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Follow-up of *Leishmania infantum* naturally infected dogs treated with allopurinol: immunofluorescence antibody test, ELISA and Western blot

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Abstract

Fourteen dogs naturally infected with *Leishmania infantum* and treated with allopurinol were monitored clinically and serologically with immunofluorescent antibody test (IFAT, amastigotes and promastigotes), enzyme linked-immunosorbent-assay (ELISA, IgG1 and IgG2) and Western blotting (WB). In all dogs therapy lead to clinical improvement together with decreasing specific antibodies in IFAT, ELISA and WB, demonstrating the usefulness of serology for follow-up. Although IgG1 and IgG2 varied considerably between individual animals, IgG2 of all dogs was predominantly in both ELISA and WB. This suggests the value of monitoring the IgG2 response (especially against 29 and 67 kDa antigens) in the follow-up of treated dogs.

Keywords: *Leishmania infantum*; Dog; Allopurinol; Follow-up; Serology

1. Introduction

Leishmania infantum is the causative agent of both human and canine leishmaniasis in Mediterranean countries. In humans, the infection was traditionally seen in children (Zuckerman and Lainson, 1977) but a notable increase has been observed for the last years related to human immunodeficiency virus (HIV) infections and to immunosuppressor treatment for the graft (Alvar et al., 1997). Canine infections are highly prevalent in some particular areas (Abranches et al., 1991; Morillas et al., 1996) and a conservative estimation of the average prevalence in dogs in the Mediterranean region could be in the range of 5–8% of the total canine population. Besides the regional interest of the disease, travelling allows the presence of the infection in virtually all European countries. Given the prevalence of the canine cases, an integrated approach to controlling leishmaniasis must include the control of infections in

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Table 1
Description of the dogs naturally infected with *L. infantum* and treated with allopurinol

Dog	Breed	Sex	Age (years)	Clinical signs	Amastigotes
1	Mongrel	Male	5	Polysymptomatic	Lymphnode
2	Mongrel	Male	5	Polysymptomatic	Lymphnode, Spleen
3	Doberman	Male	5.5	Polysymptomatic	
4	Mongrel	Male	0.5	Oligosymptomatic	
5	Teckel	Female	7	Oligosymptomatic	Skin
6	Beauceron	Female	1.5	Oligosymptomatic	
7	German Shepherd	Female	2	Oligosymptomatic	
8	Mongrel	Male	6	Polysymptomatic	
9	German Brak	Male	2.5	Oligosymptomatic	Skin
10	Mongrel	Female	6	Polysymptomatic	
11	Airdale Terrier	Male	5	Polysymptomatic	Spleen
12	Alaskan Malamute	Male	1	Oligosymptomatic	Lymphnode
13	Irish Setter	Male	9	Polysymptomatic	Lymphnode
14	Bearded Collie	Female	9	Polysymptomatic	Lymphnode

dogs. Most infected dogs from urban areas are medicated with a variety of compounds, the success being dependent on early and accurate diagnosis and monitoring systems.

A variety of methods has been used in the follow-up of medicated animals, e.g. IFAT (Alvar et al., 1994; Vercammen and De Deken, 1996), ELISA (Deplazes et al., 1995; Bourdoiseau et al., 1997), Dot-ELISA (Ferrer et al., 1995), Western blotting (WB; Fernandez-Perez et al., 1999a). However, reinfection of the animals treated in the endemic areas can not entirely be ruled out thus complicating the interpretation of the obtained results in some cases. We present here the results obtained during the follow-up of naturally infected animals treated with allopurinol in a non-endemic region without the risk of reinfection by phlebotomes.

2. Material and methods

Fourteen dogs naturally infected with *L. infantum* in the Mediterranean region, belonging to different breeds and having different ages at the beginning of the medication (Table 1), were treated with allopurinol (Zyloric, 5 mg/kg t.i.d.). All animals had been tested positively with several different serological assays and in eight dogs amastigotes had been detected by direct examination of aspirates (lymph node or spleen) or skin biopsies. Animals were monitored clinically and serologically at different times after starting medication, time schedule being variable (1–9 months interval). Animals were classified as oligosymptomatic when only one major sign of the disease was present, whereas dogs were considered polysymptomatic when several signs were observed.

