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RESEARCH ARTICLE

Coagulation Profile Alterations in Dogs Co-Infected with Visceral Leishmaniasis and Monocytic Ehrlichiosis

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ABSTRACT

Canine Visceral Leishmaniasis (CVL) and Canine Monocytic Ehrlichiosis (CME) are zoonotic diseases that cause coagulation disorders, abnormalities in various organs and systems along with vasculitis. Since it was reported that the presence of co-infection may cause more severe abnormalities, this study aimed to investigate the synergistic effects of co-infection on some coagulation analytes (prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB) and D-Dimer). Twenty dogs, all were mixed breed, aged 2-4 years, and determined to be co-infected with CVL and CME were used. As a result of the coagulation profile analyte measurements, the values of APTT, PT, and the concentration of FIB were determined to be higher in the Co-infected Group compared to the Control Group ($p<0.05$). Receiver operating characteristic (ROC) analysis revealed that PT (AUC: 0.980); APTT (AUC: 0.959), FIB (AUC: 1.000), and D-dimer (AUC: 0.929) had outstanding diagnostic discrimination. As a result, it was concluded that the presence of co-infection deteriorates the coagulation profile more severely in co-infection with CVL and CME.

Keywords: Activated partial thromboplastin time, D-Dimer, Dog, Fibrinogen, Prothrombin time

INTRODUCTION

Canine Visceral Leishmaniasis (CVL), which is described as an emerging zoonotic disease by the World Health Organization, has a poor prognosis and causes damage to various organs and systems in dogs [1,2]. It was previously reported that in CVL, not only vasculitis is present [3,4] but also abnormalities in the coagulation profile [5-7]. On the other hand, Canine Monocytic Ehrlichiosis (CME) is another vector-borne disease of dogs. It was previously reported that CME causes significant vascular and coagulation abnormalities as in CVL [8-12].

Hemostatic disorders (epistaxis, hemorrhagic diarrhea and hematuria) are reported to be common findings in both diseases [6,7,9,10]. Studies have shown that hemostatic changes in CVL-infected dogs are associated with the severity of clinical signs and thrombocytopenia [13], however, platelet dysfunction has been reported in CME-infected dogs [14].

Prothrombin time (PT) and activated partial thromboplastin time (APTT) are frequently used important biomarkers in the interpretation of the coagulation profile in dogs with bleeding tendency [15]. Fibrinogen (FIB), which is a glycoprotein, is synthesized by the liver and converted to fibrin by thrombin during homeostasis. Although FIB is one of the most important factors in the coagulation cascade, it was reported that its concentration may increase in several clinical conditions such as acute infections, hemodynamic disorders, heart and lung diseases and malignant conditions [16]. D-dimer, which is an important biomarker used primarily in the diagnosis of disseminated intravascular coagulopathy (DIC), is known as a degradation product of cross-linked fibrin that can increase with clot formation and fibrinolysis. Apart from DIC, it may increase due to infection, metabolic disorders, neoplasia and following an operation [17-19].

A study that evaluated platelet aggregation and haemostatic response in dogs co-infected with CME and CVL detected that there was a synergistic effect on haemostatic disorders in co-infected dogs with CME and CVL [20]. Therefore, this study, it was aimed to determine and validate the possible synergistic alterations in the coagulation profile

by evaluating coagulation profile analytes including PT, APTT, FIB and D-dimer in dogs co-infected with CVL and CME.

Material and Methods

Ethical Statement

This study was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 01-11, session number 2021/009).

Animals

The animal material of the present study consisted of 20 dogs, all were mixed breed, aged 2-4 years, and were admitted to animal hospital for diagnosis/treatment or routine check-up/vaccination purposes. All the dogs underwent clinical examination, including body temperature, capillary refill time, lung and heart auscultation, and assessment of palpable lymph nodes. In addition, rapid diagnostic test kits (SNAP Leishmania®, IDEXX, USA and SNAP 4Dx Plus®, IDEXX, USA) were applied to all the dogs in accordance with the manufacturer's instructions. After the clinical examinations and rapid test kit evaluations, 10 dogs (4 male, 6 female, all were intact with a median age of 3.71 years (2-4 years) and a median body weight of 20 (7-28) kg) were diagnosed to be co-infected with CVL and CME, and all were included in the Co-infected Group (n:10). The other 10 dogs (4 females, 6 males, all were intact with a median age of 2.86 (2-4) years and a median body weight of 15 (10-18) kg) were determined to be healthy as a result of the clinical examinations and rapid test kit evaluations, and included to the Control Group (n:10).

