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RESEARCH ARTICLE

Hemostatic disorders in canine monocytic ehrlichiosis

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Abstract

Objective: The aims of this study were (1) to compare platelet count, APTT (activated partial thromboplastin time) and PT (prothrombin time) between healthy dogs and dogs with canine monocytic ehrlichiosis (CME), and (2) to study whether any coagulation factor defects occur in dogs with the disease.

Materials and Methods: Blood samples were obtained from 20 clinically healthy dogs and 18 CME dogs. Some coagulation profiles and platelet count were measured by an automatic blood coagulation analyzer and an automatic analyzer, respectively. When the PT or APTT was prolonged, substitution test would be performed.

Results: Significant increases in APTT ($P<0.01$) and decreases in platelet count ($P<0.01$) for ehrlichiotic dogs were observed. However, no significant differences in PT among *Ehrlichia*-infected and healthy dogs were found. Furthermore, by substitution test (APTT), 2 of 18 cases (11.1%) possibly had revealed Factor IX defect and 1 of 18 cases (5.6%) possibly had Factor XI or XII defect, and both had factor deficiencies.

Conclusion: Defects of some coagulation factors may be involved in the pathophysiology of canine monocytic ehrlichiosis.

Keywords: canine monocytic ehrlichiosis, hemostatic disorders

Introduction

Ehrlichia canis, Gram-negative bacterium, can cause canine and human monocytic ehrlichiosis (Perez et al., 2006). They have been reported in dogs worldwide where the brown dog tick, *Rhipicephalus sanguineus*, is prevalent (Bowman et al., 2009; Labarthe et al., 2003; Mavromatis et al., 2006; Pinyoowong et al., 2008; Shipov et al., 2008; Sainz et al., 2015). This tick mainly live on dogs but occasionally found on humans (Dantas-Torres, 2010). Clinical signs of the disease are not specific. The *Ehrlichia*-infected dogs might have clinical signs includes fever, anorexia, lethargy, pale mucous membrane, anterior uveitis, retinal detachment, lymphadenopathy, splenomegaly, hepatomegaly and mostly have laboratory findings for non-regenerative anemia leading to a variety of changes in blood figures including thrombocytopenia (Bai et al., 2017; Moonarmart et al., 2014; Parashar et al., 2016; Sainz et al., 2015; Toom et al., 2016). Thrombocytopenia is the characterized finding of dogs with ehrlichiosis led to abnormal hemostatic abnormalities such as ecchymoses, petechiae, epistaxis, prolonged bleeding during estrus and hematuria or melena (Bai et al., 2017; Kottadamane et al., 2017; Sainz et al., 2015). However, the clinical signs are depended on the stage of the disease (Bai et al., 2017; Shipov et al., 2008). There are three stages of ehrlichiosis including acute, subclinical and chronic stages (McClure et al., 2010). The acute stage is usually developed within 8 to 20 days and fever, depression, lethargy, loss of appetite, ecchymoses on the skin and epistaxis are often found (Woody and Hoskins, 1991). Thrombocytopenia, mild anemia and mild leucopenia might be observed. If dogs in this stage are left untreated, or the dogs are not able to fight off the infection, the disease might go on to the subclinical stage within 40-120 days after the acute stage. In the subclinical stage, the animal might look normal or show only mild thrombocytopenia (Waner et al., 1996). Ultimately, the dog may become a carrier and either eliminate the *Ehrlichia* from its body, or the infection may progress to the chronic phase (Mylonakis et al., 2004). The clinical findings in the chronic stage are consisted of lethargy, depression and fever (Harrus et al., 1999; Mylonakis et al., 2004). Abnormal hematologic findings were anemia, severe thrombocytopenia and leucopenia (Mylonakis et al., 2004). Thrombocytopenia is considered to be the most common symptom of dogs naturally or experimentally infected with *E. canis* (Waner et al., 1995) and the level of thrombocytopenia often indicates the treatment response (Harrus et al., 1999). Platelet count should be increased within 4-8 weeks after treatment begins.

Besides platelet count, activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen concentration are essential to diagnosing secondary hemostasis (Mischke, 2000). APTT should be used for blood coagulation in intrinsic and common pathways, with PT for extrinsic and common pathways. Therefore, both parameters should be detected to identify these pathway abnormalities. The objectives of this study were 1) to compare platelet count, APTT and PT between healthy and *E. canis* infected dogs, and 2) to study whether any coagulation factor defects occur in *E. canis* infected dogs. These data will be beneficial for further pathophysiologic study, and some individualized therapeutic plans.

Materials and Methods

Ethics approval

This study was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand (Record No. ACUC-KKU-39/60, Reference No. 0514.1.75/59).

