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Original article

The diagnostic importance of coagulation parameters in cattle having natural theileriosis

V. Gunes, A.C. Onmaz, I. Keles, K. Varol, G. EkiniciErciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine,
Kayseri, 38100 Turkey

Abstract

The purpose of this study was to determine the diagnostic importance of coagulation parameters in cattle with natural theileriosis. Nine Holstein cross-breed cattle with theileriosis as infected group and 6 healthy Holstein cattle as control group were used in the present study. Mean fibrinogen level, thrombin time (TT), activated partial thromboplastin time (aPTT) and prothrombin time (PT) were not statistically different when control and infected groups compared, except for the D-dimer concentration. Quantitative D-dimer concentrations were determined by immune-turbidimetric assay. D-dimer values increased significantly ($p < 0.05$) in infected group ($631.55 \pm 74.41 \mu\text{g/L}$) compared to control group ($370.00 \pm 59.94 \mu\text{g/L}$). D-dimer sensitivity and specificity were also determined at cut-off concentrations ($372 \mu\text{g/L}$). Sensitivity and specificity of D-dimer values were determined to be 88.89% and 83.33%, respectively. D-dimer is thought to be important indicator in the evaluation of the prognosis in theileriosis cases. Analysis of D-dimer values before and after treatment in controlled case studies were suggested in future studies to enlighten the issue.

Key words: cattle, coagulopathy, D-dimer, haemolysis, theileriosis

Introduction

Theileriosis is a protozoan disease transported to the animals by *Ixodidea* ticks and caused significant economic losses in cattle breeding in all over the World (Preston 2001, Mehlhorn 2008, Orkun et al. 2012). *Theileria* (*T*) species are seen wide spreadingly, particularly in the tropical and subtropical countries (Ozkan et al. 2013, Akat et al. 2014). High fever, enlarged superficial lymph nodes, increase in heart and respiratory rate are commonly seen in animals with theileriosis. Hyperaemia or paleness in the conjunctive tissues or mucosa and petechial haemorrhages especially in mucosa and hairless regions of the skin are

common. Haemolytic anaemia, icterus and death has been reported in the advanced stages of the disease (Keles et al. 2009, Ozkan et al. 2013).

Coagulant and anticoagulant systems are balanced under physiological conditions (Noyan 2012, Dhanunjaya et al. 2013). When this balance is disrupted for any reason, fibrinolytic system is activated. Fibrin occurred as a result of coagulation is degraded by some enzymes such as plasminogen and it is converted to fibrin degradation products (FDP) (Noyan 2012). D-dimer is one of the final breakdown products of this fibrin network. It is stabilized by factor 13. D-dimer contains some cross-linked components occurring during stabilization. These parts are released from the

clot as a result of the activation of plasmin and participated in blood flow (Ver Elst 2002, Noyan 2012). It is well known that levels of D-dimer and other coagulation parameters increase in infection, tumour, after surgical operations, trauma, burn, disseminate intravascular coagulopathy (DIC), venous thromboembolism (VTE), ischemic cardiopathy, congestive heart failure, haemolysis, bleeding, acute respiratory syndrome, liver and kidney disease, inflammatory bowel disease (Noyan 2012, Dhanunjaya et al. 2013).

In theileriosis, haemolytic anaemia or haemolysis is caused by immune-mediated destruction of affected erythrocytes (Hooshmand-Rad 1976). Other possible mechanisms in inducing anaemia have been suggested as high osmotic fragility of erythrocytes, acceleration of clearance and the presence of haemolytic activity in cattle highly infected with *T. sergenti* (Shahnawaz et al. 2001, Shiono et al. 2003). El-Deep and Younis (2009) showed a significant decrease in red blood cells (RBC) and/or haemoglobin (Hb) concentrations which were determined in Egyptian buffaloes infected with *Theileria*. In another study, Grewal et al. (2005) showed that oxidative stress in erythrocytes of cattle infected with *T. annulata* might be the cause of increased erythrocyte fragility and membrane lysis.

