Original Article

Evaluation of the hematological and serum protein profiles of blood parasite coinfection in naturally infected dogs

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Abstract

Canine vector-borne diseases (CVBDs) are prevalent worldwide, including Thailand. Vectors can transmit more than one pathogen, and coinfections between blood parasites and/or filariae have been reported. Pathogenesis may affect the health status of infected dogs. Hematological, blood chemistry, and serum protein profile abnormalities can be used to screen for underlining causes of CVBDs. The aim of this study is to analyze the hematological and serum protein profiles as well as CRP in CVBD coinfection cases. In this study, 22 blood parasite coinfection cases are examined and classified into two groups: blood parasite coinfection (group 1: n = 16) and blood parasite and filaria coinfection (group 2: n = 6), both of which reveal anemia and thrombocytopenia abnormalities. The white blood cell and neutrophil count in group 2 showed slight increases. The serum protein profiles and CRP levels in both groups indicate hypoalbuminemia, increased β2 and γ globulin fractions, and increased CRP concentrations. The results of this study could be used by veterinarians to develop guidelines for the clinical diagnosis and treatment of coinfection in CVBDs.

Keywords: Blood parasites, Coinfection, Dogs, Hematology, Serum protein

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Introduction

Canine vector-borne diseases (CVBDs) are widely distributed throughout the world, and especially prevalent in tropical climates, where the conditions are ideal for the development of tick and mosquito populations. Many countries have reported CVBDs, including Thailand (Rucksaken *et al*., 2019; Thongsahuan *et al*., 2020). *Rhipicephalus sanguineus* (*sensu lato*), a brown dog tick, plays a crucial role in blood parasite transmission, while filarial worms are transmitted by mosquitoes such as *Mansonia, Anopheles, Culex,* and *Aedes* (Shaw *et al*., 2001; Tiawsirisup *et al*., 2010). Vectors are able to transmit more than one pathogen, and coinfections among blood parasites or blood parasites and filariae have been reported such as *Ehrlichia canis (E. canis)* and *Babesia vogeli (B. vogeli)* (Rawangchue and Sungpradit, 2020), *E. canis* and *Hepatozoon canis (H. canis)* (Tsachev *et al*., 2008), *Dirofilaria immitis (D. immitis), E. canis* and *Babesia canis (B. canis)* (Niwetpathomwat *et al*., 2006), *Anaplasma platys (A. platys),* and *E. canis* and *B. canis* (Lara and Conan, 2020). In Thailand, the prevalence of coinfection between *E. canis* and *B. vogeli* in Maha Sarakham Province is 2.5% (Piratae *et al*., 2015). In Bangkok, the prevalence of *dirofilariasis* and *ehrlichiosis*, *dirofilariasis* and *hepatozoonosis*, and *dirofilariasis*, *ehrlichiosis*, and *babesiosis* equates to 4, 0.4, and 0.8%, respectively (Niwetpathomwat *et al*., 2006). These CVBDs present with various clinical signs and pathology, for example: anorexia, lethargy, pale mucous membranes, fever, and hemolymphatic issues (Thongsahuan *et al*., 2020).

The evaluation of hematological alterations is important in the routine laboratory testing of many diseases and infections, including bacteria, rickettsia, mycoplasma, and protozoa. Abnormalities in hematological profiles can also be used to screen for underlying blood parasitic infection, including ehrlichiosis, babesiosis, hepatozoonosis, and anaplasmosis. Single blood parasite and filariae infections have been frequently reported in Thailand, while a lack of information exists on the hematological profiles in relation to CVBD coinfection.

In addition, the measurement of serum protein may be important to the detection, diagnosis, and health status monitoring of various diseases and pathological processes. Serum protein electrophoresis profiles (SPEPs) are used in clinical practice to identify patients with serum protein disorders, including single infection blood parasites such as *E. canis, B. canis*, *H. canis*, and *Anaplasma phagocytophilum* (Harrus *et al*., 1996; Paiz *et al*., 2016; Ravnik *et al*., 2014; Tóthová *et al*., 2020). It is an inexpensive and easy-to-perform screening procedure, whereby canine serum proteins are separated into five or six bands, including albumin, α1 globulin, α2 globulin, β globulin, and γ globulin in agarose gel electrophoresis. The β globulin fraction can be separated into β1 globulin and β2 globulin (Tóthová *et al*., 2016). Previous studies have reported single blood parasite infections such as babesiosis, ehrlichiosis, and hepatozoonosis (Harrus *et al*., 1996; Tóthová *et al*., 2020). Unfortunately, little information is available on serum protein electrophoresis in blood parasite coinfection and blood parasite and filaria coinfection.