Animals

Dogs in this study were selected from the dogs visiting the KKU Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Khon Kaen University during July 2017-October 2017. *E. canis* infected dogs had to be diagnosed by both thin blood smear to visualize the morula of *E. canis* within mononuclear cells and a commercial ELISA rapid diagnostic assay kit (SNAP® 4Dx®; IDEXX Laboratories, Inc. U.S.A.). Dogs undergoing treatment or receiving specific antibiotic for this disease were excluded. Clinically healthy dogs had to be more than 1 years old without any blood parasites or intestinal parasites and have had a normal physical examination at least 6 months before blood collection. Owner consent was obtained for all participating dogs and the study protocol was approved by the Research Ethics Committee, Khon Kaen University.

Blood sample collection, preparation and statistical analysis

Three mls of blood was drawn from the cephalic vein and divided into three parts. One ml was treated with ethylenediamine tetraacetic acid as an anticoagulant and used to determine a complete blood count and the presence of blood parasites. One ml was centrifuged at 400G at 4°C for 5 minutes to provide serum for blood chemistry tests (e.g. creatinine and alanine aminotransferase (ALT). The remainder was mixed with 3.8% sodium citrate at the ratio of 1:9. Blood samples were centrifuged at 2000G for 10 minutes at 4°C to obtain plasma. The plasma was used to measure the PT and APTT by an automatic blood coagulation analyzer (Sysmex®, Japan). Results are presented as mean ± SD (Table 1).

Packed cell volume (PCV), hemoglobin (Hb), red blood cell count (RBC), white blood cell count (WBC) and platelet count were measured using an automatic analyzer (Sysmex XT-2000iV, USA). Additionally, dried blood smears were fixed in methyl alcohol and stained with Wright-Giemsa stain and examined under the oil immersion lens of a microscope for blood parasites. Creatinine and ALT were measured using an automatic blood chemistry analyzer (Olympus® AU400, USA).

Prothrombin time test (Crocker and Burnett, 2005)

PT was detected by automated blood coagulation analyzer. Fifty microliters of plasma was pipetted into a plastic tube and incubated for 5 min then placed in the PT channel of the analyzer. After 3 min, 100 µl of thromborel (Thromborel®, Dade Behring) was added to the plasma, and then the PT was read automatically.

Activated partial thromboplastin time test (Crocker and Burnett, 2005)

Fifty microliters of plasma was pipetted into a plastic tube and incubated for 5 min then placed in the APTT channel of the analyzer. After 1 min, 50 µl of actin (Actin®, Dade Behring) was added to the plasma, and at 3 min completion, 50 µl of calcium chloride was added to the plasma. APTT was read automatically.

Substitution test

If the prolongation of the PT or APTT was due to clotting factor defect, the clotting time of the test plasma mixed with normal plasma should be reduced by more than 10% of the test plasma clotting time. The substitution experiments would be performed further using normal canine serum (aged serum) or normal adsorbed canine plasma. Aged serum was prepared by incubating normal serum for 4 hours at 37°C, then centrifuging at 3000G at room temperature for 5 min. Serum was tested by APTT and PT tests. Absorbed plasma was prepared by mixing 10 ml normal plasma with 1 ml of 25% aluminum hydroxide at 37°C for 5 min, then centrifuging at 3000G at room temperature for 5 min. APTT and PT tests were performed (Turgeon, 2005; Mukherjee and Ghosh, 2010).

In the substitution test, 0.4 ml of plasma of a dog with *E. canis* was mixed with 0.1 ml of pooled normal plasma which contains approximately 100% of all coagulation factors, 0.1 ml of adsorbed plasma which contains Factors I, V, VIII, XI and XII, and 0.1 ml of aged serum which contains Factors VII, IX, X, XI and XII. The mixing of each sample was used to measure the PT and APTT. If the abnormal result is corrected by the addition of normal plasma, a factor deficiency is indicated, whereas no correction of the abnormal result may indicate the presence of a circulating inhibitor (Crocker and Burnett, 2005).

Statistical analysis

Mean and standard deviations (SD) of the parameter exhibited during the study were calculated. These parameters were compared by Mann Whitney U tests. A value of $P<0.05$ was considered statistically significant.

Results

Of 45 dogs, 22 dogs were identified with *E. canis* infection and 23 dogs were defined as clinically healthy dogs. Four of the dogs with *E. canis* infection and three clinically healthy dogs were excluded from the analysis, as they did not fit the inclusion criteria. Therefore, 18 dogs with *E. canis* infection, 8 males and 10 females with an average age of 3.6 years (1-8 years old) and 20 clinically healthy dogs, 8 males and 12 females, with an average age of 2.9 years (1-7 years old) were used for the final analysis.