DIC is a syndrome characterized by stimulation of intravascular coagulation and degradation of haemostatic balance for various reasons (Yuksel et al. 2009). Especially, it is known to occur in protozoal infections such as theileriosis and babesiosis in cattle (Stockham et al. 2000). Therefore, it is hypothesized that serum levels of coagulation parameters may be a useful biomarker and analytical parameters in the pathogenesis of theileriosis cases. In this study, it was aimed to determine the levels of D-dimer and other coagulation markers in the diagnosis of DIC in naturally infected cattle with theileriosis. Furthermore, in the present study it was also aimed to investigate whether coagulation profile can be used as diagnostic markers or not in determining the presence of DIC which may develop in theileriosis.

Materials and Methods

The present study was conducted in the province of Kayseri where theileriosis outbreak occurred during May 2015 in a farm. Nine Holstein cross-breed cattle with clinical signs of theileriosis (Group 1: group with theileriosis) referred to the Clinics of Internal Medicine, Veterinary Faculty, University of Erciyes, were included in this study. The disease was diagnosed based on clinical examination and laboratory confirmation by Giemsa staining of blood smears.

Six clinically healthy Holstein cattle (Group 2: Control group) were also sampled during the same period as control group.

Animals

This study was conducted on cattle with theileriosis. A total of 9 Holstein cross-breed cattle from a herd in Kayseri province consisted of animal material. Death of a few individuals and some sick ones was reported in the mentioned herd. In the clinical examination of nine sick animals and after microscopic examination of the blood smears of the animals, theileriosis was diagnosed. Additionally, coagulation profiles and D-dimer levels of the cattle with theileriosis were investigated in this herd. These animals were aged 1.5 ± 0.5 years old and comprised of 2 males and 7 females which weighting from 250 kg to 500 kg (Group 1). Six healthy Holstein cattle (2 male and 4 female) aged between 2.0 ± 0.5 years old and weighting from 250 kg to 550 kg were also used from the different herd as a healthy control in the present study (Group 2). These animals were selected from those which body temperature, pulse and respiration rate was in normal references range and which were in good condition. All animals with theileriosis were treated after collecting blood specimens. For the treatment; Buparvaquone (Tailerol/Provet/Turkey) 2.5 mg/kg body weight, two doses were injected intramuscularly at 48 hours intervals. Additionally for piroplasm form of *Theileria*, two doses of oxytetracycline, (Primamycine-La[®], Pfizer) 20 mg/kg, BW/day intramuscularly was injected in 48 hours intervals and supportive treatment was carried out for 5 days.

The diagnosing of Theileriosis: Blood smears obtained from blood samples of infected animals ear tips stained with Giemsa staining were investigated for the presence of piroplasm forms of *Theileria spp* with immersion objective (x100). The pictures of smears including piroplasm forms of *Theileria* in the erythrocyte were taken by a video microscope (Zeiss AxioCam ERc5s). Ten ml blood specimens were also obtained from jugular vein of *Theileria*-positive animals and healthy animals for analysis of hemacounter into the tubes with K2 EDTA 18 mg (BD Vacutainer). Blood specimens (3.5 ml) were also taken for analysis of coagulation parameters into tubes containing citrate with vacuum (Vacutest). These tubes were blue-capped tubes containing 3.2% (0.109M) sodium citrate. Plasma specimens were harvested after centrifugation of the blood specimens for 15 minutes, at 4000 rev/min (Hettich ROTOFIX 32A). These specimens were stored at -20°C until the assay process and analysed after thawing immediately.

Table 1. Mean haematological values in *Theileria* infected and healthy cattle and P values.

