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Dynamics and Predictive Potential of Antibodies against Insect-Derived Recombinant *Leishmania infantum* Proteins during Chemotherapy of Naturally Infected Dogs

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Abstract. A predictive marker for the success treatment of canine leishmaniasis is required for the application of a more rational therapy protocol, which must improve the probability of cure and reduce *Leishmania* resistance to drugs. We investigated the dynamics and predictive value of antibodies against insect-derived recombinant *L. infantum* proteins rKMPII and rTRYP by using an enzyme-linked immunosorbent assay with retrospective serum samples from 36 dogs during treatment of canine leishmaniasis. In the entire group of dogs, concentrations of antibodies against rKMPII and rTRYP significantly decreased earlier than concentrations of antibodies against crude total *Leishmania* antigen (one versus six months), which suggested that the dynamics of antibodies against recombinant proteins may be useful for assessing clinical improvement after treatment. Interestingly, decreases in antibody concentrations against rKMPII occurred earlier in disease-free dogs than in dogs that remain clinically ill one year after beginning of treatment, which suggested that these antibodies may be useful for predicting disease-free survival one year after the beginning of therapy against canine leishmaniasis.

INTRODUCTION

Canine leishmaniasis is a fatal zoonotic disease caused by *Leishmania infantum* (*L. chagasi*) and transmitted by the bites of infected sand flies of the genera *Phlebotomus* and *Lutzomyia* in the Palearctic and Neotropic ecozones, respectively. In highly endemic foci around the Mediterranean Basin, an infection prevalence ranging from 65% to 80% has been reported in dogs.[1,2] Dogs are natural hosts and the major reservoirs of the parasite that causes human visceral leishmaniasis. Efforts to control canine leishmaniasis are a key factor in reducing transmission to humans and to other dogs.[3,4]

Infected dogs can develop a wide spectrum of *Leishmania*-specific immune responses, ranging from a predominantly cellular immune response (Th1-like) to a predominantly humoral immune response (Th2-like). In this sense, specific antibody levels against *Leishmania* antigen showed a marked correlation with parasite load and clinical status.[5,6] In *Leishmania*-infected dogs that are not able to control the infection, the parasite causes a chronic and systemic disease that culminates in death. The main clinical manifestations include poor general status, generalized muscular atrophy, enlarged lymph nodes, and nonpruritic scaling. Renal failure is the main cause of death in dogs with canine leishmaniasis.[7,8] Hypergammaglobulinemia, hypoalbuminemia, and increased concentrations of urea and creatinine are characteristic biochemical alterations of the disease.

Recently, the vaccines Leishmune® and Leish-Tec®[9,10] were granted marketing authorization against canine leishmaniasis by the Brazilian Ministry of Agriculture and Fisheries, although the Brazilian Ministry of Health recommended they should not be used in the governmental control program until phase III studies have finished. In the absence of a commercially available vaccine in other disease-endemic countries, control of the parasite is restricted to preventing sand fly bites and treating infected dogs. Several agents has been assayed to treat canine leishmaniasis,[11] although the most commonly used is a combination of meglumine antimoniate and allopurinol.[12] Successful chemotherapy has been correlated with a decrease in *Leishmania*-specific antibody levels, restoration of parasite-specific cell mediated immunity, and a reduction of parasite burden.[6,13–17] However, drugs used to treat leishmaniasis have not been able to provide a sterile cure; although temporary clinical remission is normally achieved, relapses are frequent after use of the drug is stopped,[18] thus increasing the risk of parasitic resistance.[19,20]

Research into a marker capable of predicting the success of therapy against *Leishmania* is required to develop a more rational therapy protocol, which must improve the probability of cure and reduce *Leishmania* resistance to drugs. Many studies have reported a significant decrease in levels of specific antibodies (mainly IgG) against crude *Leishmania* antigen in the follow-up of treated infected dogs.[15,16,21] This decrease in concentrations has been described in responsive and in nonresponsive dogs.[8,22] Therefore, seroreactivity against crude total parasite antigen cannot predict the outcome of treatment. However, it is unknown whether the decrease in antibody concentrations is general (all *Leishmania* antigens) or specific. Studies using recombinant proteins might make it possible to investigate this area and exploit the results to develop new and predictive tools to manage this disease.

