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SERUM PROTEIN PROFILE AS A BIOMARKER IN DIAGNOSING LEISHMANIOSIS AND MONOCYTIC EHRLICHIOSIS IN DOGS

PERFIL SÉRICO DE PROTEÍNAS COMO BIOMARCADOR NOS DIAGNÓSTICOS DA LEISHMANIOSE E DA ERLIQUIOSE MONOCÍTICA EM CÃES

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SUMMARY

Diagnosing Canine Visceral Leishmaniosis is challenging for veterinarians given that its hematological and biochemical abnormalities greatly resemble those of other illnesses of dogs, such as canine ehrlichiosis, which is caused by *Ehrlichia canis* and is highly prevalent with high pathogenicity. The aim of this study was to determine occurrences of serological positivity for the antigens of *Ehrlichia canis* and *Leishmania infantum* and its relationship to globulin concentrations in samples from dogs. Out of 93 samples tested, 12.9% were negative for the antigens of both *L. infantum* and *E. canis*; 33.3% were seropositive for both antigens. Discordant results were found from 18.3% that were positive only for *L. infantum*, while 35.5% were positive only for *E. canis.* Hyperglobulinemia was observed in 88.2% and the statistical analysis showed that there was a significant relationship between the high levels of globulins and seropositivity for the antigen of *E. canis*. However, the relationship between positivity for the antigen of *L. infantum* and hyperglobulinemia, showed that there was no statistically significant relationship between the two laboratory findings. From these results, it can be concluded that concomitant infections occur frequently and that hyperglobulinemia is more closely related to ehrlichiosis than to leishmaniosis, in dogs.

KEY-WORDS: Dogs. *Ehrlichia canis.* Globulin. *Leishmania infantum*. Protein. Serology.

RESUMO

O diagnóstico da leishmaniose canina é um desafio ao Médico Veterinário visto que anormalidades hematológicas e bioquímicas muito se assemelham à outras enfermidades dos cães, como a erliquiose canina, causada pela *Ehrlichia canis*, muito prevalente e de alta patogenicidade. O objetivo deste estudo foi determinar a positividade sorológica frente aos antígenos de *Ehrlichia canis e Leishmania infantum e* a sua relação com as concentrações de globulinas em amostras de cães. Das 93 amostras testadas, 12,9% foram negativas frente aos antígenos de *E. canis e L. infantum* e 33% foram soropositivas. Resultados discordantes foram encontradas em 18,3% positivas apenas para *L. infantum*, enquanto que 35,5% reagiram apenas frente a *E. canis.* Hiperglobulinemia foi observada em 88,2% das amostras e a análise estatística mostrou haver relação significante entre a soropositividade frente aos antígenos de *E. canis* e a hiperglobulinemia, e em contrapartida, a relação entre esse aumento de globulinas e a positividade para *L. infantum* foi insignificante. Pelos resultados apresentados, pode-se concluir que as infecções concomitantes são frequentes e que a hiperglobulinemia está mais intimamente relacionada a erliquiose quando comparada à leishmaniose em cães.

PALAVRAS-CHAVE: Cães. *Ehrlichia canis.* Globulina. *Leishmania infantum*. Proteínas. Sorologia.

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INTRODUCTION

Visceral leishmaniosis (VL) is a neglected tropical parasitic disease with high morbidity and mortality. The World Health Organization (WHO) has estimated that 1.3 million cases occur every year. It has been noted that VL is a reemerging disease, mainly in peripheral and central urban areas associated with worse infrastructure and poorer environments (MARCHI et al., 2019). The main vector responsible for transmission of the disease in Brazil is the phlebotomine sandfly *Lutzomyia* sp, which presents a broad distribution extending from Northeast to Southeast Region (ANDRADE-FILHO et al., 2017). In endemic areas, the prevalence of infection with *L. infantum* in dogs may reach 67%, but fewer than 10% of infected individuals show clinical signs (BAXARIAS et al., 2018).

The nonspecific clinical manifestations of visceral leishmaniosis in dogs may confound veterinarians, since these are common to several infectious diseases. Molecular tests can establish the correct diagnosis in symptomatic and asymptomatic dogs with VL (CARVALHO et al., 2018), but these tests are expensive and time consuming. The serum protein levels are important components of the immune system response that change in concentration due to the inflammatory process in dogs with infectious diseases (TOTHOVA et al., 2016). Determination of changes could be and indicative of potential diagnostic markers of some pathological conditions.