Hematologic Parameters	Group 1 (n=9)	Group 1 (n=6)	P values
	$\bar{x} \pm SE$ M (min-max)	$\bar{x} \pm SE$ M (min-max)	
WBC ($10^9/l$)	9.18 \pm 1.28 9.00 (1.90-15.60)	9.60 \pm 1.25 11.40 (5.80-12.00)	0.797
Lymphocyte (%)	26.71 \pm 3.54 23.30 (15.30-48.10)	52.43 \pm 4.70 54.30 (37.20-67.60)	0.002
Monocyte (%)	10.07 \pm 1.63 10.90 (2.10-18.80)	8.26 \pm 0.89 8.20 (5.60-10.90)	0.320
Granulocyte (%)	61.14 \pm 4.13 62.30 (40.00-73.70)	38.08 \pm 4.57 36.05 (24.80-52.20)	0.012
RBC ($10^6/l$)	6.90 \pm 0.89 7.08(3.66-11.98)	7.65 \pm 0.88 7.02 (5.67-11.77)	0.689
HG (g/dl)	10.96 \pm 1.31 10.90 (4.80-16.80)	10.63 \pm 0.39 10.40 (9.70-12.30)	1.000
HCT (%)	34.12 \pm 4.4 31.00 (14.60-54.50)	33.68 \pm 1.83 33.40 (27.70-41.40)	0.529
MCV (fL)	89.58 \pm 9.50 47.90 (40.20-401.00)	45.53 \pm 3.11 45.55 (35.20-56.10)	0.328
MCH (pg)	15.88 \pm 0.95 14.40 (13.10-21.20)	14.40 \pm 1.10 14.15 (10.40-18.80)	0.529
MCHC (g/dl)	32.35 \pm 0.49 31.70 (30.20-35.10)	31.73 \pm 1.04 31.00 (29.40-35.70)	0.529
RDW (%)	16.51 \pm 0.26 16.30 (15.40-17.90)	19.38 \pm 0.83 19.60 (16.70-22.50)	0.008
PLT ($10^9/l$)	371.55 \pm 52.65 372.00 (70.00-563.00)	456.33 \pm 103.46 365.00 (263.00-939.00)	1.000
MPV (fL)	5.14 \pm 0.12 5.10 (4.50-5.90)	12.81 \pm 7.51 5.25 (4.90-6.10)	0.529
PDW	16.32 \pm 0.18 16.30 (15.70-17.50)	16.15 \pm 0.22 16.05 (15.60-17.00)	0.607
PCT (%)	0.19 \pm 0.028 0.20 (0.03-0.29)	0.23 \pm 0.04 0.20 (0.13-0.46)	0.689

RBC; Red Blood Cell, HCT; hematocrit, Hgb; haemoglobin concentration, MCV; mean corpuscular volume, RDW; erythrocyte distribution change, MCH; mean corpuscular haemoglobin volume, MCHC; mean corpuscular hemoglobin concentration, WBC; White Blood Cell, PLT platelet, MPV; Mean platelet volume, PDW; platelet distribution width, PCT; plateletcrit. Values are presented as means \pm SE and M (min-max). Mean values are significantly different from each other at level of $p < 0.05$. Group 1; *Theileria* infected group, Group 2; Control group. M (min-max): Median (minimum-maximum).

Analysis of hematologic parameters

Haematological parameters were determined in a veterinary hemacounter (Mindray BC 2800 VET).

Analysis of coagulation parameters

D-dimer concentrations (cut-off value 500 ng/ml), were analysed by using a particle-enhanced, immuno-turbidimetric assay in a calibrated SIEMENS Sysmex CA-7000 having an automated coagulation

analyser. D-dimer test has been validated on this analyser. Fibrinogen, thrombin time (TT), activated partial thromboplastin time (aPTT) and prothrombin time (PT) were measured with the same device after plasma specimens being dissolved in the room temperature immediately

Statistical methods

All data were tested to determine whether obtained values show normal distribution or not using