Acute phase proteins (APPs) are useful markers for stratification of the acute phase response (Schmidt and Eckersall, 2015). The acute phase response is an innate host defense mechanism, occurring during the early stages of infection, tissue injury, or immunological disorders. During an acute phase response, the serum APPs are changed in response to cytokines such as interleukin-1 (IL-1), IL-6, and the tumor necrosis factor alpha (TNF-α) (Cerón and Eckersall, 2005). The CRP is one such APP and synthesized by hepatocytes, macrophages, endothelial cells, lymphocytes, adipocytes, and smooth muscle cells. The CRP is a major APP in dogs and part of the γ-globulin fraction. The CRP concentration is used as a predictive marker for disease risk and monitoring the response to treatment (Sproston and Ashworth, 2018).

There are insufficient data on hematological profiles, SPEPs, and the CRP of CVBD coinfection. The aim of this study is to determine the hematological profiles, SPEPs, and CRP concentrations in dogs coinfected with various blood parasites and/or filariae.

Materials and Methods

Blood sample collection and diagnosis of blood parasites and filariae: The protocol compiled for the care of animals in this study has been approved by Chulalongkorn University Animal Committee (Approval No. 1931052). The canine blood samples were collected from small animal hospitals and clinics in Bangkok and its vicinity from October 2019 to October 2020. Samples were collected in EDTA and serum collection tubes, with blood parasite infections confirmed by the buffy coat thin blood smear and polymerase chain reaction (PCR) techniques (Rucksaken *et al*., 2019). The 22 cases with naturally infected CVBD coinfection (n = 22) were classified into two groups: group 1 blood parasite coinfection (n = 16) consisting of *B. canis* and *E. canis* (n = 12), *H. canis* and *E. canis* (n = 1), *H. canis, B. canis,* and *E. canis* (n = 1), *A. platys* and *E. canis* (n = 2); and group 2 blood parasite and filaria coinfection (n=6) consisting of *D. immitis* and *E. canis* (n = 1), *Brugia pahangi (B. pahangi)* and *E. canis* (n = 1), *D. immitis* and *H. canis* (n = 3), and *B. pahangi* and *B. canis* (n = 1). The criteria for normality were healthy dogs with no clinical signs of blood parasite infection. Blood chemistry profiles of normal dogs (n=6) were in the normal range. All serum samples were kept at -20 ^{o}C until analysis.

Hematology, blood chemistry, and serum protein determination: The hematology and blood chemistry of all coinfected samples were measured using automated analyzers (the Sysmex XN-10 hematology and Olympus AU400 clinical chemistry analyzers). The hematological and blood chemistry profiles were recorded and analyzed, consisting of red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), red blood cell distribution width (RDW), white blood cell (WBC) count, platelets, WBC differential count, alanine

aminotransferase (ALT), and creatinine. The total protein was determined by the Biuret colorimetric test (Human®, Wiesbaden, Germany). All serum samples were separated using agarose gel electrophoretic techniques (SPIFE® split beta SPE kit, Helena Laboratories, TX, USA). Twenty microliters of serum samples were placed in each well and electrophoresis performed at 400 volts for six minutes. The gel was pre-dried at 53 °C for 12 minutes, stained with an acid blue solution, and destained in a citric acid solution. All steps were performed by an automated machine (Spife®3000, Helena Laboratories). The band density of the serum proteins was measured and analyzed using the QuickScan Touch program (Helena Laboratories).

Laboratory measurements of CRP concentrations: The positively coinfected serums were determined for CRP concentrations by fluorescent immunoassay (Vcheck Canine CRP 2.0 test kit, Bionote, South Korea). Five microliters of each sample were diluted in a diluent buffer from the test kit. One hundred microliters were mixed and added to the test device. The CRP concentration was displayed on the screen after five minutes, with CRP concentration above 30 mg/L considered abnormal.

