Evaluation of acute phase protein indexes in dogs with leishmaniasis at diagnosis, during and after short-term treatment

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ABSTRACT: An acute phase index based on a combination of acute phase proteins which permitted monitoring the response to therapy of canine leishmaniasis was developed and evaluated in this study. Six dogs naturally infected by *Leishmania infantum* were treated with meglumine antimoniate (Glucantime®, Merial, Lyon, France) 100 mg/kg/day *sc*, given concurrently for 20 days with allopurinol (Zyloric®, Glaxo Wellcome, Madrid, Spain) 30 mg/kg/day *po* and then, allopurinol alone for one month at the same dosage. Blood samples for acute phase proteins were obtained on different days before and after the beginning of treatment and two groups of indexes were calculated: (1) Indexes that combined one positive and one negative acute phase protein and (2) Indexes that combined two positive and one negative acute phase proteins. All calculated indexes were significantly higher in animals with leishmaniasis compared with clinically healthy dogs (n = 8) and a decrease was observed in all dogs tested during the treatment. Indexes that combined C-reactive protein (CRP) and ceruloplasmin (CP) with other proteins showed greater percentages of decrease that were statistically significant. Among these, the index CRP*CP/Alb was selected as the optimum since it showed a larger and faster decrease compared with the others as well as with individual proteins alone. These results would support the use of selected acute phase indexes, especially the CRP*CP/Alb index, to suspect about a leishmaniotic dogs and to monitor their response to treatment.

Keywords: acute phase; indexes; leishmaniasis; dogs

The term Acute Phase Response reflects all the changes that occur in an organism shortly after any tissue injury in order to restore itself to the state of homeostasis. During this response, there is an increase in the rate of synthesis and release of certain proteins such as haptoglobin, C-reactive protein and ceruloplasmin collectively called "Positive acute phase proteins", and concurrently a decrease in the rate of production of other plasma proteins such as albumin and prealbumin called "Negative acute phase proteins" (Eckersall and Conner 1988; Toussaint et al., 1995; Eckersall, 2000). Studies in veterinary medicine have demonstrated that the quantification of these proteins provides valuable clinical information in the diagnosis, prognosis and treatment monitoring of different pathologic processes (Martinez-Subiela et al., 2001). It has been postulated that their predictive value increases considerably when values of positive and negative acute phase proteins are combined in a mathematical formulation. Researchers have developed in bovine and porcine species a ratio called the "Acute Phase Index" (Toussaint et al., 1995, 2000) that has been used to evaluate the animals' health status and to predict the response to the treatment.

Canine leishmaniasis is a systemic disease caused by a protozoan of the genus *Leishmania*. It is endemic in the Mediterranean area (Guy et al., 1993; Nash, 1993) and has the additional importance of being a zoonosis in which the dog is considered to be the chief reservoir of the parasite (Alvar et al., 1994). It is transmitted by sandflies of the subfamily *Phlebotominae* and, after the inoculation, amastigotes of *Leismania infantum* multiply inside macrophages and others cells of the mononuclear phagocytic system causing inflammatory processes and immune-mediated lesions (Chang et al., 1985).

One of the most important problems of this disease is related to the treatment monitoring since protocols used for the treatment that induce clinical remission rarely achieve a parasitological cure and relapses are very frequent and consequently several cycles of treatment are necessary (Slapendel and Ferrer, 1998). These circumstances make difficult to decide when to withdraw the treatment and thus a useful method to monitor the response to canine leishmaniasis therapy and to predict possible relapses of the disease is clearly needed. Most frequently used parameters to evaluate the response to therapy include the albumin-globulin ratio and the study of the electrophoretical pattern since normalisation in the electrophoretogram and therefore in this ratio indicates a good response to treatment (Romdane et al., 1992; Denerolle, 1996; Bourdoiseau et al., 1997; Amusategui et al., 1998). However, *Leishmania* parasites do not always cause modifications in the serum protein patterns (Martinez-Subiela et al., 2002a), and in some cases abnormal electropherograms have been found after successful treatment of leishmaniasis (Romdane et al., 1992).

It has been reported that measurements of selected acute phase proteins such as C-reactive protein (CRP) and/or ceruloplasmin (CP) could be used to evaluate the initial response to treatment in canine leishmaniasis since these proteins significantly decreased in concentration when the treatment is successful and it has been postulated that acute phase proteins could have a greater potential as biomarkers for monitoring the response to treatment compared to the albumin-globulin ratio (Martinez-Subiela et al. 2003a). The aim of this study, was the evaluation of acute phase protein indexes in dogs with leishmaniasis before, during and after short-term treatment.