We recently analyzed the usefulness of enzyme-linked immunosorbent assays (ELISAs) based on insect-derived rKMPII, rTRYP, and rLACK antigens for the serodiagnosis of leishmaniasis in dogs; these assays showed a sensitivity of 93% when used in parallel.[23] The aim of the present study was to describe the dynamics of the antibodies against the two antigens (rKMPII and rTRYP) and to investigate their usefulness in monitoring the therapeutic response and predictive potential in naturally infected dogs treated with meglumine antimoniate and allopurinol.

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MATERIALS AND METHODS

Recombinant KMPII and TRYP proteins. Recombinant proteins were obtained in baculovirus-infected *Trichoplusia ni* larvae as described.[23] Briefly, recombinant bacmids carrying *KMPII* and *TRYP* genes and an additional bacmid with non insert clones produced by the Bac-to-Bac® system (Invitrogen, Carlsbad, CA) were used to transfect *Spodoptera frugiperda* Sf21 cells to obtain to the recombinant and the wild-type baculovirus, respectively. *Trichoplusia ni* larvae were injected with the recombinant baculovirus preparations and incubated at 28°C for 96 hours. Thereafter, infected larvae were frozen immediately at −20°C and total protein was extracted. Larvae infected with the wild-type baculovirus were used to obtain the control raw protein extract (Ni) for the ELISA. Specific KMPII and rTRYP proteins in the raw larvae extracts were detected by sodium dodecylsulfate–polyacrylamide gel electrophoresis on a 15% polyacrylamide gel stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA) and quantified using a Tina 2.0 image analyzer software package (Raytest, Straubenhardt, Germany). Both recombinant proteins were observed as bands of the expected molecular mass: 11 kD for rKMPII, and 22 kD for rTRYP. Concentrations of specific recombinant proteins in raw larvae extracts were 1% for rKMPII and 0.5% for rTRYP.

Canine serum samples. Retrospective serum samples from 36 *Leishmania*-infected dogs were included in this study. These serum samples were kept in the LeishLab bank at −20°C until use. The animals were originally brought to the Veterinary Teaching Hospital of our institution during 2006–2008 with clinical manifestations compatible with canine leishmaniasis. The dogs were of different breeds, 19 males and 17 females, from 6 months to 11 years of age with a mean ± SD age of 4.7 ± 3.0 years. The diagnosis of leishmaniasis was confirmed by visualization of *L. infantum* organisms on bone marrow smears and by detection of specific antibodies by using a crude total *Leishmania* antigen (CTLA)–based ELISA performed as described.[15] Dogs were treated with meglumine antimoniate (Glucantime®; Sanofi-Aventis, Barcelona, Spain) at a dose of 50 mg/kg every 12 hours for 28 days and allopurinol (Zyloric; Faes Farma, Lieioa, Spain) at a dose of 10 mg/kg every 12 hours for 8 months.

The clinical status of the dogs was monitored at diagnosis and at 1, 6, and 12 months after the beginning of treatment. Clinical signs were obtained from clinical records maintained at the Veterinary Teaching Hospital.

At the same intervals, a sample of blood was collected from each dog for urea and creatinine determination and serum protein electrophoresis. Biochemical analyses were performed according to standard procedures at the Veterinary Clinical Biochemistry Service of our institution. Bone marrow smears were not evaluated during follow-up or at the end of the study, and parasitologic status one year after treatment was started was not available.

One year after the beginning of treatment, the 36 dogs were classified into 2 groups based on the presence of clinical signs. Group A was composed of 18 dogs that did not show clinical signs and that were considered disease-free survivors. Group B was composed of 18 dogs that showed clinical signs compatible with leishmaniasis at the end of the study.