Inclusion/Exclusion Criteria

In order to rule out the diseases (*Dirofilaria sp., Anaplasma phagocytophilum, Anaplasma platys* and *Borrelia burgdorferi)* that may cause similar clinical findings similar to CVL and CME infections, rapid diagnostic test kits (SNAP 4Dx Plus®, IDEXX, USA) were applied to the dogs of the Co-infected Group of the study. Dogs with any comorbid disease were excluded from the study. The same examinations and tests were applied to the dogs of the Control Group. All examination and test results were negative in context of differential diagnosis.

Clinical Examination

In accordance with the anamnestic data which were obtained as a result of face-to-face interviews with the animal owners, it was learned that all of the dogs were living at home, were taken to a walk 2-3 times a day and were fed on commercial dry dog food. Heart rate, respiratory rate, body temperature and capillary refill time (CRT) measurements, evaluation of dehydration status and palpable lymph nodes were assessed in all the dogs within the scope of clinical examinations. In addition, to detect pulmonary (pulmonary edema, dyspnea, etc.) clinical pathological changes secondary to cardiac disorders and cardiac clinical pathological changes (arrhythmia, valvular leaks, etc.), auscultation of the lungs and heart (mitral valve, in line with the costochondral junction of the left 5th intercostal space; aortic valve, just above the costochondral junction of the left 4th intercostal space; pulmonary valve, just above the sternum in the left 2nd-4th intercostal space region; tricuspid valve, right 3rd-5th intercostal space near the costochondral junction) was performed as previously reported [21].

Positive cases included in the study were not hospitalized. The routine treatment procedures reported for CVL [22] and CME [23] were followed.

Collecting Blood Samples

Venous blood samples (5-10 mL) were obtained via *vena cephalica* venepuncture with minimal restraint in order to not cause stress to all the dogs. The blood samples were taken into tubes without anticoagulant (BD Vacutainer® SST™ II Advance, BD, United Arab Emirates) and citrated anticoagulant tubes (BD Vacutainer® Citrated Tube, BD, United Arab Emirates), all were centrifuged at 3000 rpm for 10 minutes and their serum and plasma were extracted. Some portions of the serum and plasma samples (0.5-1 mL) were used for rapid diagnostic test kit evaluations (serum D-dimer and plasma PT, APTT, FIB, respectively). Centrifugation and rapid diagnostic test kit evaluations were performed within 10-15 min after the blood sampling.

Rapid Diagnostic Test Kit Applications

Confirmation of CVL and CME infections in the dogs, which were included in the Co-infected Group, was based on the clinical findings such as anorexia, lethargy, exercise intolerance, and arrhythmia, and as a result of rapid ELISA-based diagnostic test kits (SNAP 4Dx Plus®, IDEXX, USA) evaluations from the serum samples of the dogs of the Co-infected Group in accordance with the manufacturer's instructions. CVL and CME infections were confirmed according to the rapid diagnostic test kit results along with the compatible clinical findings as previously reported [24]. The same examinations and diagnostic tests were also applied to the dogs of the Control Group and all were determined to be negative.

Coagulation Profile Analyte Measurements

Plasma APTT, PT, FIB and serum D-dimer concentrations were measured using a commercial analysis system (Precil® C2000-4 four channel semi-automatic coagulometer and Wondfo Finecare® Fluorescent Immunoassay, respectively). The linear range (min-max) of the Finecare®

Table 1. *Temperature (T), Pulsation (P), Respiration (R) findings of all animals (according the groups)*