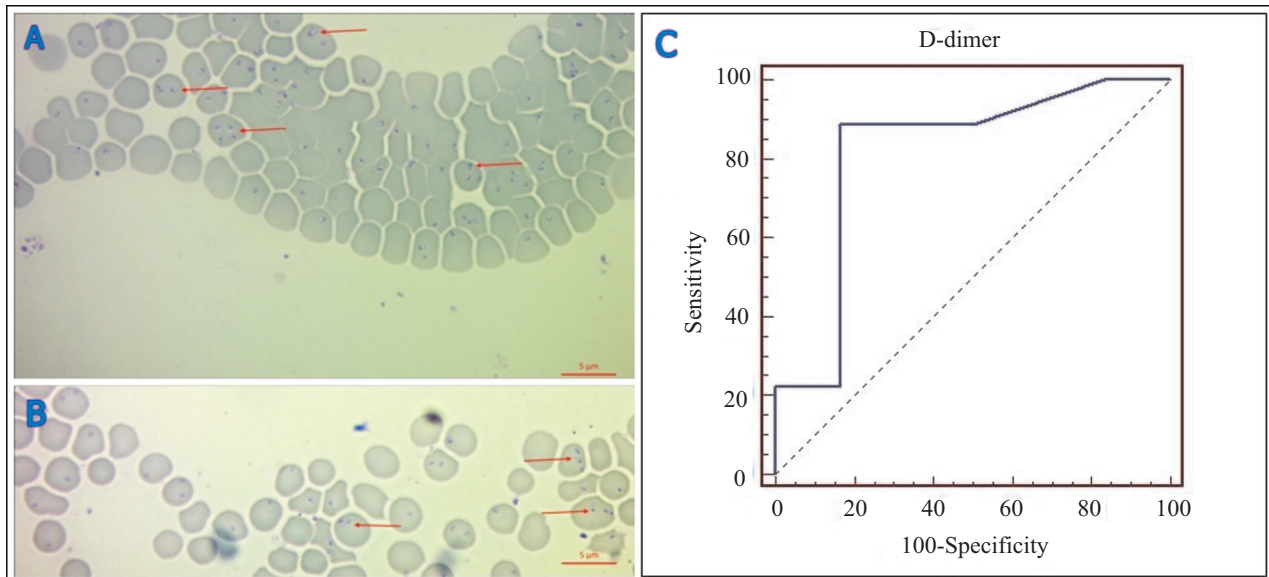


Fig 1. A – *Theileria* piroplasms (arrows). Blood smear was prepared from periferal blood of animal with Theileriosis (Giemsa stain). x100 B – *Theileria* piroplasms (arrows). Blood smear was prepared from periferal blood of animal with Theileriosis (Giemsa stain). 100x C – Receiver operating characteristics curve for D-dimer findings in theileria positive cattle.

the Kolmogorov-Smirnov test. As a result of Kolmogorov-Smirnov test the values found to have abnormal distribution, so Mann-Whitney *U*-test applied to compare the two groups. The IBM SPSS 16.0 software statistical package program was used for these tests. ROC curve, sensitivity, specificity and cut-off values for D-dimer values, were determined by using 9.1 MedCalc statistical software. The Data were expressed as mean \pm standard error and Median, Minimum-Maximum. The level of $p < 0.05$ was used to determine the statistical significance.

Results

Clinical examination findings

Loss of appetite, depression, paleness in conjunctival mucosa, increase in capillary refill time (>2 sec.), high body temperature ($>40^{\circ}\text{C}$), single-sided or double-sided enlargement and asymmetry in pre-scapular and subiliac lymph nodes, decrease in rumen movements, increase in heart ($>75/\text{min}$) and respiration rate ($>20/\text{min}$) were observed in clinical examination of the cattle with Theileriosis.

Hematologic analyse findings

Mean lymphocyte, granulocyte and erythrocyte distribution change (RDW) measurements showed significant differences between groups. Mean haema-

tological values in *Theileria* infected and control cattle were given in Table 1.