According to Castro et al. (2012), in case of canine VL, the albumin-globulin ratio in symptomatic animals is lower than in asymptomatic animals. However, several infectious diseases caused by agents that invade blood cells present similar clinical manifestations and this may lead to diagnostic confusion. There is therefore a need to seek serological tests of greater specificity, along with refinement of interpretation of these examinations. This is especially so, with regard to diagnosing canine visceral leishmaniosis (CVL), given that animals diagnosed with this disease in Brazil will be euthanized as a control measure, determined by the health authorities.

MATERIAL AND METHODS

Samples

Ninety-three blood samples from asymptomatics dogs were collected for an epidemiological survey of Canine Leishmaniosis in the municipality of Sorocaba, SP, Brazil (23° 30' 7" South; 47° 27' 28" West). This municipality is considered to present canine transmission of VL. All dogs had been naturally exposed to infection by *Ehrlichia canis* and *L. infantum*, because vectors *Rhipicephalus sanguineus* and *Lutzomyia longipalpis* are commom in this area. The present study had previously been approved by the Ethics Committee for Use of Animals in Research (CEUA-UNISO no. 129/2019). Blood samples were collected intravenously and serum was then obtained through centrifugation of the whole blood for 15 minutes at 1500 rpm. These samples were then subjected to biochemical assays on serum proteins and to serological tests to detect anti-*Ehrlichia canis* and anti-*Leishmania infantum* antibodies.

Assays on serum proteins and fractions

Protein assays were performed on the serum samples using the biuret method (Bioclin® k-030) and albumin concentrations were assessed using the bromocresol green method (Bioclin ® k-040), in accordance with the kit manufacturer's instructions. Globulin values were obtained by subtracting the albumin value from the total protein total (KERR, 2002). Samples with concentrations greater than 5.1g/dl were considered to be hyperglobulinemic.

Serological tests

Indirect enzyme-linked immunosorbent assay (ELISA)

Anti-*E. canis* antibodies were detected using a canine immunological ELISA test kit for *Ehrlichia* (Imunodot Diagnósticos®). The technique was conducted in accordance with the manufacturer's instructions.

TR-DPP® Dual Path Platform

To detect anti-*Leishmania infantum* antibodies, the qualitative immunochromatographic test produced by BioManguinhos (FIOCRUZ, BP) was used. The antigen for this test is the recombinant proteins K26/K39. The method recommended by the manufacturer was used in the present study.

The samples were divided into four groups (G1, G2, G3 and G4), as described below:

- G1: samples that were seronegative for the antigens of both *L. infantum* and *E. canis*
- G2: samples that were seropositive for the antigens of *L. infantum* and *E. canis*
- G3: samples that were seropositive for the antigen of *L. infantum* and seronegative for the antigen of *E. canis*
- G4: samples that were seronegative for the antigen of *L. infantum* and seropositive for the antigen of *E. canis*.

Statistical analysis

The analysis on relationship of serological results to *E. canis* and *L. infantum* antigens, and the relationship of seropositive and increased globulins samples was done using the Fisher's Exact Test, in StatPlus from AnalystSoft Statistical Analysis Program. Version v7. The relationship was considered significant when p-value≤0.05.