Parameters	Control Group (n:10)		Co-infected Group (n:10)	
	No	Value	No	Value
T (°C)	1	38.6	1	40.2
	2	38.5	2	40.6
	3	39.1	3	40.7
	4	38.5	4	39.3
	5	38.5	5	39.0
	6	39.0	6	38,9
	7	38.6	7	39.0
	8	39.1	8	38.8
	9	39.0	9	39.2
	10	39.3	10	39.0
P (beat/min)	1	80	1	175
	2	76	2	175
	3	84	3	180
	4	76	4	104
	5	100	5	88
	6	88	6	100
	7	96	7	96
	8	72	8	96
	9	104	9	84
	10	100	10	80
R (breaths/min)	1	16	1	62
	2	16	2	60
	3	24	3	68
	4	28	4	24
	5	18	5	28
	6	16	6	26
	7	15	7	28
	8	30	8	16
	9	16	9	24
	10	20	10	24

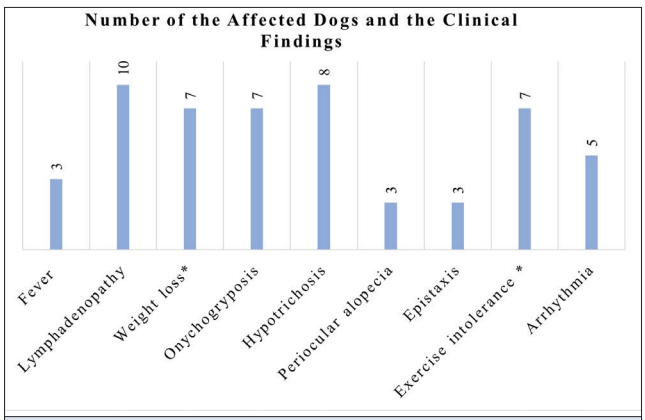

Fig 1. The clinical findings and their distribution of the co-infected group
*Based on body condition score and daily diet intake [25]

Fluorescent Immunoassay was 0.1-10 mg/L for D-dimer. Measurements below the lower detection limit (0.1 mg/L for D-dimer) were used for statistical analysis.

Statistical Analysis

All the data were evaluated using SPSS 25.00 (SPSS for Windows®) statistical software. One sample Kolmogorov-Smirnov test was applied to determine whether all data were parametric or non-parametric. It was found that the data were not normally distributed. Therefore, non-parametric data were evaluated with the Mann Whitney U, Kruskal-Wallis test. In addition, Receiver operating characteristic curve (ROC) analysis was used to distinguish the healthy dogs from the co-infected ones using coagulation profile analytes. Within the scope of ROC analysis, Area Under Curve (AUC), standard error (std. error), cut-off, sensitivity, and specificity parameters were evaluated. Within the scope of ROC analysis, it was accepted that an AUC of 0.5 suggests no discrimination (i.e., ability to diagnose patients with and without the disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding. Moreover, the Spearman correlation test was performed to determine possible correlations between PT, APTT, FIB, and D-Dimer. Statistical significance was accepted as $P<0.05$ for all the data.

Results

Clinical Examination Results

As a result of clinical examinations, among the dogs of the Co-infected group, three had elevated body temperature (40.2°C, 40.6°C and 40.7°C) and three had prominent tachycardia (175, 175 and 180 beats/min). Temperature (T), Pulsation (P), and Respiration (R) findings of all animals (according to the groups) were given in *Table 1*. In addition, lymphadenopathy, hypotrichosis, exercise intolerance and onychogriposis were prominent clinical findings in the dogs of the Co-infected Group. The distribution of the clinical findings of the Co-infected Group are presented in *Fig. 1*.

Coagulation Profile Analyte Measurement Results

Within the scope of coagulation profile analyte measurement, the values of APTT, PT and the concentration of FIB were determined to be higher in the Co-infected Group compared to the Control Group ($P<0.05$). All coagulation profile analyte measurement results are presented in *Table 2*.