MATERIAL AND METHODS

Animals

Six dogs of different breeds (One German braco; three German shepherd; one Boxer and one Collie), naturally infected by *Leishmania infantum* were used for the study. The group comprised four males and two females aged four to eight years and come from clinical cases received in different veterinary clinics of southern Spain

Additionally eight clinically healthy dogs (four German braco and four Beagle), four males and four females aged five to eight years, from the Murcia University Animal Resources Centre were used as control animals in order to determine the normal values of the different acute phase indexes.

Diagnosis

The diagnostic tests applied to the dogs were: bone marrow aspirates for direct observation of the parasite and ELISA for detection of antibodies to *Leishmania infantum*. Bone marrow aspirates were obtained from the costochondral junctions with a 22 G needle, after local anaesthetic had been infiltrated around the site. Smears were made and stained with Giemsa. A commercial ELISA test (Ingenzim, Ingenasa, Spain) was used to detect antileishmania antibodies following manufacturer's instructions, with serum samples considered positive at 1/100 titers

Treatment protocol

Dogs were treated with meglumine antimoniate (Glucantime®, Merial, Lyon, France) 100 mg/kg per day *sc*, given concurrently for 20 days with allopurinol (Zyloric®, Glaxo Wellcome, Madrid, Spain) 30 mg/kg/day *po* and then, allopurinol alone for one month at the same dosage following the protocols of Ginel et al. (1998).

Sampling protocol and clinical and laboratory assessment

Blood samples for the different determinations were obtained on day 0 (pre-treatment sample) and subsequently on days 10, 20, 35 and 50 after the beginning of treatment.

Clinical and laboratory assessment in all dogs included a physical examination, a complete blood count performed on automated animal blood counter (Vet Abc, ABX diagnostic, Montpellier, France), a blood smear examination and basic serum chemistry profile using an automated chemistry analyzer (Cobas Mira Plus, ABX diagnostic, Montpellier, France).

Albumin-globulin ratio

In order to determine the albumin-globulin ratio, capillary zone electrophoresis was performed in the Paragon CZE™ 2000 clinical capillary elec-

trophoresis system (Beckman instruments, Brea California). This system has been previously validated for analysis of serum proteins in canine samples (Martinez-Subiela et al., 2002b). Total protein concentrations were determined by refractometry (Atago mod. SPR-T2, Tokyo, Japan).

Acute phase proteins and acute phase indexes

Four positive and one negative acute phase proteins were measured:

Positive acute phase proteins:

(1) Haptoglobin (Hp). The commercial kit Tridelta Phase^{TM} range serum haptoglobin (Tridelta Development Limited, Ireland), was used to determine this protein. All determinations were done in duplicate in the automated biochemistry analyzer Cobas Mira Plus (ABX Diagnostic Montpellier, France).

(2) C reactive protein. Levels of this protein were determined with the Tridelta Phase^{TM} range canine CRP kit (Tridelta development limited, Ireland). Final absorbance of samples was measured by use of a microtiter plate reader (Powerwave XS, Biotek Instruments, USA) at 450 nm using 630 nm as reference.

(3) Serum Amyloid A (SAA). Concentration of this protein were measured with the Tridelta Phase Range Serum Amyloid A Assay (Tridelta Development Limited, Ireland). Final absorbance of samples was measured by use of a microtiter plate reader (Powerwave XS, Biotek Instruments, USA) at 450 nm using 630 nm as reference.

(4) Ceruloplasmin. Levels of this protein were determined with the method described by Ceron and Martinez-Subiela (2004). All determinations were performed in duplicate in the automated biochemistry analyzer Cobas Mira Plus (ABX Diagnostic Montpellier, France).

All these methods have been validated in our laboratory to measure acute phase proteins in canine serum samples showing a good within-run precision and accuracy. However CRP and SAA assays had a high between-run imprecision, so that in the present study, all samples were stored and assayed for acute phase proteins on the same day (Martinez-Subiela et al., 2003b).

Negative acute phase protein:

(1) Albumin. Concentrations of this protein were measured by capillary zone electrophoresis in the Paragon CZE^{TM} 2000 clinical capillary electrophoresis system (Beckman instruments, Brea California) as it has been described above.