RESULTS

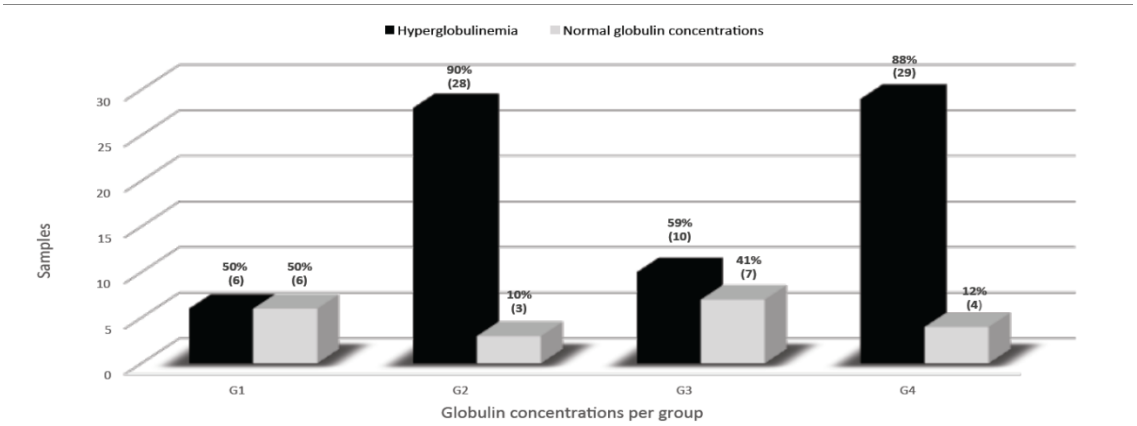
Among all the samples analyzed, 12 (12.9%) were negative for the antigens of both *L. infantum* and *E. canis* (G1); and 31 (33.3%) were seropositive for both antigens (G2). Discordant results were found from 17 samples (18.3%) that were positive only for *L. infantum* (G3), while 33 (35.5%) were positive only for *E. canis* (G4) (Table 1). No significant correlation was found between *L. infantum* and *E. canis* infection (p=0.381).

Table 1 - Ninety-three blood samples from asymptomatic dogs that had been naturally exposed to infection by *Ehrlichia canis* and *Leishmania infantum* distributed into four groups (G1, G2, G3 and G4) according to serological reactions.

		E. canis							
		Negative			Positive				
		G	No.	%	G	No.	%	Total	p
L. infantum	Negative	G1	12	12.9	G4	33	35.5	45 (48.4%)	0.38109
	Positive	G3	17	18.3	G2	31	33.3	48 (51.6%)	
Total			29	31.2		64	68.8	93 (100%)	

G1: samples that were seronegative for the antigens of both *L. infantum* and *E. canis* ; G2: samples that were seropositive for the antigens of *L. infantum* and *E. canis*; G3: samples that were seropositive for the antigen of *L. infantum* and seronegative for the antigen of *E. canis;* G4: samples that were seronegative for the antigen of *L. infantum* and seropositive for the antigen of *E. canis*.
p = p-value by Fisher's Exact Test

High globulin concentrations were observed in 73 (78.49%) of the 93 serum samples tested. Among the 12 samples of group G1 (co-negative samples), hyperglobulinemia was observed in 6 (50%) of them. This was also seen in 28 (90%) of the samples in group G2 (positive for both antigens tested). Analyses on groups G3 and G4 showed hyperglobulinemia in 10 (59%) among the samples positive only for *L. infantum* (G3), while in the 33 samples positive only for *Ehrlichia canis* the globulin levels were high in 29 (88%) of them, as shown in Figure 1.



G1: samples that were seronegative for the antigens of both *L. infantum* and *E. canis* ; G2: samples that were seropositive for the antigens of *L. infantum* and *E. canis* ; G3: samples that were seropositive for the antigen of *L. infantum* and seronegative for the antigen of *E. canis;* G4: samples that were seronegative for the antigen of *L. infantum* and seropositive for the antigen of *E. canis*.

Figure 1 - Comparision between hiperglobulinemic and normal samples tested for the antigens of *Ehrlichia canis* and *Leishmania infantum*, divided into four groups (G1, G2, G3 and G4) according to serological results.

The statistical analysis was done to evaluate if there is a relationship between seropositivity, to *L. infantum* or *E. canis* antigens, and globulins measurement. The results suggested that hyperglobulinemia was not correlated with the detection of anti-*L. infantum* antibodies (p=1.000). On the other hand, the statistic result pointed out that the hyperglobulinemia was dependent of anti-*E. canis* antibodies detections (p=0,00064) (Table 2).

Table 2 - Ninety-three blood samples from asymptomatic dogs that had been naturally exposed to infection by *Ehrlichia canis* and *Leishmania infantum* divided into hyperglobulinemic and normal globulinemic samples.