Crude and recombinant antigen–based ELISA. Serum samples obtained at diagnosis, 1, 6, and 12 months after the beginning of treatment were analyzed for the presence of antibodies against CTLA, rKMPII, and rTRYP by ELISA. The ELISAs were performed as described.[15,23] Briefly, microtiter plates were coated with CTLA, raw protein larva extracts containing rKMPII or rTRYP, and the corresponding control antigen Ni prepared at the same concentration (Ni_{KMPII} and Ni_{TRYP}), respectively. Serum samples were diluted 1:400 for detection of antibodies against CTLA, 1:200 for antibodies against KMPII, and 1:800 for antibodies against TRYP in phosphate-buffered saline, 0.05% Tween 20, 1% dried skimmed milk. Horseradish peroxidase–conjugated Protein A was used as a secondary antibody. Absorbance values were read at 492 nm. Serum samples were tested under identical conditions against protein raw larva extracts containing rKMPII and rTRYP and control antigen Ni. A pool of known CTLA-positive serum samples and a pool of negative serum samples from nonendemic area were included in all plates as a positive control and a negative control, respectively. A known positive serum sample used as calibrator (approximately one optical density value) was included in all plates, and plates with interassay variations ≥ 10% were not used.

Results were expressed as optical densities. For ELISAs using recombinant antigens, absorbances were corrected by subtracting the absorbance achieved by the serum sample on the control antigen Ni extract from that achieved on the protein larva extract containing specific recombinant antigen.

Data analysis. Statistical analysis was performed by using nonparametric tests with SPSS version 14.0 (SPSS, Inc., Chicago, IL). Differences in antibody median levels were analyzed by using the Wilcoxon test for paired samples and the Mann-Whitney test for unpaired samples. The Spearman rank test was used to evaluate correlations between variables. The chi-square and Fisher's exact tests were used to compare discrete variables. A P value < 0.05 was considered statistically significant.

RESULTS

Serorecognition of recombinant *Leishmania* proteins by dogs with canine leishmaniasis at diagnosis. Twenty-nine (80.5%) of the 36 serum samples were positive for at least one of the recombinant proteins at diagnosis. Individually, 28 serum samples were positive for rKMPII (77.7%), and 20 to rTRYP (55.5%). Concentrations of antibodies against CTLA, rKMPII, and rTRYP in seropositive dogs to each antigen at diagnosis are shown in Figure 1.

No statistically significant association was found between the presence or absence of seroreactivity against these antigens at the time of diagnosis and presence or absence of clinical signs one year after the beginning of therapy (P = 0.067 for rKMPII and P = 0.056 for rTRYP, by chi-square test). No significant differences were found in concentrations of specific antibodies against recombinant antigens or CTLA at the time of diagnosis between dogs from group A or B (P = 0.372 for rKMPII, P = 0.521 for rTRYP, and P = 0.293 for CTLA, by Mann-Whitney U test).

Description and evolution of clinical signs recorded in dogs at diagnosis and one year after initiation of treatment. The number of clinical signs recorded at diagnosis are shown in Table 1. The mean ± SD number of clinical signs recorded in each dog at diagnosis was 4 ± 2. Post-diagnosis examinations showed that all animals improved. The mean ± SD number of

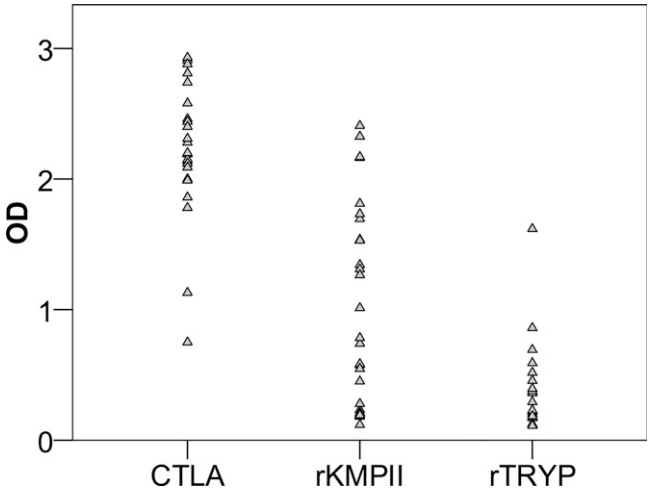

FIGURE 1. Concentrations of antibodies against crude total *Leishmania* antigen (CTLA), and *Trichoplusia ni*-derived rKMPII and rTRYP in serum samples from 36 seropositive dogs with canine leishmaniasis at diagnosis. The cut-off values (mean + 3 SD for 76 dogs from non-endemic areas) were optical densities of 0.180 for CTLA, 0.100 for rKMPII, and 0.082 for rTRYP.