Standard immunofluorescent antibody test with promastigotes (IFATp) was performed as described by Vercammen and De Deken (1996). IFAT titration with amastigotes (IFATa) was carried out according to Fernandez-Perez et al. (1999b) employing macrophage-like cell line J774.G8 infected with stationary phase promastigotes (MCAN/ES/88/ISS441, DOBA; Mendez et al., 1996). Cut-off titre was 1/32 and 1/100 in IFATp and IFATa, respectively.

ELISA conditions were determined in a checkerboard manner. Microplates were coated with soluble antigen of a strain of *L. infantum* (MCAN/ES/00/UCM-1) at a final concentration of 5 μg/ml in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. Canine sera were tested at the concentration of 1/1500 for IgG1 and 1/6000 for IgG2. Second antibodies (goat anti-dog IgG1, 1/1000 and sheep anti-dog IgG2, 1/16 000) were from Bethyl Lab and Horseradish peroxidase labelled and color was developed with 2,2′-azino-bis (3-

Table 2
Serological follow-up (IFAT, ELISA-IgG1, IgG2) of infected and treated dogs with allopurinol

Dog		Reciprocal IFATp[a]	ELISA IgG1 OD ($\times 10^{-3}$)[d]	ELISA IgG2 OD ($\times 10^{-3}$)[e]	Allopurinol months
1	a[b]	256	56	64	1
	b	128	84	57	3.5
	c	128	88	40	6.5
2	a	256	57	71	0
	b	64	80	59	11
	c	32	39	47	20
	d	8	55	77	24
3	a	8192	579	663	0
	b	512	318	657	4
4	a	32	65	64	0
	b	16	12	50	7
	c	8	9	49	14
5	a	512	68	520	3
	b	128	24	338	9
	c	64	12	144	11
6	a	1024	70	617	0
	b	128	11	177	5
	c	16	26	115	12
7	a	8192	104	517	1
	b	256	6	218	13
	c	64	1	192	20
8	a	4096	515	819	0
	b	256	184	517	6
	c	512	294	664	13
9	a	512	93	326	2
	b	512	133	471	–[c]
	c	512	92	437	–
	d	256	114	469	–
	e	512	118	500	–
	f	64	56	328	13
10	a	1024	343	731	0
	b	256	168	669	4.5
11	a	2048	185	660	0.5
	b	128	68	252	4.5
	c	8	144	105	9.5
12	a	4096	412	746	0
	b	1024	147	632	7
13	a	256	21	180	0
	b	64	14	123	3
14	a	4096	392	772	0
	b	2048	297	729	1.5

[a] IFATp, Immunofluorescent antibody test performed with promastigotes.
[b] Letters within each dog correspond with the date of serum samples.
[c] The owner gave the medication irregularly.
[d] Cut-off OD = 0.139.
[e] Cut-off OD = 0.092.

ethyl-benzthiazoline-6-sulphonic acid). Antibody levels were given as optical density values (OD) at 405 nm and as IgG1/IgG2 ratios. Cut-off OD (average of non-infected population +3 S.D.) were 0.139 (IgG1) and 0.092 (IgG2). All sera from the same animal were tested in parallel.

Late log phase promastigotes of a local strain (Madrid, Spain) of *L. infantum* (MCAN/ES/00/

Table 3
Relationship between IFATp (promastigotes) and IFATa (amastigotes) in dogs with visceral leishmaniasis and treated with allopurinol

Dog		Reciprocal IFATp titre	Reciprocal IFATa titre
2	a[a]	256	50
	d	8	0
9	a	512	100
	f	64	50
11	a	2048	400
	c	8	0
12	a	4096	>400
	b	1024	400
13	a	256	100
	b	64	50
14	a	4096	>400
	b	2048	400

[a] Letters within each dog correspond with serum samples of Table 2.

UCM-1) were extensively washed in phosphate buffered saline. Soluble protein extracts were obtained by freezing-and-thawing cycles (−80 °C, room temperature). Homogenates were centrifuged (15 000 × *g*, 15 min, 4 °C) and the protein concentration in the supernatants determined (Bradford, 1976). Proteins were fractionated in slab polyacrylamide gels (12.5%) under denaturing and reducing conditions (SDS-PAGE) at a concentration of 1 mg/ml loaded over the gel. WB was carried out as described by Cuquerella et al. (1991), using Horseradish peroxidase labelled anti-dog IgG1 and anti-dog IgG2, both diluted 1/1000. Nitrocellulose membranes were incubated with 1:100 diluted canine sera for 2 h. Low molecular weight mass markers were included as standards. Color was developed with 4-chloro-1-naphtol.

