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Balikci, Canberk; Ural, Kerem
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Artículos Originales

Evaluation of Cardiopulmonary biomarkers during different stages of Canine Visceral Leishmaniasis

Evaluación de biomarcadores cardiopulmonares durante diferentes etapas de Leishmaniasis visceral canina

Canberk Balikci
University of Adnan Menderes, Turquía
canberkbalikci@gmail.com

Kerem Ural
University of Adnan Menderes, Turquía
canberkbalikci@gmail.com

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Abstract:

Objective. The purpose of the present study was to test the hypothesis that cardiac alterations participate within different stages of CVL. **Materials and methods.** Dogs were diagnosed with CVL, were classified as follows; group I (mild disease), group II (moderate disease), group III (severe disease), group IV (very severe disease) and group V included healthy controls. **Results**. Ig G antibodies against Leishmaniasis in group as tested by IFAT, were deemed 1/64 to 1/16000 among infected groups. Considering the cTnI levels, there were significant differences (p=0.018) between stage IV (group IV) and healthy control group, besides between group IV and group I. Considering D-dimer levels, there was difference between healthy control group and group II, III and IV (p=0.005). Regareing NT-pro BNP levels, there were differences between healthy control group and stage III, IV, besides between stage I with stage III, IV (p=0.000). **Conclusions.** The results showed that levels of cTnI, Nt pro-BNP and D-dimer were higher in dogs infected with CVL in contrast to healthy dogs, in which levels of those biomarkers were below detection limits. Obtained results suggested the possibility of cTnI and NT pro-BNP as markers for cardiac damage and D-dimer as a supportive tool for a diagnosis of probable thromboembolism in dogs with CVL.

Keywords: Canine Visceral Leishmaniasis, cTnI, D-dimer, NT-proBNP.

Resumen:

Objetivo. El propósito del presente estudio fue probar la hipótesis de que las alteraciones cardíacas participan en diferentes estadios de CVL. **Materiales y métodos.** Perros fueron diagnosticados con CVL, se clasificaron de la siguiente manera; Grupo I (enfermedad leve), grupo II (enfermedad moderada), grupo III (enfermedad grave), grupo IV (enfermedad muy grave) y grupo V controles sanos incluidos. **Resultados**. Los anticuerpos Ig G contra la leishmaniasis en el grupo como probado por IFAT, se consideraron 1/64 a 1/16000 entre los grupos infectados. Considerando los niveles de cTnI, hubo diferencias significativas (p=0.018). Entre el grupo IV y el grupo control sano, además entre el grupo IV y el grupo I. Considerando los niveles D-dímero, hubo diferencia entre el grupo control sano y el grupo II, III y IV (p=0.005). Teniendo en cuenta los niveles de NT-proBNP, hubo diferencia estadística entre el grupo de control sano y el estadio III, IV, además entre la etapa I con estadio III, IV (p=0.000). **Conclusiones.** Los resultados mostraron que los niveles de cTnI, Nt pro-BNP y D-dímero fueron mayores en perros infectados con CVL en contraste con perros sanos, en los que los niveles de estos biomarcadores estaban por debajo de los límites de detección. Los resultados obtenidos sugirieron la posibilidad de que cTnI y NT pro-BNP como marcadores de daño cardíaco y D-dímero como una herramienta de apoyo para un diagnóstico de tromboembolismo probable en perros con CVL.

Palabras clave: Leishmaniasis Visceral Canina, cTnI, D-dímero, NT-proBNP |.

INTRODUCTION

Canine Visceral Leishmaniasis (CVL), one of the most significant zoonotic disease caused by Leishmania parasite (1), is transmitted to the canine species by the bite of blood-sucking sand flies (Phlebotomus species) (2). This systemic disease probably involve any organ, tissue/body fluid, that might have manifestations

within nonspecific clinical sings or by frequent clinical abnormalities (3). Clinical findings in relationship with cardiac involvement participated within the literature due to CVL (4), myocardial lesions might exist, thus the parasite could be detected in cardiac tissue (4,5).

According to the authors' knowledge none of the previous studies investigated intravital diagnosis of cardiac alterations. Therefore, the purposedof this study involving dogs naturally exposed to CVL were to a) in cardiopulmonary biomarkers such as cTnI, D-dimer and NT-proBNP concentrations, b) establish if different stages of CVL is contributing to cardiac failure (5).

