 dogs from group C, while A/G ratio was impaired in 7 dogs from group A, 6 from group B and 10 from group C.

Results and discussion

After the beginning of each treatment a progressive improvement of clinical signs was observed in all animals. At day 720 anemia was normalized in 5/9 dogs from group A, 4/10 from group B and 6/7 from group C, respectively, while A/G ratio was improved in 5/7 and 5/6 subjects from groups A and B, and in 9/10 dogs from group C. Adverse effects were never noticed.

Data regarding parasitological parameters showed a general decrease of IFAT titres, at day 720, while 7 animals maintained the initial value (3 from group A, 1 from group B and 3 from group C). One dog from group C registered an increase of the titre (from 320 to 640) with positive PCR results on all examined tissues. Three animals from group B resulted negative both to serologic and molecular determinations (2 animals since day 90, the other after a further course of treatment). One dog from group C scored negative to all parasitological tests at day 180, 1 at day 360, and the other 3 subjects showed negative parasitological

Tab. 1. Serological and molecular data of examined animals at days 0 and 720 after the beginning of the treatments

	Dog N	day 0*	day 720			
		IFAT titre	IFAT titre	PCR b**	PCR c**	PCR l***
Group A	1	320	320	pos	pos	pos
	2	80	80	neg	pos	pos
	3	160	40	neg	neg	pos
	4	640	80	neg	pos	pos
	5	640	160	neg	pos	pos
	6	160	80	pos	pos	pos
	7	160	40	neg	pos	pos
	8	320	80	pos	pos	pos
	9	320	80	pos	pos	pos
	10	5120	80	pos	pos	pos
	11	80	80	pos	pos	pos
	12	640	160	pos	pos	pos
	13	320	80	pos	pos	pos
	14	80	40	neg	pos	pos
Group B	15	320	neg	neg	neg	neg
	16	320	80	neg	pos	pos
	17	80	neg	neg	neg	neg
	18	80	80	pos	pos	pos
	19	160	80	neg	neg	pos
	20	1280	320	pos	pos	pos
	21	320	160	pos	pos	pos
	22	160	80	neg	neg	pos
	23	160	80	pos	pos	pos
	24	1280	160	pos	pos	pos
	25	1280	320	pos	pos	pos
	26	640	320	neg	neg	pos
	27	160	neg	neg	neg	neg
Group C	28	80	neg	neg	neg	neg
	29	640	neg	neg	neg	neg
	30	80	neg	neg	neg	neg
	31	1280	80	pos	pos	pos
	32	1280	640	pos	pos	pos
	33	160	80	neg	pos	pos
	34	80	neg	neg	neg	neg
	35	2560	80	neg	neg	pos
	36	160	160	pos	pos	pos
	37	320	80	pos	pos	pos
	38	80	neg	neg	neg	neg
	39	320	640	pos	pos	pos
	40	640	80	neg	pos	pos
	41	80	80	neg	pos	pos
	42	640	640	neg	pos	pos

Explanations: * at day 0, all tissues scored positive to PCR assay; ** blood; *** conjunctiva; **** lymph node

results after the further course of treatment. Furthermore 3 subjects from group A, 4 from group B and 7 from group C yielded PCR negative results in at least 1 examined tissue at day 360. All parasitologically negative subjects did not relapse. All these subjects became clinically asymptomatic. More detailed results are reported in Tab. 1.

The results of the present study show that miltefosine did not allow any treated dog to become negative to both clinical and parasitological tests. This finding agrees with data reported by Manna et al. (2009) (13), who detected residual parasite load after administration of this drug. Three out of 13 animals treated with difloxacin and 5 out of 15 subjects treated with meglumine antimoniate plus allopurinol underwent to both clinical and parasitological cure at day 720.

Difloxacin was never employed in the treatment of CanL; this treatment allowed a faster clinical and parasitological recovery. Previous *in vitro* studies demonstrated a direct and indirect leishmanicidal activity of marbofloxacin via the TNF-a and NO synthase pathways, and correlated with NO_2 production (22), while enrofloxacin, has recently been tested as a single treatment in leishmaniosis but without any significant results (3). Difloxacine seems to promote a good response in affected dogs. The different treatments used in this study did not completely eliminate the parasite, but the use of meglumine antimoniate and allopurinol would improve dogs clinical condition and reduce or eliminate the parasite from the skin significantly reduces the infectivity of reservoir towards sandflies, decreasing the epidemiological risk (10, 14).

The risk of induce the selection of drug-resistant parasites after pharmacological pressure is reported in literature. Cases of resistance to antimonials, both *in vitro* and *in vivo* are reported (7, 9). Parasite resistance to miltefosine can be induced experimentally, and the long half-life of this drug *in vivo* makes it vulnerable to the development of resistance in endemic regions (21). Furthermore a possible marker of miltefosine resistance in leishmaniasis has been identified (4). To the best of our knowledge resistance was never claimed with quinolones, when administered against this parasite.

Our results suggest that, apart from miltefosine, the other two therapeutic regimens could be evaluated to treat animals with CanL in medium-endemicity area.

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