3. Results and discussion

No correlation was found between the clinical status of the dogs and the observation of *L. infantum* amastigotes in biopsies. In fact, parasites were found in eight out of 14 infected animals, this representing slightly more than 50%. Moreover, no clear relationship between symptomatology and parasite presence in lymphnodes, spleen and skin was observed. In a similar way, although all dogs were positive with IFATp at the beginning of the treatment, no relationship was observed between IFAT titre and either clinical signs or detection of amastigotes (Tables 1 and 2).

Treatment provoked clinical improvement of all animals and a parallel decrease in IFAT titres using either promastigotes (IFATp) or amastigotes (IFATa) and the results obtained with both life cycle stages were generally comparable (Table 3). Reduction of specific antibody levels determined by IFATp was related to the period allopurinol treatment was administered although the slope of the decrease was variable among individuals (Table 2).

Similarly, determination of specific IgG subclasses (IgG1 and IgG2) showed that except for three animals (dogs 1, 2 and 4) there was a good agreement between the initial (prior treatment) results obtained in ELISA and IFATp (Table 2). Surprisingly, two dogs that were clearly positive with IFAT (dogs 1 and 2) and one borderline case (dog 4) were ELISA negative, in spite of ELISA generally being a more sensitive technique.

High variation was found in the IgG subclasses although IgG2 showed higher levels than IgG1 in most cases (13 out of 14) in the first control carried out in the animals. Moreover, of the 11 ELISA positive dogs for leishmaniasis, all of them were IgG2 positive, whereas only six dogs (dogs 3, 8, 10, 11, 12 and 14) were IgG1 positive. Allopurinol treatment provoked a reduction in OD values for both IgG subclasses although the pattern was variable among individual dogs. ELISA did not allow the determination of the actual levels of specific IgG1 and IgG2, but the reduction kinetics showed that in all dogs (14) during the entire treatment period with allopurinol and clinical recovery there was a tendency towards lower OD values for both IgG1 and IgG2 (Table 2). Comparable results were obtained considering the IgG1/IgG2 ratio, although in two animals (dogs 6 and 11) the pattern was inverted. Similar results have been obtained in the follow-up of allopurinol treated dogs (Solano-Gallego et al., 2001) and Glucantime treated dogs with clinical improvement (Deplazes et al., 1995). Considering shorter

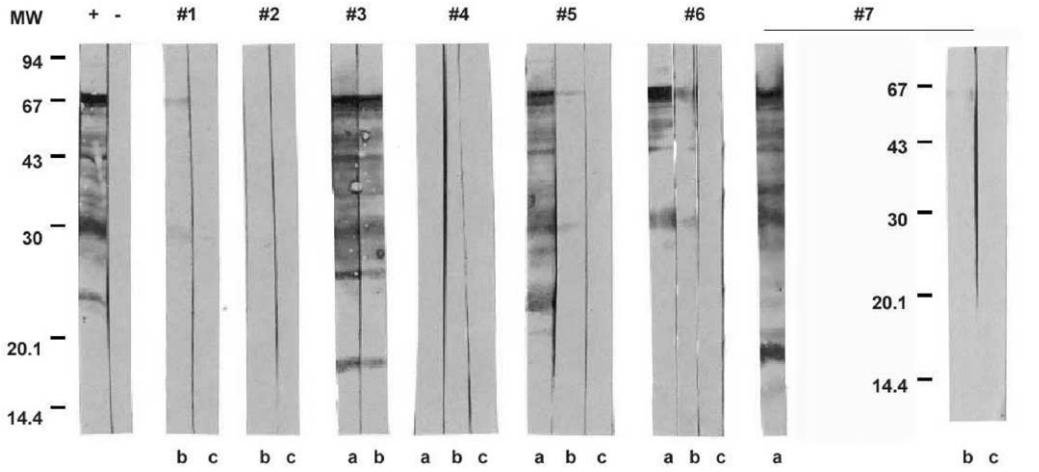

Fig. 1. Serum IgG immunodetection patterns of *L. infantum* infected dogs (dogs 1–7) treated with allopurinol. Positive (+) and negative (−) control sera, and molecular weight (MW) markers in kDa. Letters within each dog correspond with serum samples of Table 2.