MATERIALS AND METHODS

Site of study. The study was performed at Eagean part of Turkey in Aydin to those of dogs referred to clinics CVL disease associated symptoms, which were then subjected to further classification.

Grouping and classification of dogs with CVL. At the beginning of the study ethical guidelines were applied, and a written owner consent was available for all dogs involved. The research protocole was approved by the instutional laboratory animals ethics committee of Adnan Menderes University HADYEK (no: 64583101/2014/118, 29.08.2014). In total 35, 28 with CVL and 7 healthy, dogs were enrolled. CVL diagnosis based on was one or some of the clinical signs in association with the disease condition, verified to ELISA test kits and immune fluorescence antibody test. Dogs diagnosed with CVL, based on clinical, serological, hematological and biochemical findings, were classified into 4 different groups (n=7), as reported by Leishvet Group (6). In addition with healthy dogs, research groups were assigned in a subset of 5 major groups as follows;

group: stage I (mild disease)
group: stage II (moderate disease)
group: stage III (severe disease)
group: stage IV (very severe disease)
group: healthy controls.

Laboratory analysis performed necessary for classification of CVL were shown in table 1.

Cardiac evaluation.

Special reference to cardiopulmonary markers. Cardiac examination was performed [D-dimer, NT-proBNP and cnTI analysis] in dogs infected with leishmaniasis [for diagnosing, detecting presence/severity of cardiac injury] and to those of healthy dogs [comperatively].

Serum cTnI, D-dimer and NT-proBNP concentrations. Serum cTnI, D-dimer and NT-proBNP concentrations were determined at the Department of Internal Medicine, Faculty of Veterinary, University of Adnan Menderes, with a commercial analysis system (Wondfo Finecare Fluorescent Immunoassay) previously validated. Serum aliquots were stored till study analysis, and were thawed just prior to the moment of the analysis. Measurements below the lower limit of detection (0.1 ng/mL, 0.1 mg/L and 18 pg/L for cTnI, D-dimer and NT-proBNP respectively) were assigned this value for the statistical analysis. Linear range (min-max) of the Finecare Fluorescent Immunoassay was 0.1-50 ng/mL, 0.1-10 mg/L and 18-35000 pg/mL, respectively.

Statistical analysis. Statistical analysis was performed via SPSS 18.0 for Windows (SPSS, 2009). Arithmetic mean (X), standard deviation (s) and minimum-maximum (Xmin-Xmax) values were calculated. The present authors checked if its normally distributed. Two tests, Shapiro-Wilk or Kolmogorov–Smirnov, were run for normality. Even if parameters were not normally distributed were then verified with nonparametric methods. Comparison of parameters in more than 2 groups were checked by Kruskall-Wallis test, post-hoc pairwise comparisons.

RESULTS

Signalment. Dogs of 11 different breeds were enrolled (In group I; one Labrador retriever, Spanial cooker, Golden retriever and four crossbred. In group II; one Pointer, English mastiff, Terrier, German shepherd and three crossbred. In group III; Kangal, Terrier, Presa canario, Boxer, crosbred and two Dogo argentino. In group IV; Dogo argentino, Spaniel cocker, Boxer, Golden retriever, Cooker, Kangal and Golden retriever. In control group; Terrier, Dogo argentino, Kangal and four crossbred). Twenty three dogs were purebred, whereas 12 were of crossbred. Enrolled dog population consisted of 26 males and 9 females, distributed as following: Group I dogs: 3 males and 4 females; Group II: all male; Group III and IV each 6 males and 1 female and controls: 4 males and 3 females. The body weight ranged from 7 to 41 kg and did not vary significantly among the groups.

Clinical findings. Control group of dogs were subjected to physical examination and laboratory analysis aforementioned above and suggested as healthy. Based on laboratory parameters (Table 1) and presented clinical signs as shown in Table 2.