Two different types of acute phase indexes were calculated:

– Acute phase index-1, was calculated as the ratio between a positive and a negative acute phase protein. An index was determined for each positive acute phase protein measured.

$$\text{APP-Index-1} = \frac{\text{Positive APP (g/100 ml)}}{\text{Negative APP (g/100 ml)}} \times 10^6$$

– Acute phase index-2, was calculated as the ratio between two positive and one negative acute phase proteins. An index was determined for each pair of positive acute phase proteins measured.

$$\text{APP-Index-2} = \frac{\text{Positive APP (g/100 ml)} \times \text{Positive APP (g/100 ml)}}{\text{Negative APP (g/100 ml)}} \times 10^6$$

Statistical analysis

Albumin-globulin ratio and Acute Phase index values obtained during treatment were compared with those obtained before the therapy by using the Wilcoxon test. Acute phase index values obtained for control animals were compared with those obtained in Leishmaniotic dogs at the moment of diagnosis by using the Mann Whitney test of significance. Differences were considered significant at $P < 0.05$.

RESULTS

Diagnosis

Dogs included in the group of leishmaniotic animals showed the presence of amastigotes of *Leishmania sp.* within the macrophages on cytologic examination of bone marrow aspirates and all infected dogs had ELISA titers >1/100.

None of the healthy dogs were positive to bone marrow examination or ELISA test.

Additionally all dogs were tested for *Ehrlichia canis* and *Babesia canis* infection and presented negative results.

Clinical and laboratory examinations

At the moment of diagnosis the most common manifestations of the disease were weight loss, different degrees of generalized lymphadenopathy and skin lesions. Haemogram and serum biochemistry alterations most commonly found were mild non regenerative anemia, hyperproteinaemia and hypoalbuminaemia. No dogs with renal insufficiency or liver damage/insufficiency were included in this study.

A clinical improvement was observed in all animals after beginning of treatment. Main clinical signs began to disappear with a progressive normalisation of the haematological and biochemical parameters. All animals exhibit a good health status at the end of the study period.

Albumin-globulin ratio

Before therapy, the animals had an inversion of the albumin-globulin ratio, with mean value of 0.55. A slight, non-significant increase in the albumin-globulin ratio was observed during the treatment (Table 1).

Acute phase indexes

Results obtained for the different acute phase indexes in control animals are presented in Table 2.

Tables 3 and 4 show the values obtained for the different acute phase indexes that combined one positive and one negative acute phase protein.

Table 1. Albumin-globulin ratio obtained in dogs treated with meglumine antimoniate + allopurinol

Day	Albumin/Globulin	
	$\bar{x}$ ± SD	*P*
0	0.55 ± 0.35	
10	0.53 ± 0.30	0.60
20	0.49 ± 0.21	0.24
35	0.59 ± 0.21	0.46
50	0.58 ± 0.22	0.46

$\bar{x}$ = mean; SD = standard deviation; *P* = comparison between the values for the albumin-globulin ratio obtained in different days during therapy and those values on day 0

Table 2. Indexes obtained in control dogs

Index	$\bar{x}$ ± SD
CRP/ALB	94.38 ± 88.39
SAA/ALB	3.29 ± 2.28
HP/ALB	67 495.7 ± 26 700.9
CP/ALB	1 729.6 ± 180.8
CRP*SAA/ALB	0.0006 ± 0.0005
CRP*HP/ALB	14.5 ± 7.5
CRP*CP/ALB	0.44 ± 0.38
SAA*HP/ALB	0.78 ± 0.73
SAA*CP/ALB	0.01 ± 0.01
HP*CP/ALB	339.8 ± 154.6

$\bar{x}$ = mean; SD = standard deviation

Acute phase index values obtained for leishmaniatic animals on day 0 were significantly higher than control values. A gradual decrease was observed during the therapy in all acute phase indexes calculated except in the index HP/ALB. Wilcoxon test showed statistically significant decreases on day 20 after the commencement of treatment, in both indexes calculated: (1) CRP (CRP/ALB) decreased 67 per cent, and (2) ceruloplasmin (CP/ALB) had a decrease of 66 per cent on day 20. No significant changes were observed with SAA and a significant increase appeared when haptoglobin was used in the index.

Tables 5 and 6 show the values obtained for the different acute phase indexes that combined two positive and one negative acute phase protein. Significantly higher acute phase index values were observed in diseased dogs compared with control animals. A decrease in all indexes was observed during the treatment. This decrease was statistically significant on day 10 for the index CRP*CP/ALB with a 78 per cent decrease on this day and on day 35 for the indexes CRP*HP/ALB and HP*CP/ALB with a 80 and 78 per cent decrease respectively.