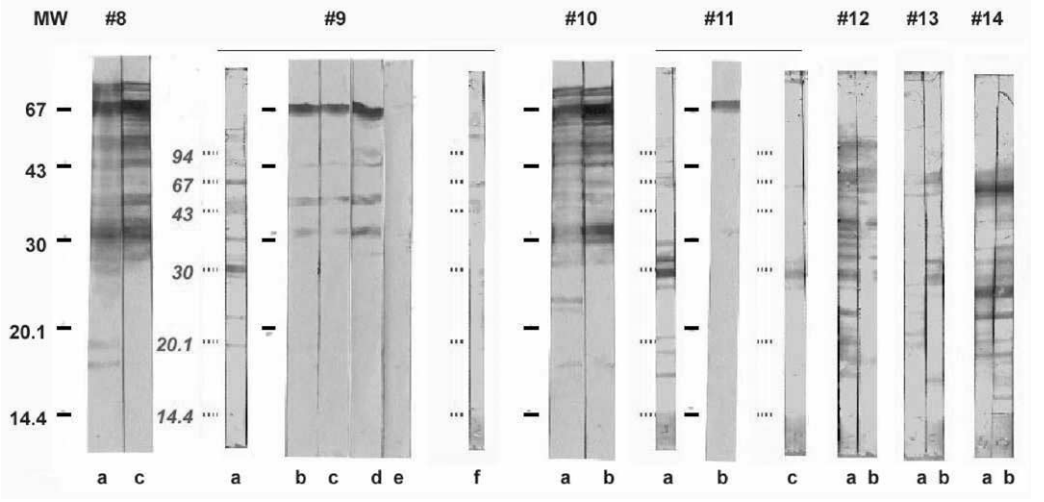

Fig. 2. Serum IgG immunodetection patterns of *L. infantum* infected dogs (dogs 8–14) treated with allopurinol. MW markers in kDa. Letters within each dog correspond with serum samples of Table 2.

period treatments (90 days) with the same antimonial compound Bourdoiseau et al. (1997) found that recovery of the dogs was accompanied by a very marked specific IgG2 decrease, and partly a rise in IgG1, thus yielding higher values of IgG1/IgG2 ratio. We can not compare their results with ours, since only few serum samples were available.

WB showed that sera from infected dogs reacted with a variety of low molecular weight antigens of *L. infantum* promastigotes (17, 19, 26, 29, 34, 35.4, 50–57, 67, 68–94, >94 kDa). Despite WB not being a quantitative technique, a certain correlation was observed between ELISA, IFAT and immunodetection patterns with simpler profiles in WB in those cases with low antibody levels (i.e. dogs 4 and 13). As expected, no direct correlation between immunoreactivity and clinical status (oligosymptomatic vs. polysymptomatic) or parasite

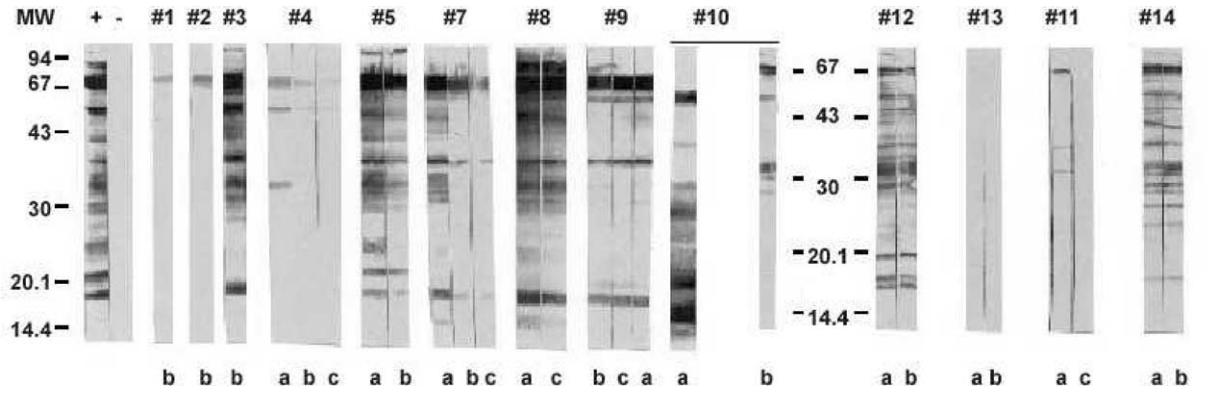

Fig. 3. Serum IgG2 immunodetection patterns of *L. infantum* infected dogs (dogs 1–5 and 7–14) treated with allopurinol. Positive (+) and negative (−) control sera, and MW markers in kDa. Letters within each dog correspond with serum samples of Table 2.