clinical signs for the 36 dogs decreased after treatment and were 2.4 ± 1.9 at 1 month, 2.1 ± 2.6 at 6 months, and 2.7 ± 1.9 at 12 months. However, a total remission of clinical signs was not observed in all animals, and, one year after treatment was started, dogs from group B still showed nonpruritic scaling (n = 10), lymphadenomegalia (n = 6), weight loss (n = 2), asthenia (n = 2), abnormal locomotion (n = 3), eye disease (n = 1), and signs of urinary disease (n = 4). Two of these four dogs also had uremia and creatinemia, which indicated renal failure.

Correlation between concentrations of specific antibodies against recombinant or CTLA antigens, number of clinical signs and biochemical parameters at diagnosis. A significant correlation was found between concentrations of antibodies against rKMPII and rTRYP and the number of clinical signs at diagnosis (Spearman's $\rho = 0.420$, $P = 0.011$ and $\rho = 0.435$, $P = 0.008$, respectively). Conversely, the correlation between concentrations of antibodies against CTLA and the number of clinical signs was not significant (Spearman's $\rho = 0.213$, $P = 0.213$).

Concentrations of antibodies against rKMPII were significantly correlated with concentrations of gammaglobulins (Spearman's $\rho = 0.404$, $P = 0.030$) and significantly negatively correlated with concentrations of serum albumin (Spearman's $\rho = -0.415$, $P = 0.022$). Likewise, concentrations of antibodies against TRYP were also significantly correlated with concentrations of gammaglobulins (Spearman's $\rho = 0.463$, $P = 0.011$) and significantly negatively correlated with the A:G ratio (Spearman's $\rho = -0.399$, $P = 0.032$). No other significant correlation was found between concentrations of antibodies against rKMPII or rTRYP and the other biochemical parameters evaluated. Concentrations of antibodies against CTLA did not show a significant correlation with any of the biochemical parameters evaluated.

TABLE 1
Clinical signs recorded in 36 *Leishmania*-infected dogs at diagnosis

Clinical sign	No. (%) dogs
Skin involvement	22 (61.1)
Weight loss	20 (55.5)
Lymphadenomegalia	18 (50)
Asthenia	13 (36.1)
Eye disease	10 (27.7)
Abnormal locomotion	9 (25)
Urinary disease	7 (19.4)
Hyperthermia	6 (16.6)
Vomiting or diarrhea	6 (16.6)
Epistaxis	3 (8.3)
Pale mucoses	2 (5.5)

Dynamics of specific antibodies against recombinant *Leishmania* proteins and CTLA in infected dogs during follow-up. Dynamics of specific antibodies against recombinant *Leishmania* proteins were analyzed in dogs showing specific antibody concentrations against these proteins at diagnosis (36 dogs for CTLA, 28 dogs for rKMPII, and 20 dogs for rTRYP).

When the entire group of dogs was analyzed, a significant decrease in the concentrations of antibodies against rKMPII and rTRYP was detected from the first month of treatment and thereafter (rKMPII, diagnosis versus first month after beginning of treatment [T1]; $P = 0.004$, diagnosis versus sixth month after beginning of treatment [T6]; $P < 0.001$, and diagnosis versus 12th month after beginning of treatment [T12]; $P < 0.001$, by Wilcoxon signed-rank test; rTRYP, diagnosis versus T1; $P < 0.001$, diagnosis versus T6; $P = 0.002$, and diagnosis versus T12; $P < 0.001$, by Wilcoxon signed-rank test).