ROC Analysis Results

ROC analyses (CI: 95%) were performed to evaluate the diagnostic performances/efficacies of PT, APTT, FIB and

Table 2. *Coagulation profile analyte measurement results between the co-infected and healthy dogs*

Parameters	Control Group (n:10) median (min-max)	Co-infected Group (n:10) median (min-max)	P-Value
PT (sec)	7.97 (7.66-8.91)	9.84 (8.59-10.9)	0.000
APTT (sec)	11.7 (11-12.1)	18.4 (11.8-38.2)	0.025
FIB (mg/dL)	120 (103.3-189.2)	272 (201-752.1)	0.019
D-Dimer (mg/L)	0.09 (0.09-0.09)	0.7 (0.09-5.1)	0.083

PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen

Table 3. *ROC analyses data*

Parameters	AUC	Std. Error	P-Value	Asymp. 95% CI		Cut-off	Sensitivity	Specificity
				Lower Bound	Upper Bound			
PT (sec)	0.980	0.032	0.003	0.917	1.000	8.51	100%	85.7%
APTT (sec)	0.959	0.050	0.004	0.861	1.000	11.95	85.7%	85.7%
FIB (mg/dL)	1.000	0.000	0.002	1.000	1.000	195.4	100%	100%
D-Dimer (mg/L)	0.929	0.082	0.007	0.767	1.000	0.29	85.7%	100%

PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen, AUC: Area under curve, Std. Error: Standard Error, Asymp: Asymptotic tests, CI: Confidence interval

Table 4. *Spearman correlation test result*

Method	Parameters	Relation	Parameters			
			PT	APTT	FIB	D-Dimer
Spearman's rho	PT	Correlation Coefficient	1	0.647*	0.691**	0.836**
		Sig. (2-tailed)		0.012	0.006	0.000
	APTT	Correlation Coefficient		1	0.827**	0.869**
		Sig. (2-tailed)			0.000	0.000
	FIB	Correlation Coefficient			1	0.860**
		Sig. (2-tailed)				0.000
	D-Dimer	Correlation Coefficient				1
		Sig. (2-tailed)				

** Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed). PT: Prothrombin time, APTT: Activated partial thromboplastin time, FIB: Fibrinogen*

D-dimer in the determination of the coagulation disorders and the distinguishment of the healthy dogs from the diseased ones. As a result of the comparative ROC analysis, it was determined that PT (AUC: 0.980, sensitivity: 100%, specificity: 85.7%); APTT (AUC: 0.959, sensitivity: 85.7%, specificity: 85.7%), FIB (AUC: 1.000, sensitivity: 100%, specificity: 100%) and D-dimer (AUC: 0.929, sensitivity: 85.7%, specificity: 100%) had outstanding diagnostic discrimination. Comparative ROC analysis results are presented in *Table 3*.

Spearman Correlation Test Results

Spearmen correlation tests were performed for the analytes evaluated within the scope of coagulation profile. As a result, a strong positive correlation was found between PT and D-dimer, APTT and D-dimer, APTT and FIB,

FIB and D-dimer and a moderate positive correlation was found between PT and APTT, PT and FIB. The results are presented in *Table 4*.

Discussion

Bleeding disorders caused by CVL and CME are reported to cause significant clinical problems that cannot be ignored [5-12]. As expected, prominent clinical findings such as fever, lymphadenopathy, weight loss, onychogryposis, hypotrichosis, periocular alopecia, epistaxis, arrhythmia, and exercise intolerance which were previously reported in cases of CVL and CME [1,7,8] were also observed in the Co-infected Group of the present study.

There are studies evaluating the abnormalities in the coagulation profile of CVL-infected dogs. A previous study conducted on 28 CVL-infected dogs [7] evaluated the changes in the coagulation profile of dogs which were classified according to the disease stage. As a result of the aforementioned study, a statistically significant difference in PT, APTT, FIB and D-dimer concentrations between the dogs with an advanced stage (stage IV) disease and the healthy ones were determined. In another study conducted on 33 dogs infected with CVL, it was reported that PT, APTT and D-dimer concentrations were statistically significantly different in the dogs with an advanced stage (stage IV) disease compared to the previously reported findings [26]. They were also reported that this change might be related to the altered vitamin D concentrations. In a study investigating the cause of epistaxis, which could develop in dogs naturally infected with CVL, it was reported that no statistical difference was determined in the coagulation profile parameters including PT, APTT, FIB, von Willebrand Factor deficiency. It was reported that epistaxis may be associated with a secondary homeostasis mechanism or the presence of DIC [26]. In the present study, statistically differences in the PT, APTT and FIB concentrations (prolongation of PT and APTT duration, increase in FIB concentration) were determined similar to the dogs with an advanced stage (stage IV) disease in the previous studies [7,26]. On the contrary to the previous report [27], prolonged PT and APTT, and increased FIB concentrations were observed in the present study. The most prominent difference between the studies [7,26] with abnormalities in the coagulation profile and the present study that did not is the use of the CVL staging system as an inclusion criterion that was defined in the previous article [1]. the staging system was not used in the group whose coagulation parameters did not alter, it is noteworthy that the cases included in our study may not be in the initial stages (Stage I, II) and/or in the advanced stage (Stage IV). Also, Juttner et al.[27] reported that there may be a possibility of the presence of DIC, but fibrin degradation products such as D-dimer, which may increase in the presence of DIC, were not included in the study. Although there was no statistically significant difference in the present study, the concentration of the measured D-dimer was above the reported [17] cut-off value (0.3 mg/L). In the present study, the reason for the more severe alteration in the coagulation profile may be related to the presence of co-infection with CME.