When the hemostatic value of healthy dogs and *E. canis* infected dogs were compared, the result demonstrated the decreased platelet count, APTT and PT shown in Table 1. Platelet counts in *E. canis* infected dogs were

Table 1. Platelet count, APTT and PT of E. canis infected and healthy dogs.

Parameters	E. canis infected dogs (Mean ± SD)	Healthy dogs (Mean ± SD)	P value
Platelets (× 109/L)	95 ± 133.02	355 ± 57.59	<0.01
APTT* (seconds)	20.55 ± 5.02	14.04 ± 0.78	<0.01
PT** (seconds)	7.61 ± 1.38	7.47 ± 1.52	0.26

*Activated partial thromboplastin time

**Prothrombin time

significantly decreased (P<0.01), and the APTT was significantly increased (P<0.01). When substitution tests were assayed, two out of 18 cases (10.5%) had coagulation Factor IX deficiencies, and one case (5.3%) had coagulation Factor XI and/or XII deficiency in *E. canis* infected dogs. However, the PT between healthy and infected dogs was not significantly different.

There were no significant differences in age and body weight between the two groups (p=0.28 and 0.10, respectively). As a result, PCVs were significantly lower in *E. canis* infected dogs (27.66±8.97%) than in clinically healthy dogs (46.25±5.95%) (P<0.01), Hb was significantly lower in *E. canis* infected dogs (9.16±3.15 g%) than in clinically healthy dogs (15.75±2.13 g%) (P<0.01), RBC counts were significantly lower in *E. canis* infected dogs ($4.91±1.83×10^6$ μL^{-1}) than in clinically healthy dogs ($8.26±1.04×10^6$ μL^{-1}) (P<0.01) and WBC counts were significantly lower in *E. canis* infected dogs ($8.71±5.65×10^3$ μL^{-1}) than in clinically healthy dogs ($14.32±4.02×10^3$ μL^{-1}) (P<0.01). No blood parasites were found and creatinine and ALT levels were within normal ranges and not significantly different between the two groups (p=0.74 and p=0.27, respectively).

Discussion

When comparing hematology and coagulation tests between healthy dogs and *E. canis* infected dogs, *E. canis* infected dogs possessed significantly lower platelet count, lower RBC count, lower Hb, lower WBC count and higher APTT. However, the PT between healthy dogs and *E. canis* infected dogs was not significantly different, and other hematologic values and blood chemistry were not significantly different between both groups.

Prognostic factors for *E. canis* infected dogs were previously studied. Major indicators of poor survival were severe anemia, leucopenia, pancytopenia, hypokalemia and thrombocytopenia (Harrus et al., 1997; Shipov et al., 2008). In the previous study, the reduction in platelet count was observed in canine monocytic ehrlichiosis (Cortese et al., 2006). Therefore the decreasing platelet count involved in the pathogenesis of CME can be attributed to several mechanisms, acting together or alone. Generally, there are three stages of the ehrlichiosis including acute, subclinical and chronic stages. Each stage is associated with a different degree of systemic pathogenesis, clinical manifestations, and complicated treatment (Duffy et al., 2010). The reduction of platelet count was relied on stage of the disease. In the acute stage, mild to moderate thrombocytopenia, lower WBC counts, and non-regenerative anemia are often observed (Buhles et al., 1974; Codner and Farris-Smith, 1986; Iqbal et al., 1994; Rikihisa, 1991). In the subclinical stage, chronic pancytopenia might be occurred in infected dogs resulting from bone marrow suppression. Thrombocytopenia, leucopenia, and severe non-regenerative anemia may follow (Waner et al., 1996). In the chronic stage, secondary infection and hemorrhage are often reported stated that abnormal coagulation associated with thrombocytopenia often is occurred in *E. canis* infected dogs (Cortese et al., 2006).

In this study, *E. canis*-infected dogs had clinical signs including have anemia, leucopenia and thrombocytopenia, which is similar to previous studies. Several mechanisms might involve with thrombocytopenia including dysfunction of platelets, platelet consumption during vasculitis, destruction of shorter life span platelets by the spleen, bone marrow suppression causing lower platelet production, and platelet kinetics in canine ehrlichiosis. There is an evidence to increase platelet destruction as the cause of thrombocytopenia, reduction of platelet adhesiveness, and immunologic reactions (Breitschwerdt, 1988; Harrus et al., 1999; Harrus et al., 1996; Kakoma et al., 1978;

Lovering et al., 1980; Palacios et al., 2017; Pierce et al., 1977; Reardon and Pierce, 1981; Smith et al., 1975; Waner et al., 1995; Waner et al., 2000; Woody and Hoskins, 1991).