As for concentrations of antibodies against CTLA, the first significant decrease was detected six months after the beginning of treatment (diagnosis versus T1; $P = 0.647$, diagnosis versus T6; $P = 0.006$, and diagnosis versus T12; $P < 0.001$, by Wilcoxon signed-rank test).

The number of dogs that seroreverted one year after the beginning of treatment was 16 (57.14%) of 28 for rKMPII, 17 (85%) of 20 for rTRYP, and 2 (5.55%) of 36 for CTLA. The proportion of dogs that seroreverted to recombinant antigens was significantly higher than the proportion that seroreverted to CTLA ($P < 0.001$ for rKMPII and rTRYP).

Relationship between disease-free survival and dynamics of specific antibodies against recombinant *Leishmania* proteins and CTLA during follow-up. Only dogs showing specific antibodies against these proteins at diagnosis (36 dogs for CTLA, 28 dogs for rKMPII and 20 dogs for rTRYP) were included in the analysis. Concentrations of specific antibodies against rKMPII decreased significantly one month after the beginning of treatment in dogs from group A ($P = 0.028$, by Wilcoxon signed-rank test). In contrast, the first significant reduction detected in dogs from B group occurred six months after treatment was started ($P = 0.005$). Concentrations of specific antibodies against rTRYP decreased significantly one month after the beginning of treatment in both groups of dogs ($P = 0.012$ and $P = 0.010$, respectively). As for concentrations of specific antibody against CTLA, these concentrations did not decrease significantly until six months after the beginning of treatment in disease-free survivor dogs and in dogs that remain clinically ill ($P = 0.028$ and $P = 0.016$, respectively). Results are shown in Figure 2.

In the disease-free survivor group (A), 7 of 13, 6 of 9, and 2 of 18 dogs seroreverted one year after the beginning of treatment to rKMPII, rTRYP, and CTLA, respectively. In group B, serorevertion was observed one year after the beginning of treatment in 9 of 15, 11 of 11, and 0 of 18 dogs to rKMPII, rTRYP, and CTLA, respectively. The proportion of dogs that seroreverted was not significantly different