There was a statistically significant increase was found in APTT [9,10] and D-dimer [28], while no statistically significant change was found in PT [9,10] value in studies conducted in dogs co-infected with CME compared to healthy dogs. In the present study, a statistically significant difference in APTT value and a higher cut-off value of D-Dimer than the previously reported concentrations were determined. Unlike the previous studies, a statistically significant increase in PT value was observed in the present study. This finding was thought to be related to the synergistic effect of co-infection.

In co-infected dogs with CVL and CME, a statistical increase in APTT values was detected, while no difference in PT value and FIB concentration was reported, which did not support the expected hypothesis. It was reported that the low sensitivity of the commercial PT kit that was used in the study may be a reason for not detecting an increase [20]. In the present study, prolongation in PT and an increase in FIB concentrations were determined in the co-infected dogs. The increase in FIB concentrations was thought to be related to the presence of acute inflammatory changes [29]. The prolongation of PT and APTT can be explained by the synergistic effect of CVL and CME on the coagulation profile.

In the ROC analysis of the present study, the sensitivity and specificity of PT, APTT, FIB and D-dimer (100% and 85.7%; 85.7% and 85.7%; 100% and 100%; 85.7% and 100%, respectively) were determined to have outstanding diagnostic discrimination/performance. These findings may be related to the low number of animals in the present study. The cut-off value of the D-dimer concentration was determined to be 0.29 mg/L, similar to the previously reported cut-off value (0.3 mg/L) [17]. The cut-off values of PT, APTT and FIB were 8.51 sec, 11.95 sec and 195.4 mg/dL, respectively *(Table 3)*. Since there is no study reporting the cut-off values of PT, APTT and FIB in dogs infected with CVL and/or CME, the cut-off values determined in the present study are promising in terms of diagnosis.

In the present study also demonstrated that a strong positive correlation was found between PT and D-dimer, APTT and D-dimer, APTT and FIB, FIB and D-dimer and a moderate positive correlation was found between PT and APTT, PT and FIB. The positive correlations identified in the study were expected due to the disturbances in both the extrinsic and intrinsic coagulation cascade caused by

the CVL [7,13,20,26] and CME [6,9,10,12,20]. We believe that this correlation data makes an unique contribution to the scientific literature.

This study has some limitations. The low number of animals, along with the use of laboratory analyses including hemogram and serum biochemistry profiling for inclusion criteria and to form a differential diagnosis list, and the fact that the laboratory analysis results were not discussed, can be considered as limitations. For this reason, it is recommended to evaluate the coagulation profile along with laboratory analysis results.

In the present study, promising findings were determined. These included prolonged PT, APTT and higher FIB value which was interpreted that the presence of co-infection deteriorates the coagulation profile more severely in dogs co-infected with CVL and CME. In addition, these promising findings validated the previous study.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (C. Balıkçı) at the University of Harran, Şanlıurfa, Türkiye.

Financial Support

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Competing Interest

The authors declared that there is no conflict of interest.

Ethical Statement

This study was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 01-11, session number 2021/009).

Author Contributions

C. B. and E. G. designed, planned and drafted the research and converted into the manuscript. C. B., E.G. A.Ş. and İ.G. conducted and collected the data. C. B., E. G. analysed the data. C.B., E.G. and A. Ş. performed interpretation of data, conception and reviewed the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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