TABLE 1.
Table 1. Laboratory methods used in the present study

Symptoms	Groups				
	I	II	III	IV	V
High body temperature	0	0	1	2	0
Lymphadenopathy	7	7	7	7	0
Weight loss	3	5	7	7	0
Onychogripozis	2	3	3	4	0
Hypotrichosis	7	7	7	7	0
Periocular alopecia	0	1	1	1	0
Skin lesions	7	7	7	7	0
Epistaxis	0	0	0	2	0

TABLE 2.
Table 2. Number of cases showing clinical manifestations in infected groups.

Symptoms	Groups				
	I	II	III	IV	V
High body temperature	0	0	1	2	0
Lymphadenopathy	7	7	7	7	0
Weight loss	3	5	7	7	0
Onychogripozis	2	3	3	4	0
Hypotrichosis	7	7	7	7	0
Periocular alopecia	0	1	1	1	0
Skin lesions	7	7	7	7	0
Epistaxis	0	0	0	2	0

IFAT titers. The IFAT was positive for all dogs in the infected groups. IFAT titers according to groups and individual cases were shown in Table 3.

TABLE 3
Table 3. IFAT analysis results of groups

Group	I	II		III				IV					V
Case	All	1	2 3 4 5 6 7	1	2 3	4 5 6	7	1	2 3 4	5	6	7	All
IFAT	1/64	1/512	1/128	1/512	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8000	1/16000	0

cTnI results. There was no change in cTnI level observed in the control and I. group of the study, whereas 2 cases in the second group (0.09-0.01 ng/mL), 3 cases in the III. group (0.09-0.16 ng/mL) and 5 cases in the IV. (0.09-0.8 mg/L) group (Table 4) revealed elevated values. There was significant differences (p=0.018). between stage IV (group IV) and healthy control group, besides between group IV and group I.

TABLE 4.
Table 4. cTnI, D-dimer and NT-proBNP values of CVL-infected and control dogs according to groups

Data	Groups					
	I	II	III	IV	V	P
cTnI (ng/mL)	0.09±0a (0.09)	0.092±0.01a,b (0.09-0.01)	0.11±0.03a,b (0.09-0.16)	0.23±0.28b (0.09-0.8)	0.09±0a (0.09)	0.018
D-dimer (mg/L)	1.55±2.22a,b (0.09-6.1)	2.77±3.45b (0.09-10)	1.96±2.1b (0.4-5.7)	1.78±1.43b (0.4-4.2)	0.09±0a (0.09)	0.005
NT-pro BNP (pmol/L)	67.82±7.51a,b (61.9-78.9)	140.1±53.7a,b,c (80.2-225.6)	224.1±51.3c (152.6-272)	1355.2±791c (315.6-1939.8)	62.4±0.6a (61.9-62.9)	0.000
Mean ± standard deviation (minimum-maximum), Difference between groups according to letters a and b are shown						

D-dimer results. No change in D-dimer level was observed in the control group of the study, whereas 5 cases in the first group (0.2-6.1 mg/L), 6 cases in the II. group (0.6-10 mg / L), was detected in all cases in the III. (0.4-5.7 mg/L) and IV. (0.4-4.2 mg/L) group (Table 4). There was statistical difference between healthy control group and group II, III and IV (p=0.005).

NT-proBNP results. Considering the evaluation of pmol/L in studies on NT-proBNP in dogs, it was evaluated based on the conversion factor of 1 pg/mL = 0.118 pmol/L (7). No increase in NT-proBNP level was found in the I., II., III. and control groups of the study. An increase of 4 cases in the IV. group (1808.3-1939.73 pmol/L) was determined (Table 4). There was statistical difference between healthy control group and stage III, IV, besides between stage I with stage III, IV (p=0.000).

Urinalysis. UPC analysis revealed control group values were <0.1. On the other hand UPC values were <0.1-0.3 in G I, 0.5-1 in G II, 2-3 in G III, 5-10 in G IV. All values were shown in Table 5. Statistical difference was evident among control group and stage II to IV dogs (p=0.000)

TABLE 5.
Table 5. Values and statistical evaluation of the UPC analysis

	Groups					
Data	I	II	III	IV	V	P
UPC	0.15±0.1a,b (0.1-0.3)	2.4±0.5b (2.0-3.0)	2.4±0.5b (2.0-3.0)	6.9±2.2b (5.0-10.0)	0.09±0.0a (0.09-0.09)	0,000
Mean ± standart deviation (minimum-maximum) The differences between the groups according to letters a and b are shown.						