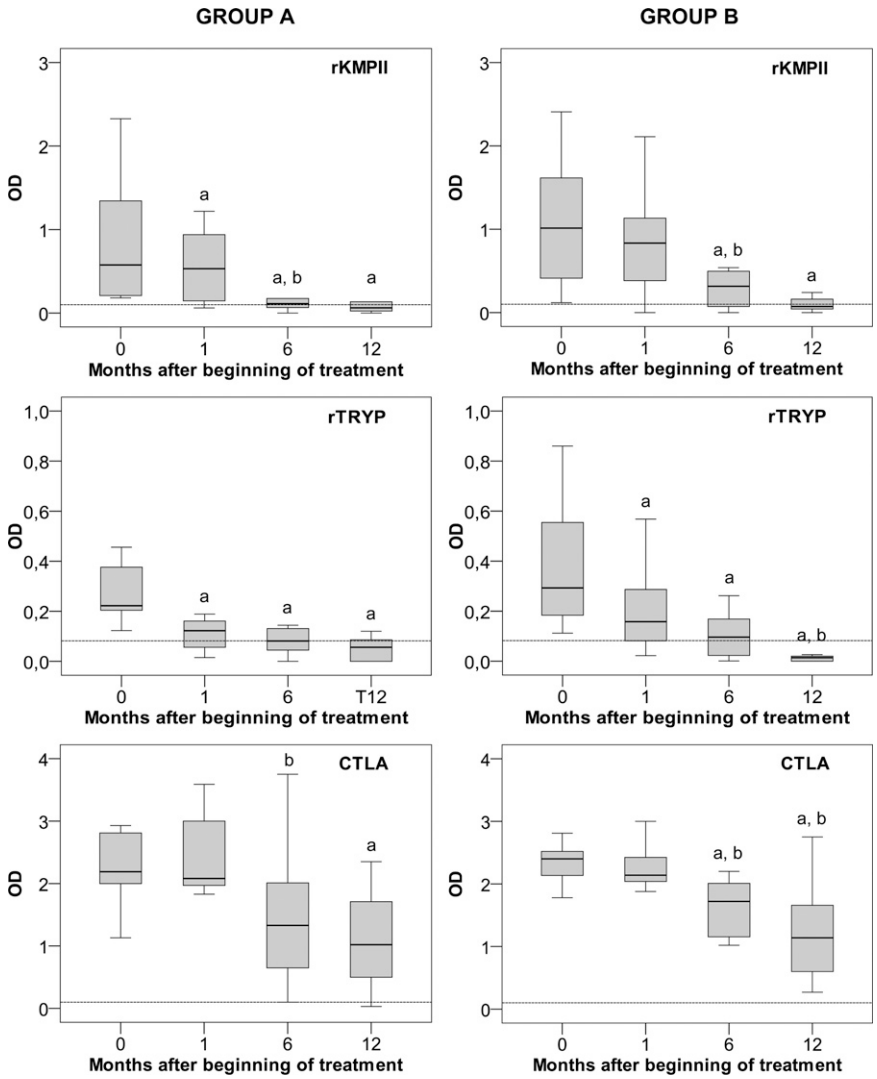

FIGURE 2. Dynamics of antibodies against recombinant proteins rKMPII, rTRYP, and crude *Leishmania* antigen (CTLA) in 18 dogs from group A (disease-free survivor dogs) and 18 dogs from group B (dogs remaining clinically ill) at one year after beginning of treatment for canine leishmaniasis with meglumine antimoniate and allopurinol. The x-axis represents months after beginning of treatment. a, $P < 0.05$, by Wilcoxon test, when compared with concentrations obtained at diagnosis. b, $P < 0.05$, by Wilcoxon test, when compared with concentrations obtained at the previous check-up. The dotted lines represent the cut-off values for each enzyme-linked immunosorbent assay.

between groups A and B ($P \geq 0.050$, by chi-square test for all comparisons).

DISCUSSION

Canine leishmaniasis is a widespread and severe zoonotic disease caused by *L. infantum*. The inefficacy of drugs used to treat this disease by completely eliminating the parasite stresses the need for follow-up and predictive markers that enable treatment to be tailored to the patient. In this study, we analyze the usefulness of two recombinant antigen-based ELISAs in monitoring and predicting the clinical success of the most frequently used therapy against leishmaniasis, the combination of meglumine antimonite and allopurinol, in dogs with canine leishmaniasis.

At diagnosis, antibodies against rKMPII showed the highest seroprevalence among the *L. infantum* antigens studied, thus supporting previous results that indicated that this antigen acts as a potent B-cell immunogen in dogs.[23,24] Seropositivity against rTRYP was also significant because it was detected in half of the dogs studied, as found elsewhere.[23] When the two recombinant antigen-based ELISAs were evaluated in parallel, 80% of dogs with leishmaniasis showed seroreactvity against rKMPII or rTRYP. In addition, concentrations of antibodies against rKMPII and rTRYP correlated significantly with canine leishmaniasis–related biochemical parameters (hypergammaglobulinemia and hypoalbuminemia) and with the number of clinical signs recorded in dogs. These results suggest that detection of antibodies against KMPII and TRYP antigens could be components of a standardized tool for the diagnosis of canine leishmaniasis.

When the entire group of dogs was considered, the results show that treating dogs with leishmaniasis leads to a clinical improvement and a parallel significant decrease in concentrations of specific antibodies against rKMPII, rTRYP, and CTLA one year after the beginning of treatment. However, concentrations of antibodies against rKMPII and rTRYP decreased significantly as early as one month after treatment was started,

at the same time that the first reduction in the number of clinical signs, whereas the decrease in concentrations of antibodies against CTLA occurred at six months. In addition, the proportion of dogs that seroreverted one year after the beginning of treatment to recombinant proteins was significantly higher than those that seroreverted for CTLA. Thus, our results suggest that the dynamics of antibodies against recombinant proteins may be more useful than the dynamics of antibodies against CTLA for assessing clinical improvement after treatment, as described in human patients treated for visceral leishmaniasis. In accordance with our results, antibodies against the recombinant proteins rKMPII[25] and *Lic*TXNPx, a protein similar to TRYP,[26] decreased significantly after treatment of patients with visceral leishmaniasis, conversely to serologic results with crude *Leishmania* antigen.