From a retrospective study for canine monocytic ehrlichiosis, the APTT of non-surviving dogs were significantly longer than those of surviving dogs (>18.25 sec and <14.5 sec, respectively) (Shipov et al., 2008). Additionally, severe anemia, severe leucopenia and hypokalemia were often observed in dogs that cannot survive. In this study, 3 out of 19 cases with more than 18.25 sec of APTT were survived.

Hemorrhage of *E. canis*-infected dogs is associated with a hemostatic disorder. In this study, some parameters of the hemostatic process were compared between healthy and *E. canis* infected dogs. Disorders of primary and secondary hemostasis were screened by performing platelet counts, and APTT and PT, respectively. APTT is the primary parameter for coagulation Factors II, V, VIII, IX, X, XI and XII of intrinsic and common pathways, whereas PT is the primary parameter for coagulation Factors II, V, VII and X of extrinsic and common pathways (Triplett, 2000). The common pathway is working process for coagulation Factors I, II, V and X (Rizzo et al., 2008). In this study, disorders of hemostasis including lower platelet counts and prolonged APTT were detected in *E. canis* infected dogs. In addition, the cause of prolonged APTT is still unknown and the PT level was not significantly different between healthy and infected dogs.

Since APTT influence the intrinsic pathway and PT affect the extrinsic pathway, it is possible that prolonged APTT and normal PT are related to defect some factors in the intrinsic pathway, such as Factors VIII, IX, XI, XII (Smith, 2005) and to defect these coagulation factors might leading to the pathogenesis of canine monocytic ehrlichiosis. In this study, 2 of 18 cases with prolonged APTT but normal PT were corrected with normal plasma but not corrected with normal adsorbed plasma by the substitution test. So the cases were possibly from Factor IX deficiency. One of 18 cases was corrected by both normal serum and adsorbed plasma. Therefore, this case was possibly a Factor XI or XII defect, and both factor deficiencies.

In all factor deficiencies, males and females are equally affected (Cotter, 2007). Hemophilia is the most common inherited coagulation in humans and dogs. Molecular diagnosis will enhance the detection of carriers with hemostatic defects in dogs (Brooks, 1999). Factor IX deficiency (hemophilia B) may result from either reduced production of the functional Factor IX protein or production of a defective protein (Giannelli et al., 1997). Hemophilia B is a recessive bleeding disorder due to mutations of the Factor IX gene on the X chromosome (Tsai et al., 2007). The incidence of hemophilia B is lower than hemophilia A (factor VIII deficiency). Disorder of coagulation Factor IX is one of the common inherited blood clotting disorders in dogs (Slutsky et al., 2013). Canine hemophilia B is highly similar to the hemophilia in human. Therefore, dogs are one of the most common animal models for hemophilia (Yen et al., 2016). Factor XI deficiency (hemophilia C) is an infrequent autosomal-recessive form of canine hemophilia. Factor XII deficiency (Hageman factor) is an uncommon autosomal-dominant form of canine hemophilia. However, it is rarely associated with any symptom in humans (Cotter, 2007; Conner, 2016).

Coagulation disorders in hepatic disease are often caused by reduced synthesis of these coagulation factors or qualitative defects in factor production (Kemkes-Matthes et al., 1991). Defects of coagulation factors in *E. canis*-infected dogs might be caused by the liver damage. Acquired hemophilia is a bleeding disorder caused by the spontaneous development of the autoantibody against endogenous coagulation factors in individuals with previous hemostasis (Collins et al., 2010). This study was not suggestive of this disease due to APTT correction after doing the substitution test in this study.

Clotting factor deficiencies are leading to bleeding into soft tissue, joints, and muscles. There is a severe bleeding tendency in Factor IX deficiency, mild to moderate bleeding tendency in Factor XI deficiency and no clinical signs in Factor XII deficiency (Cotter, 2007). However, there were no clinical signs of bleeding into joints, soft tissue, and muscles in this study. This reveals that APTT and PT are useful to detect these factor deficiencies. Factor XII has not affected the fibrin formation, since these dogs had no clinical signs except prolonged APTT (Stokol, 2010). In treatment, fresh frozen plasma should be used for Factor XI deficiency and Cryo poor plasma or cryosupernatant for Factor IX and XI deficiency (Cotter, 2007). Patients with Factor XII deficiency have no specific therapy (Brooks, 2010). At present, there are studies on the use of gene therapy in hemophilia, especially in humans, where-

as researchers have been studying canine hemophilia (Mischke, 2012; Cantore et al., 2015). Prognosis is good for dogs with Factor IX deficiency and is excellent in dogs with Factor XII deficiency (Conner, 2016).

List of abbreviations

ALT: Alanine aminotransferase; APTT: activated partial thromboplastin time; Hb: Hemoglobin PCV: Packed cell volume; PT: prothrombin time; RBC: Red blood cell counts; WBC: White blood cell counts.

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