DISCUSSION

The present study was conducted with the financial and scientific (Adnan Menderes University Research Projects Funding Unit) support, in which updated staging of the disease was taken into consideration based on the crietaria by Leishvet Guidelines (6). Therefore it should not be unwise to draw conclusion that cardiological examination based on cnTI, D-dimer and NT-proBNP analysis according to the stages of CVL was not reported previously.

Taking into account Leishvet Guidelines serological (IFAT titers and additional ELISA tests), clinical findings and laboratory evaluation (TP, Alb and creatinine) dogs enrolled in this study were participated in 4 different infected groups (stage I to IV). Stage I included (low positive antibody titers: 1/64), to stage IV (high positive antibody levels: 1/1024-1/16000). In agreement with the proposed staging and the relevant literature (6), stage I and II (to those of groups denoted with same numbers) revealed normal renal profile, whereas stage III dogs presented chronic kidney disease (CKD) IRIS stage I or stage II, and finally stage IV dogs showed CKD) IRIS stage III or stage IV.

Cardiopulmonary biomarkers, namely biological processing markers easily be analyzed, thus might be used as indicators of response to treatment interventions and detecting pathogenic conditions (8). Regarding veterinary medicine different cardiac biomarkers might be analyzed in an attempt to diagnose and interpret the extent and severity of cardiopulmonary disease (9). Visceral leishmaniasis, a global parasitic zoonosis, may cause myocarditis and heart rate changes in canine and human hosts. In CVL, myocarditis has already been already described (5,10). In the present study, we measured levels of troponin I, NT-proBNP as indicative of the myocardial damage and D-dimer levels as a marker of thrombolysis in CVL.

Cardiac troponins (cTn) are used as blood biomarkers with high specificity and sensitivity for detecting myocardial degeneration. The latter contractile proteins, might be released from myocardium in relation with the severity of tissue injury and myocyte membrane disruption (11). Speficifically detectable elevations in circulating cTnI were reported in Canine Monocytic Ehrlichiosis (11). Regarding the relation between cTnI and leishmaniasis limited portion of study took place in the literature. Detailed literature search revealed case reports (12), and some limited original studies (13,14).

A recent study reported that myocarditis in CVL could be in association within immunological alterations due to Leishmania infection (4,5). Another study inspected the hearts of 30 dogs naturally infected with Leishmania infantum chagasi, detected myocardial lesions in all dogs, and the parasite was found in the cardiac tissue (20/30 dogs). According to the results of that study cardiac lesions might be prevalent in dogs with naturally occurring CVL even in the absence of clinical signs related to heart failure (5).

A prior study was the subject of an evaluation for serum cTnI as an indicator of myocardial injury in dogs with leishmaniasis (13). In that study in 40 dogs with leishmaniasis, median cTnI concentration was significantly ($p=0.011$) higher in contrast to 11 control dogs. Sixteen dogs (40%) with CVL presented elevated cTnI concentration. There was moderately to weak correlation among cTnI with decreased positive Leishmania titer, and increased age, whereas cTnI concentration did not differ between azotemic animals and control dogs (13). However serum cTnI concentration might have interaction with age, thus could be elevated with marked azotemia, a finding frequently associated with canine leishmaniasis (14). In the present study 2 cases in the second group (0.09-0.01 ng/mL), 3 cases in the III. group (0.09-0.16 ng/mL) and 5 cases in the IV. (0.09-0.8 mg/L) group (Table 4) revealed elevated values, evidencing that as stage progressed cases with elevated cTnI values increased. There was significant differences ($p=0.018$) between stage IV (group IV) and healthy control group, besides between group IV and group I, indicating that myocarditis might be participated in advanced stages of CVL. Although it is not possible to make interpretation solely analyzing cnTI levels, it was an interesting finding. The severity of elevation was in correlation with the extent of myocardial damage (15). Taking into account this data, elevated cnTI levels observed in this study, in advanced stages of the infection, could be due to myocardial injury.