A significant reduction in concentrations of antibodies against CTLA has been extensively reported in dogs with leishmaniasis after treatment.[8,15,21,22] This reduction is thought to be related to attenuated antigenic stimulation resulting from the decrease in parasite load. Our results might indicate that antigens such as rKMPII or rTRYP could be more sensitive than CTLA in detecting early reduction of *Leishmania* load in treated dogs, although the presence of seropositive dogs for these recombinant proteins at the end of the study could indicate persistence of subclinical infection.[14,15,21] Unfortunately, parasitologic data for these dogs during follow-up or at the end of the study were not available.

Because of the lack of parasitologic data, dogs were split in two groups according to only their clinical status one year after the beginning of therapy. We evaluated the value of rKMPII, rTRYP, and CTLA as predictive markers for long-term disease-free survival in dogs with canine leishmaniasis. One month after treatment was started, concentrations of antibodies against rKMPII had decreased significantly in disease-free survivor dogs. Conversely, in those remaining clinically ill, concentrations of antibodies against rKMPII did not decrease significantly until six months after the beginning of therapy. Thus, the dynamics of antibodies against rKMPII might be an early predictive marker for long-term disease-free survival after therapy for canine leishmaniasis.

Different profiles of antibodies against rKMPII between the two groups of dogs could be caused by different parasite loads related to different immune responses, but further studies are needed to confirm this possibility. Quantification of parasite burden in bone marrow samples by real-time polymerase chain reaction and further immune characterization could be helpful for answering this question. Information from other studies that used bone marrow aspiration showed that there was no sterile cure in most treated dogs, despite clinical improvement.[14,15,21,27] Thus, the possibility of relapse as an epidemiologic risk[28,29] still exists in disease-free survivor dogs, although to a lesser extent. For this reason, it has been recommended to use preventative measures against parasite transmission in animals and to monitor them on a regular basis to assess future clinical relapse.[30]

Concentrations of antibodies against CTLA did not decrease significantly until six months after the beginning of therapy, and this decrease occurred in disease-free survivors and dogs that remained clinically ill. Regarding concentrations of antibodies against rTRYP, although the decrease occurred one month after treatment was started, this decrease was observed in both groups of dogs. Consequently, ELISAs for CTLA[8,22] and rTRYP could not predict long-term clinical success of therapy.

Some parameters have been suggested as predictive markers of clinical outcome in canine leishmaniasis,[8,31,32] although, to date, none has been used in veterinary clinical practice. Dogs showing high levels of IgG against *Leishmania* at diagnosis showed a poor prognosis after chemotherapy.[8] In addition, several studies have reported different dynamics of antibodies against *Leishmania*-specific antigens between responsive and nonresponsive dogs. In accordance with our results, Western blot showed a reduction in the intensity of low molecular mass (12–30 kD) bands in serum samples of dogs that showed clinical improvement after treatment against *Leishmania*. In contrast, persistence of 14-kD, 24-kD, and 29-kD bands has been related to persistence of the parasite and a potential unfavorable prognosis.[32] Interestingly, two studies have described specific seroreactivity against a band of 26 kD, approximately the molecular mass weight of rTRYP, in untreated dogs in the acute phase of disease and in unsuccessfully treated dogs,[31,33] thus suggesting that such seroreactivity can be a marker for active canine leishmaniasis and a potential prognostic marker.

The results reported in the present study suggest that ELISAs based on the insect-derived antigens rKMPII and rTRYP from *L. infantum* may be useful for assessing clinical improvement after treatment of canine leishmaniasis. Also, dynamics of antibodies against rKMPII could be useful for predicting long-term disease-free survival after one year of the beginning of therapy against this parasitic disease in dogs.

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