Blood smear analysis findings

Piroplasm forms of *Theileria* were determined in blood smear obtained from cattle with theileriosis and two examples of Giemsa staining pictures were given in Fig. 1A and Fig. 1B.

Coagulation analysis findings

The mean D-dimer concentration in healthy cattle and cattle with theileriosis cases were 370.00 ± 59.94 $\mu\text{g/L}$ and 631.55 ± 74.41 $\mu\text{g/L}$, respectively, which showed significant increase in diseased group ($P = 0.049$). D-dimer levels ranged from 300-960 $\mu\text{g/L}$ in cattle with theileriosis and 230-650 $\mu\text{g/L}$ in healthy ones. On the other hand, fibrinogen, thrombin time, active partial thromboplastin time (aPTT) and prothrombin time (PT) values were not different significantly. Furthermore, sensitivity of D-dimer value was determined to be 87.5%. The D-dimer measurements of blood plasma specimens and the other coagulation factors, D-dimer's sensitivity (SN) and specificity (SP) and 95% Confidence Interval (CI) are shown in Tables 2 and 3, respectively. The cut-off values of the D-dimer in predicting theileriosis in cattle based on the microscopic analyses was 372 $\mu\text{g/L}$, SN was 88.89% (95% CI: 51.7-98.2%) and the SP was 83.3% (95% CI: 36.1-97.2%), respectively (Table 3). Results

Table 2. Mean values of coagulation parameters in *Theileria* infected and healthy cattle and P values.

Coagulation Factors	Group 1 (n=9)	Group 2 (n=6)	P values
	$\bar{x} \pm SE$ M (min-max)	$\bar{x} \pm SE$ M (min-max)	
D-dimer ($\mu\text{g/L}$)	631.55 \pm 74.41 590.00 (300.00-96.00)	370.00 \pm 59.94 334.00 (230.00-650.00)	0.049
Fibrinogen (mg/dl)	233.33 \pm 9.58 240.00 (176.00-275.00)	216.50 \pm 10.55 213.00 (181.00-259.00)	0.271
TT(sec)	20.94 \pm 0.54 20.90 (18.70-23.80)	24.80 \pm 1.77 22.90 (19.90-30.40)	0.066
PT (sec)	21.87 \pm 1.91 24.60 (14.00-31.00)	27.53 \pm 1.55 24.60 (18.50-37.50)	0.224
aPTT (g/dl)	28.92 \pm 1.53 27.30 (23.80-36.40)	27.43 \pm 3.35 26.70 (23.90-31.90)	0.607

TT; thrombin time, aPTT; activated partial thromboplastin time, PT; prothrombin time.

Values are presented as means \pm SE. Mean values are significantly different from each other on level of $p < 0.05$. Group 1; *Theileria* infected group, Group 2; Control group. M (min-max): Median (minimum-maximum).

Table 3. Sensitivity, specificity, and positive and negative predictive value for D-dimer cut-off point.

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	-LR	+PV	-PV
>372*	88.89	51.7 – 98.2	83.33	36.1 – 97.2	5.33	0.13	88.9	83.3

CI: Confidence Interval, +LR: Positive Likelihood Ratio, -LR: Negative Likelihood Ratio, +PV: Positive Predictive Value, -PV: Negative Predictive Value

of ROC curve analyses for the D-dimer that was statistically different between Groups 1 and 2 are shown in Table 2 and Fig. 1C.

Discussion

Findings of the present study showed that only analysis of D-dimer may potentially play role for its pathogenesis, because statistically significant differences were found between cattle with theileriosis and without theileriosis (Table 2). Theileriosis was individually diagnosed by blood smear examination in nine Holstein cross-breed cattle. The clinical signs of theileriosis, such as fever, anaemia and swelling of lymph nodes, were observed in each of animals. In blood smears of all animals microscopically detectable piroplasm forms at different stages were observed as an indication of high or low parasitemia. The diagnosis of theileriosis was based on clinical findings and Giemsa staining which is considered as gold standard for diagnosis of theileriosis in cattle (Chauhan et al. 2005, Burgu and Karaer 2005). Thus, in the present study, piroplasm forms of *Theileria* were easily and safely determined in blood smear specimens obtained from each animal (Fig. 1A and 1B). Small number of infected animals were the limitation of present study.