DISCUSSION

Monitoring the plasma concentrations of acute phase proteins have been reported to be useful in the evaluation of the response to therapy

Table 3. Acute phase indexes that combine one positive and one negative acute phase protein obtained in dogs treated with meglumine antimoniate + allopurinol

Day	CRP/ALB			SAA/ALB		
	$\bar{x}$ ± SD	%	*P*	$\bar{x}$ ± SD	%	*P*
0	1 126.3 ± 536.1			1 697.5 ± 2075.6		
10	616.1 ± 293.7	45	0.11	270.5 ± 266.1	84	0.34
20	375.8 ± 245.7	67	0.02	194.8 ± 230.9	88	0.34
35	185.0 ± 142.0	83	0.02	119.5 ± 233.5	93	0.34
50	309.4 ± 283.77	72	0.04	88.3 ± 55.5	94	0.34

$\bar{x}$ = mean; SD = standard deviation; % = percentage of decrease compared to day 0; *P* = comparison between the values for the different indexes obtained in different days during therapy and those values on day 0

Table 4. Acute phase indexes that combine one positive and one negative acute phase protein obtained in dogs treated with meglumine antimoniate + allopurinol

Day	HP/ALB			CP/ALB		
	$\bar{x}$ ± SD	%	*P*	$\bar{x}$ ± SD	%	*P*
0	188 939.2 ± 79 519.9			231 672.6 ± 102 317.1		
10	345 380.7 ± 161 808.1	87**	0.04	100 601.5 ± 89 324.4	56	0.07
20	316 119.1 ± 101 378.0	67**	0.17	79 415.7 ± 78 479.7	66	0.02
35	173 334.8 ± 93 600.8	8	0.24	46 792.8 ± 32 697.9	79	0.02
50	135 157.8 ± 71 222.7	28	0.07	47 607.2 ± 31 617.7	79	0.02

$\bar{x}$ = mean; SD = standard deviation; % = percentage of decrease compared to day 0; *P* = comparison between the values for the different indexes obtained in different days during therapy and those values on day 0
**increase rather than decrease

Table 5. Acute phase indexes that combine two positive and one negative acute phase protein obtained in dogs treated with meglumine antimoniate + allopurinol

Day	CRP*SAA/ALB			CRP*HP/ALB			CRP*CP/ALB		
	$\bar{x}$ ± SD	%	P	$\bar{x}$ ± SD	%	*P*	$\bar{x}$ ± SD	%	*P*
0	5.79 ± 7.96			489.5 ± 238.9			67.4 ± 51.9		
10	0.42 ± 0.38	93	0.34	488.2 ± 342.5	0.2	0.91	14.5 ± 13.0	78	0.02
20	0.19 ± 0.33	97	0.34	309.1 ± 244.2	37	0.34	6.9 ± 6.5	90	0.02
35	0.08 ± 0.15	99	0.24	97.6 ± 115.6	80	0.02	2.2 ± 2.3	97	0.02
50	0.05 ± 0.04	99	0.17	114.0 ± 137.4	76	0.02	4.8 ± 6.2	93	0.02

$\bar{x}$ = mean; SD = standard deviation; % = percentage of decrease compared to day 0; *P* = comparison between the values for the different indexes obtained in different days during therapy and those values on day 0

Table 6. Acute phase indexes that combine two positive and one negative acute phase protein obtained in dogs treated with meglumine antimoniate + allopurinol

Day	SAA*HP/ALB			SAA*CP/ALB			HP*CP/ALB		
	$\bar{x}$ ± SD	%	*P*	$\bar{x}$ ± SD	%	*P*	$\bar{x}$ ± SD	%	*P*
0	673.5 ± 891.9			109.9 ± 161.73			10 341.6 ± 5 015.9		
10	212.7 ± 290.0	68	0.46	6.67 ± 8.73	94	0.24	6 011.9 ± 4 685.1	42	0.24
20	172.5 ± 257.9	74	0.46	3.65 ± 4.44	97	0.34	5 069.4 ± 3 662.8	51	0.11
35	48.6 ± 83.2	93	0.34	0.82 ± 1.28	99	0.11	2 276.8 ± 1 814.9	78	0.02
50	30.2 ± 28.7	95	0.24	0.93 ± 0.58	99	0.17	1 740.3 ± 1 307.5	83	0.02