A total of 30 non-uremic dogs with leishmaniasis in Greece, were prospectively enrolled as 20 in Group A were treated with a combination of meglumine antimonate and allopurinol for 28 days, whereas 10 dogs participated in Group B were treated with allopurinol alone (14). Blood samples were collected at timepoint 0 (before treatment) and at 14 and 28 days after the initiation of treatment. None of the dogs treated with meglumine antimonate in group A presented a serum cTnI concentration above the upper limit of the reference range (>0.5 ng/mL) nor cardiotoxicity at at 2 and 4 weeks after the initial therapy (14). In the present study none of the dogs involved received medical treatment at the beginning of the study, till analysis were performed.

As aforementioned above limited literature evaluated intravital diagnosis of cardiac alterations in leishmaniasis. Cardiac involvement in dogs (4,5,13) revealed that this protozoan might be also involved in heart, which promptly support the necessity of cardiac evaluation in cases of suspected leishmaniasis.

As a well recognized fibrin degradation product, D-dimer elevations in plasma concentrations might probably denote the oocurence of thrombi and their degradation. Elevated values of D-dimer in dogs with clinical evidence of thromboembolism (16,17) and dirofilariosis (18) have been reported. In the present study, these elevated values might probably be associated with thromboembolic complications caused by the CVL infection. Pulmonary thromboembolism is a common complication in animals infected with CVL (19), due to the nephrotic syndrome and aortic and caudal vena cava thrombosis (20), the three main predisposing factors for thrombus formation, [recognized as Virchow's triad, such as hypercoagulability, endothelial injury or abnormal blood flow, disseminated intravascular coagulation, as was described in dogs with leishmaniasis (21).

Lomtadze et al (22) assessed intravascular active markers before and after treatment for the assessment of 45-man coagulation with VL, and found that D-dimer levels increased 95.6% in severe and advanced forms of the disease. Even if the results of the last mentioned study are examined, it was concluded that the increased D-dimer levels might be in association with activation of DIC involved, probably at severe forms of the disease. The researchers also claimed D-dimer presented diagnostic and prognostic importance (22).

Studies show that serum concentrations of NT-proBNP are higher in dogs with mitral valve disease and dilate cardiomyopathy compared to healthy dogs, using NT-proBNP to assess cardiac disease and severity of the disease. It has also been reported that NT-proBNP concentrations correlate with heart rate, respiratory rate, echocardiographic changes and renal function in cardiac diseased dogs and that NT-proBNP concentrations may be useful in diagnosing cardiac diseases as well as in assessing severity (23,24).

In one study, BNP concentration was assessed to distinguish cardiac and noncardiac dyspnoea. BNP concentrations in congestive heart failure dogs (mean 34.97 pg/ml) were reported to be higher than those of non-cardiac dyspnea (mean 12.8 pg/ml) compared to 22 dogs with dyspnea due to congestive heart failure and 26 dogs with noncardiac dyspnea (35). In the present study no increase in NT-proBNP levels was found in the I., II., III. and control groups of the study. An increase of 4 cases in the IV. group (1808.3-1939.73 pmol/L) was determined. There was statistical difference between healthy control group and stage III, IV, besides between stage I and stage III, IV (p=0.000). Although echocardiographic examination and related data was not shown in this study 6 out of 28 CVL cases revealed decreased fractional shorthening indicating systolic function besides 2 cases in group II, 3 cases in group III and 4 cases in group IV revealed decreased LVIDd and LVIDs probably showing hypertrophic cardiomyopathy which all could contributed to increased NT-proBNP well used detected in this study.

In conclusion based on Leishvet guidelines serological (IFAT titers and ELISA test kits), clinical and laboratory findings (especially Tp, Alb and UPC) cases were enrolled into four diffrent stages/groups (stage I to IV) presented cardiopulmonary alterations which must be taken into consideration. Cardiac status should be established which could contribute to intravital diagnosis and hasten additional therapy protocoles directed to cardiovascular system in dogs with CVL. In summary, available data suggested the possibility of using cTnI and NT-proBNP as markers for cardiac damage and D-dimer as a marker supportive for

thromboembolism in dogs with CVL. Detailed studies with additional biomarkers, and repeated analysis should be warranted to achieve more interesting data on the marking capacity of those molecules, as was also denoted in dogs with dirofilariosis (18).

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