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Real-Time PCR in Dogs Treated for Leishmaniasis with Allopurinol

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Abbreviations: A/G, albumin/globulin ratio; BID, *bis in die*; IFAT, immunofluorescent antibody test; PCR, polymerase chain reaction; PO, *per os*

INTRODUCTION

The difficulty of pharmaceutical treatment for canine leishmaniasis (Pennisi, 2000) means it is necessary to monitor its effects both during and after therapy. For this purpose, the antibody titre and serum protein pattern are routinely monitored. The determination of the amount of parasitic DNA using real-time PCR can be used to estimate the parasitic loads in the samples (Nicolas *et al*., 2002) and can therefore contribute to the assessment of the efficacy of treatment (Bossolasco *et al.*, 2001). The aim of the present work was to apply quantitative real-time PCR in blood, lymph node and skin samples during the clinical follow-up of dogs with symptomatic natural infection assuming allopurinol.

MATERIALS AND METHODS

Six adult dogs (five mongrels and one beagle; three males and three females, weighing between 15 and 27 kg) affected by leishmaniasis (i.e. with pathognomonic clinical signs of the disease and diagnosis confirmed by both antibody titre IFAT and PCR) were enrolled for this study. The animals started their treatment with allopurinol (10 mg/kg BID PO) at time 0. The severity of the disease was expressed according to a score of clinical signs (score from 1 to 3) by considering nutritional condition, appetite, sensorium, rectal temperature, cutaneous lesions, eye lesions, lymphadenomegaly, epistaxis, albuminaemia, globulinaemia, haemoglobinaemia and IFAT. On the first and the 90th day of treatment, the clinical score was determined, a blood sample was taken, fine-needle aspiration from a lymph node and a cutaneous biopsy were performed. The cutaneous biopsy was always performed in the left shoulder with a 3 mm biopsy punch, in a zone with no evident cutaneous lesions.

TABLE I
Clinical score, antibody titre (IFAT), albumin and globulin concentrations (g%) and ratio (A/G), quantitative PCR (qPCR) on blood, lymph node (LN) and skin in each dog at the beginning of therapy (T_0) and after 90 days (T_{90})

Dog	239	262	410	432	478	3585
T_0						
Score	13	13	14	10	19	19
IFAT	5,120	5,120	80	80	5,120	1,280
Albumin	2.07	2.09	2.26	2.92	1.66	1.98
Globulins	5.83	5.25	5.56	3.94	5.29	4.55
A/G	0.35	0.4	0.4	0.74	0.31	0.43
qPCR blood	5,000	1,500	4,000	3,000	1,000	300
qPCR LN	130	2,000	600	300	1,000	6,000
qPCR skin	1,000	200	20,000	20,000	400	9,200
T_{90}						
Score	9	7	12	4	16	14
IFAT	1,280	1,280	160	80	2,560	640
Albumin	2.71	1.79	2.23	3.38	2.23	1.86
Globulins	5.24	6.27	5.12	3.26	5.01	5.1
A/G	0.52	0.28	0.43	1.03	0.44	0.36
qPCR blood	500	1,500	1,500	1,500	1,200	200
qPCR LN	200	1,200	300	600	1,000	850
qPCR skin	1	40	20	20	20	10

IFAT was performed with antigen produced by the National Reference Centre for Leishmaniasis (CReNaL) of Palermo according to the OIE method modified by the CReNaL.

For quantitative PCR, DNA extraction was performed using the GenElute Kit (Sigma) according to the manufacturer's instructions. The amplification of a 78-bp sequence in the constant region of the minicircles of kinetoplast was done using Applied Biosystems 7700; the DNA concentration in unknown samples was calculated by comparison with the calibration curves obtained by the analysis of serial dilutions of *Leishmania* amastigotes in a range between 1 and 10^6.

Results are expressed as absolute numbers of parasite present in 1 ml of blood, in the lymph node fine-needle aspirate and in the cutaneous biopsy. Changes in each parameter (see Table I) for each dog were evaluated using the Wilcoxon test.

RESULTS

Clinical scoring, antibody titre, albumin (g%), globulins (g%), albumin/globulin ratio (A/G) and quantitative PCR on blood, lymph node and skin at the beginning of therapy (T_0) and after 90 days (T_{90}) are reported in Table I. Changes recorded between the two time points were only statistically significant for clinical scoring ($p < 0.032$) and quantitative PCR on skin samples ($p < 0.032$). At T_{90}, the clinical score was in fact lower for all dogs—even

if some dogs registered only a slight decrease—and the parasitic loads on skin biopsies were reduced (up to 2 log). The antibody titre recorded a maximum reduction of only serial dilutions and no dog was seronegative (not even the two dogs starting with a very low titre). The serum protein pattern was only slightly improved and for two dogs it worsened (dog 262 and dog 3585). The same pattern was registered in the parasitic loads from the blood and lymph node, with only slight decreases and an increase at T_{90} in the blood (dog 478) or lymph node (dog 239 and dog 432).

DISCUSSION

Treatment with allopurinol induces short-term clinical improvements in dogs affected by leishmaniasis, but the antibody titre and serum protein pattern do not change in the same way. Quantitative PCR carried out on blood and lymph node samples seems to express a similar trend; quantitative PCR from skin biopsies seems to be a more sensitive marker of the clinical efficacy of treatment. Moreover, this study confirms that quantitative PCR gives positive skin samples from infected dogs, even in the absence of cutaneous signs. The efficacy of allopurinol in reducing the parasitic load in the skin is confirmed, as also indicated in a study based on xenodiagnosis (Baneth *et al*., 2000).

These results show that further studies on a larger number of animals and a long-term follow up are warranted; the efficacy of different therapeutic protocols can also be analysed using the quantitative PCR method.

The possibility of calculating a wide range for parasitic load using a rapid method with a reduced risk of cross-contamination, could have a high potential for clinical application.

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