$\bar{x}$ = mean; SD = standard deviation; % = percentage of decrease compared to day 0; *P* = comparison between the values for the different indexes obtained in different days during therapy and those values on day 0

in canine leishmaniasis (Martinez-Subiela et al., 2003a). Their interpretative benefit can be further enhanced by the acute phase index calculated as a ratio between positive and negative reacting acute phase proteins (Skinner, 2001). Acute phase indexes have been determined in processes such as the *S. suis* infection in pigs or different clinical problems in cattle (Toussaint et al., 1995), but there are no reports about these indexes in dogs. Therefore, different acute phase indexes based in combination of acute phase proteins (APPs) and their possible utility in the detection and monitoring the response to therapy of canine leishmaniasis were investigated in this study.

All calculated indexes were significantly higher in animals with leishmaniosis compared to control dogs therefore, they could be of help to detect possible infected animals.

A decrease in all indexes was observed in all animals during the treatment. The highest percentage of decrease was observed in the indexes that combine SAA with other acute phase proteins although none of these indexes showed a significant decrease. An explanation for this situation could be that SAA did not experience a consistent increase in all dogs tested as opposed to the other acute phase proteins. Additionally, previous studies have reported that this protein is less sensitive for detecting infected animals than others acute phase proteins such as CRP, haptoglobin and ceruloplasmin (Martinez-Subiela et al., 2002a).

It must be pointed out that the use of indexes based on haptoglobin measurements should not be recommended since an increase in this protein, and thus in the corresponding indexes, was detected during the treatment administration. These results are in agreement with previous reports in which a possible stimulation in the synthesis of this protein caused by different drugs such as antihelmintics (Tosa et al., 1993), meglumine antimoniate (Martinez-Subiela et al., 2003a), glucocorticoids (Martinez-Subiela et al., 2004) and cytotoxic drugs (McGrotty et al., 2003) was suggested.

Indexes that combine CRP and CP with other proteins were the indexes that showed a great percentage of decrease and were statistically significant. Among these, the index CRP*CP/Alb was selected as the optimum since it showed a greater and faster decrease (78% decrease on day 10) compared with the others. Moreover, the magnitude of the decrease of that index on day 10 was greater than that reported for individual acute phase proteins (44% for CRP and 58% for CP)(Martinez-Subiela et al., 2003a). This finding suggest that the combination of both positive acute phase proteins (CRP and Cp) with a negative one could have a greater potential as a biomarker to evaluate the early response to therapy in canine leishmaniasis compared to the individual proteins alone.

It should be pointed out that limitations in sensitivity and specificity have been described for acute phase proteins. It has been reported that although most dogs with leishmaniosis have high concentrations of the distinct acute phase proteins, sensitivities do not reach 100% (Martinez-Subiela et al., 2003a). However, the use of indexes in which different acute phase proteins are combined could provide better sensitivity. Regarding specificity, results of

individual APPs and acute phase indexes should be interpreted with caution due to the variety of disease conditions that can lead an acute phase response and therefore produce a change in the indexes calculated that masks the response to therapy in a dog.

In addition to the modifications reported in the acute phase proteins values, other abnormalities observed in the biochemical profile of dogs infected by *Leishmania* is a remarkable dysproteinemia related to hyperglobulinemia causing a pronounced albumin-globulin ratio inversion (Romdane et al., 1992; Denerolle, 1996; Ciaramella et al., 1997). The study of these modifications, has been reported to be useful for assessing the clinical course of the disease during and after suitable therapy (Romdane et al., 1992). In the present study, a dysproteinemia with a decrease in the albumin-globulin ratio was also observed before the treatment, however during the therapy the ratio only raised to 0.58 (5%) similar to the results obtained by Bourdoiseau et al. (1997) but in contrast with other studies that found a significant increase in this ratio after treatment (Amusategui et al., 1998). These results suggest that the albumin-globulin ratio should be used with caution to monitor the initial response to treatment and to establish its effectiveness, and confirm the need of new biochemical indexes, or ratios, for this purpose.

Results obtained in this study suggested that the use of the selected acute phase indexes, especially the CRP*CP/Alb index, could be an aid to suspect about leishmaniotic dogs and also to monitor their response to treatment. Further studies involving the use of more negative acute phase proteins and a larger number of dogs treated with other commonly used anti-leishmanial drugs, would be necessary to check the ability of the acute phase indexes for monitoring disease activity and responses in long-term treatment in canine leishmaniasis, and also to determine treatment cessation.

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