The number of animals with theileriosis used in the present study may not be sufficient. However, the calculation of adjusted values for SN and SP in this model believed to minimize these limitations and caused more accurate estimations. In the present study, it was seen that D-dimer concentrations can safely be measured with a particle-enhanced, immuno-turbidimetric assay in bovine serum. The findings of the study described the potential for D-dimer concentrations to diagnose disseminated intravascular coagulation (DIC) in dairy cows with theileriosis, because significant difference was found between cattle with theileriosis (631.00 \pm 74.41 $\mu\text{g/L}$) and healthy cattle (370.00 \pm 59.94 $\mu\text{g/L}$) ($p = 0.049$), respectively.

In a study, the coagulation profile was investigated in cattle with displacement of abomasum. D-dimer levels were determined with the chromogen substrate method by relying on the colorimetric reaction kinetics at a wavelength of 405 nm (Sobiech et al. 2008). In another study on the cattle infected with BVD virus, coagulation and fibrinolysis tests were carried out using the Coag-Chrom 3003 device and reagents (Bio-Ksel, Poland) (Radwinska 2010). ELISA techniques are also conventional method for D-dimer investigations. An Asserachrom D-dimer kit (Diagnostica Stago, Parsippany, New Jersey) was used to measure fibrin D-dimer using an ELISA procedure

(Mcdermott et al. 2007). It has also been found that increased plasma D-dimer level can be useful in the diagnosis of dogs with thromboembolic disease (TE) and DIC (Stokol et al. 2000, Griffin et al. 2003, Nelson and Adnreasen 2003, Dewhurst et al. 2008). Results of this study demonstrate that the D-dimer analysis is sensitive and specific in cattle with theileriosis. These results are consistent with those described by other researchers within the physiological range (Sobiech et al. 2005, Sobiech et al. 2008). Radwyńska (2010) found that D-dimer concentrations in BVD-MD infected cows were higher ($1180.47 \pm 62.81 \mu\text{g/L}$) than those of non-infected cows ($159.27 \pm 26.81 \mu\text{g/L}$). Blood concentrations of D-dimer and some coagulation parameters have been described as accurate indicators of cattle with left displacement of abomasum ($688.07 \pm 168.26 \mu\text{g/l}$) and diarrhoeic calves ($587.25 \mu\text{g/l}$) (Sobiech et al. 2013, Radwinska 2010). Wittek et al. (2010) also stated that peritoneal fluid D-dimer was most accurate in diagnosis of cattle with peritonitis compared to the other some parameters. High mean D-dimer levels ($631.55 \pm 74.41 \mu\text{g/L}$) determined in present study were statistically more significant than those of control, this difference was in accordance with the above studies. D-dimer showed accuracy in positive and negative prediction of theileriosis. However, D-dimer concentration may be modified also by blood coagulation during DIC, inflammation, thrombosis, or pulmonary embolism. High levels in D-dimer in sick animals most probably resulted from the activation of intravascular fibrinolysis. Other coagulation parameters obtained from healthy cattle such as fibrinogen ($216.50 \pm 10.55 \text{ mg/dl}$); TT ($24.80 \pm 1.77 \text{ sec}$); PT ($27.53 \pm 1.55 \text{ sec}$) and aPTT ($27.43 \pm 3.35 \text{ g/dL}$) were concomitant with the results of earlier studies (Gentry et al. 1979, Sobiech et al. 2013, Karakurum et al. 2009). Although different analysers have been used in the above studies, these results were very close to each other and reinforced the accuracy of the data obtained from the present study.