	Serology	Hyperglobulinemic samples		Normal globulinemic samples		Total		p
		No.	%	No.	%	No.	%	
L. infantum	Positive	38	40.85	10	10.75	48	51.60	1.00000
	Negative	35	37.65	10	10.75	45	48.40	
Total		73	78.50	20	21.50	93	100	
E. canis	Positive	57	61.30	7	7.52	64	68.82	0.00064
	Negative	16	17.20	13	13.98	29	31.18	
Total		73	78.50	20	21.50	93	100	

p = p-value by Fisher's Exact Test

DISCUSSION

Similar clinical signs and laboratory test abnormalities between canine leishmaniosis and canine ehrlichiosis frequently give rise to diagnostic confusion and cause difficulty in differentiating between cross-reactions and coinfections. In the present study, the concomitant positive reaction to *E. canis* and *L. caninum* antigens was observed in 33.3% samples, which was close to the prevalence that had been found in other areas from Brazil (OLIVEIRA et al. 2008; RIBEIRO et al., 2019). Despite the serpositivity to both antigens and the frequent presence of arthropod vectors *Lutzomyia longipalpis* and *Rhipicephalus sanguineus* (responsible for transmission of VL and canine ehrlichiosis respectively) in this region (DANTAS-TORRES & OTRANTO, 2014; ANDRADE-FILHO et al, 2017) there was not significant correlation between *L. infantum* and *E. canis* infection and therefore one disease do not increase the risk of another. However, it is important to emphasize that all dogs seemed to be asymptomatic then they could be infected previously or co-infected but in the incubation period at the time of the test. Both of these diseases are systemic and, when they occur together, the pathogenicity becomes worse. There is higher prevalence of clinical development of VL when it occurs together with canine ehrlichiosis because of the alterations to the host's immunological response that are observed (TOEPP et al., 2019).

Tests to detect IgG antibodies could not determine if the infection is in course or if it has already been resolved (PALTRINIERI et al, 2016), and cross-reactions with other agents have already been demonstrated through using ELISA (SANTAREM et al., 2020). However, Brazilian Health Programs considerer the serological tests results to determine the control strategy of canine leishmaniosis. KASZAK et al. (2015) compared serological tests for *E. canis*, *Babesia canis* and *L. infantum* and proved statistically that coinfection was more plausible than cross-reactions. They suggested that the explanation for this lay in the taxonomic classification of these species of pathogens, considering that *E. canis* is a bacterium while *B. canis* and *L. infantum* are protozoa. Coinfections were also explained by seropositivity found in other serological tests for *L. infantum* and *E. canis* (OLIVEIRA et al., 2008; MEKUZAS et al., 2009).

Evaluation of serum proteins is a method that aids in diagnosing and monitoring various infections. In dogs with naturally-occurring VL albumin is considered a negative acute phase protein (APP), and when analyzed with others APP levels could help to identify animals in transition from one stage of leishmaniosis to another (CERON et al., 2018). Among the samples tested in the present study, it was observed that 78,50% presented serum protein concentrations that had been increased through hyperglobulinemia, and this finding was common to all the groups, but no relationship between this hyperglobulinemia and seropositivity for the antigen of *L. infantum* was observed. Some studies have indicated that hyperglobulinemia is frequently found due to polyclonal humoral immune response from B lymphocytes in *L. infantum* however, increased gamma-globulin levels may be due to other circulating antibodies, immune complexes or, additionally, other molecules of similar mass (PALTRINIERI et al, 2016).

It should be emphasized that 90% of the sample that were concomitantly positive for *L. infantum* and *E. canis* presented hyperglobulinemia, which may have been associated with the immune response to the two agents or, especially, to infection by *E. canis*. High globulin concentrations presented a significant relationship with seropositivity for *E. canis*, corroborating with the findings of previous studies (MARCONDES et al. 2006) and may be associated with a monoclonal or polyclonal gammopathy in *E. canis* infections leading to a diminished albumin-globulin ratio (NETO, 2011; KATAOKA et al., 2008).

CONCLUSION

Diagnosing VL remains a challenge, despite the various direct and indirect detection tests that are available. The nonspecific clinical signs, prolonged incubation period and high prevalences of vector-borne diseases in Brazil give rise to diagnostic confusion, especially in regions that are endemic for canine VL. From these results, it follows that concomitant infections occur frequently and that hyperglobulinemia is more closely related to infection by *E. canis* than to infection by *L. infantum*, however studies in other endemic area with another serologic and molecular tests are necessary.

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