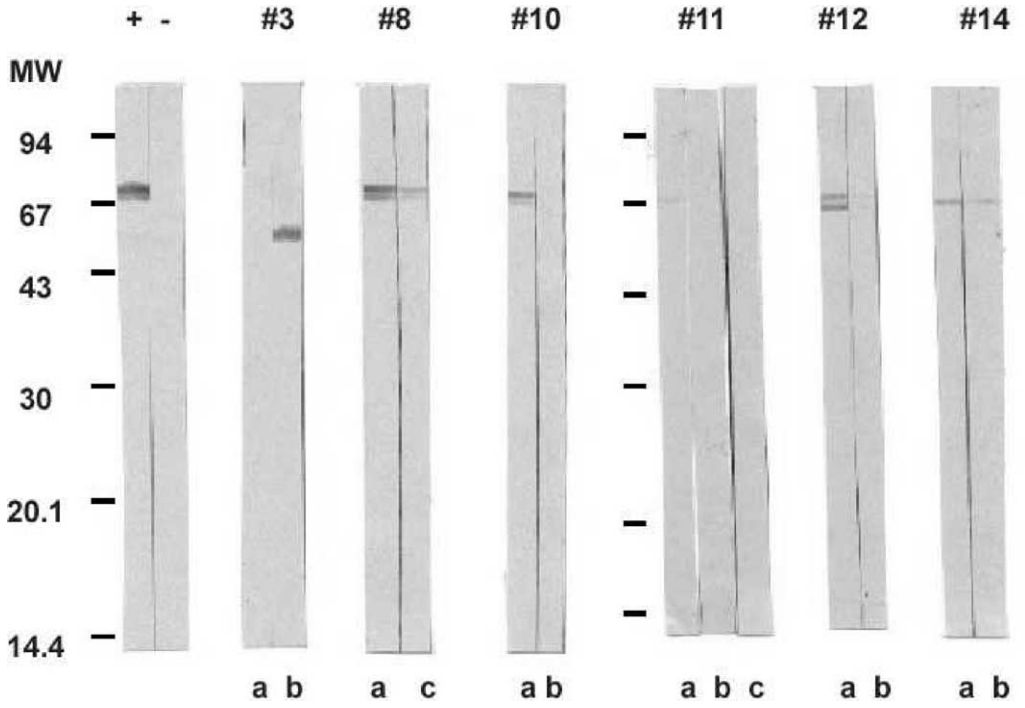

Fig. 4. Serum IgG1 immunodetection patterns of *L. infantum* infected dogs (dogs 3, 8, 10–12, 14) treated with allopurinol. Positive (+) and negative (−) control sera, and MW markers in kDa. Letters within each dog correspond with serum samples of Table 2.

isolation was observed. However, clinical recovery of the animals treated with allopurinol was associated with a clear reduction of reactivities (Figs. 1 and 2) particularly visible when immunodominant regions (ca. 29 and 67 kDa) were considered, corresponding with previous observations in animals treated with antimonials (Fernandez-Perez et al., 1999a).

Determinations of IgG subclasses in WB showed that total IgG reactivity was especially related to the IgG2 subclass (Fig. 3) against a high number of antigens, whereas the reactivity by

IgG1 was much less intense (Fig. 4). These results were comparable to those obtained by Deplazes et al. (1995) and seem to indicate that the reduction in WB complexity in infected and treated dogs with clinical recovery is propably the rule and unrelated to the compound employed (antimonials, allopurinol).

In conclusion, the present study shows that IFAT (with amastigotes and promastigotes), ELISA (IgG1 and IgG2), and WB can be used in the follow-up of dogs naturally infected with *L. infantum* and treated with allopurinol. This treatment provoked the clinical improvement and the recovery was accompanied by a lower level of specific circulating antibodies. There was a considerable degree of individual variation (IgG1, IgG2) among dogs, but all animals showed a higher IgG2 reactivity in both ELISA and WB. Serial WB suggests its value in monitoring medicated dogs, particularly the IgG2 response against 29 and 67 kDa antigens.

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