Haematological parameters in cattle with theileriosis remained within physiological ranges except for mean granulocyte number, lymphocyte and RDW levels (George et al. 2010). In this study we observed an increase in the level of mean granulocyte number which has a direct relationship with disturbances occur in response to infections. A decreased lymphocyte level and RDW value in blood was determined in this study. Haematological results showing lymphopenia and granulocytosis were interpreted as a response to *Theileria* infection. The immune mechanism starts due to lymphocytes infected with schizont form of parasites and TNF α and nitric oxide produced by lymphocytes destroys the cells infected with

schizont and piroplasm. This destruction may cause lymphopenia (Sandhu et al. 1998). The life cycles of *Theileria* spp. are completed in RBC and WBC and it leads to destruction of these cells. Most pathogenic species are *T. parva* and *T. annulata* and they multiply in the WBC of host. Less pathogenic species also show, the growth in RBC (Morrison 1998, Burgu and Karaer 2005). High number of parasitized cells increase in the lymphoid system is associated with widespread lymphocytolysis, marked lymphoid depletion and leukopenia (Morrison 1998). However, leukopenia was not observed in this study. In fact, a non-statistical numerical increase in the WBC of the infected animals have seen. These conditions are considered due to non-statistical numerical increase in the levels of granulocyte and monocyte in patients. In this study, anaemia or massive destruction of RBC has not been seen as a major diagnostic sign, the results were not in agreement with the findings of some other studies (EL-Deep and Younis 2009, Khan et al. 2011), since *Theileria* shows minimal replication in RBCs (Morrison 1998). The reason of high mean granulocyte count might be associated with the increase in the number of neutrophil or high eosinophil level seen in the parasitic infection identified.

In this study, D-dimer results derived from the control group were determined to be consistent with those of obtained from healthy cows in other studies. However, in the present study, D-dimer cut-off value, specificity and sensitivity in cattle with theileriosis were determined for the first time. The absence of this data in earlier veterinary literature has prevented us to compare our values to the same animal species. But, especially the cut-off level ($0.30 \mu\text{g/mL}$) and specificity (77%) obtained from immuno-turbidometrical and D-dimer analyses used latex-agglutination and immuno-turbidometric method in dogs with DIC and healthy dogs were quite close to the result of the present study ($> 372 \mu\text{g/L}$ cut-off in the 88.89% sensitivity and specificity of 83.33%). But, a low specificity has been observed in dogs with DIC (Stokol et al. 2000). Additionally, it is pointed out that D-dimer analysis was an important marker in the diagnosis of clinically suspected acute pulmonary embolism in human (PE) (Righini et al. 2000, Righini et al. 2014). Mean D-dimer levels obtained from human and dog were higher than the findings in the present study. To compare the D-dimer cut-off values in dogs and humans with the results obtained from cattle is not a valid approach. However, a partial evaluation can be made. Therefore, especially D-dimer levels are needed to be investigated in further studies on cattle with bleeding disorders or disease characterized by anaemia.

Haematological changes are a controversial issue in cattle with theileriosis. Settlement of *Theileria* in

erythrocytes varies according to the type of it. The reason for high D-dimer levels in animals with theileriosis may be due to haemolytic factors within the erythrocyte. However, activating of clotting may be due to activation of certain factors. This study had some limitations, because animals were naturally infected with *Theileria* and the animals might not completely reflect the uniform response due to differences in incubation period of infection in each animal. This limitation may lead to a large range of coagulation data. However, to our best knowledge, this is first study in which D-dimer concentrations were analysed in natural bovine theileriosis.

Conclusions

The D-dimer analysis is a beneficial test to determine coagulopathy status in the cattle suffering from theileriosis and also could be used to observe response to treatment and prognosis of disease management in an outbreak under field conditions.

As a result, in cattle with theileriosis cases, analysis of D-dimer may give more specific information than the others and it may be an important indicator in the evaluation of the prognosis in such cases. Analysis of D-dimer before and after treatment may also be suggested to enlighten the issue in future controlled case studies

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