Statistical analysis: The SPEPs data in both coinfection groups were compared with normal dog (n = 6) values using the paired T-test. Significance was set at $P < 0.05$.

Results

The results of the hematological profiles, blood chemistry, total protein, albumin, α1-, α2, β1-, β2-, γ-globulin, and A/G ratio in CVBD coinfections are presented in Tables 1 to 3. The hematological profiles and blood chemistry results for the two groups of CVBD coinfections indicated anemia and thrombocytopenia. In group 2, the average WBC and neutrophil counts showed slight increases and high ALT levels.

The total protein, albumin, α1-, α2-, β1-, β2, γ-globulin levels, and A/G ratio were compared between CVBD coinfections and normal dogs. The results for the total protein level indicated that two groups of CVBD coinfections were significantly higher than normal but exhibited lower A/G ratios. The results for albumin in both groups indicated lower than normal levels. The globulin level of both groups was higher than normal, especially the β2- and γ-globulin fractions. Moreover, in group 2, especially in *B. pahangi* and *E. canis* coinfection and *B. pahangi* and *B. canis* coinfection, a higher α2-globulin level than normal was exhibited. The SPEPs were determined by agarose gel electrophoresis, with the representative electrophoretograms presented in Figure 1. The average CRP levels in both groups were 132.46 and 77.23 mg/L, respectively. Most CVBD coinfection cases showed higher than normal CRP levels.

Table 1 Results of hematological profiles, ALT, and creatinine in dogs with CVBD coinfection (mean ± SEM).

Parameters	Units	Blood parasite coinfection (n=16)	Blood parasite and filaria coinfection (n=6)	Reference range (Kaneko, 1997; Latimer, 2011)
RBC	10^6 cells/mm^3	4.388 ± 0.4334	3.717 ± 0.9478	5.5–8.5
Hb	g/dl	9.638 ± 0.8986	8.933 ± 2.103	12–19
Hct	%	28.50 ± 2.585	26.33 ± 6.296	37–57
MCV	fL	66.18 ± 1.249	72.25 ± 2.792	66–77
MCH	Pg	22.13 ± 0.5296	24.17 ± 0.5031	19.5–24.5
MCHC	g/dl	33.46 ± 0.4466	33.65 ± 0.8943	32–36
RDW	%	14.21 ± 0.4258	15.68 ± 1.465	12–15
WBC	cells/mm^3	9,281 ± 2114	17,700 ± 4840	6,000–17,000
Neutrophil	cells/mm^3	7121 ± 1852	13085 ± 3319	3,000–11,500
Eosinophil	cells/mm^3	86.31 ± 32.99	395.2 ± 142.4	100–1,250
Lymphocyte	cells/mm^3	1,835 ± 296.0	3,725 ± 1372	1,000–4,800
Monocyte	cells/mm^3	230.9 ± 51.17	393.8 ± 172.0	150–1,480
Platelet count	10^3 cells/mm^3	120.13 ± 10.75	134.00 ± 13.10	200-500
ALT	U/liter	46.06 ± 5.756	311.7 ± 238.8	21–102
Creatinine	U/liter	0.9625 ± 0.07899	1.367 ± 0.2629	0.5–1.5

Table 2 Results for the relative concentrations of serum protein fractions (%) and albumin/globulin ratio (A/G) in dogs with CVBD coinfection (mean ± SEM). The *P* value refers to the analysis of variance significance, while n.s. is not significant. *Significance level of the difference between normal and CVBD coinfections at $P<0.05$.

Parameters	Normal (n = 6)	Blood parasite coinfection (n = 16)	Blood parasite and filaria coinfection (n = 6)	*P* value
Albumin	39.53 ± 1.69	28.30 ± 2.610*	23.98 ± 3.72*	<0.05
α1-globulin	6.22 ± 0.92	5.96 ± 0.38	5.63 ± 0.67	n.s.
α2-globulin	6.78 ± 1.99	6.425 ± 1.137	8.58 ± 2.57	n.s.
β1-globulin	12.10 ± 1.69	11.11 ± 1.42	13.78 ± 3.92	n.s.
β2-globulin	10.00 ± 1.81	23.29 ± 3.179*	20.52 ± 5.69	<0.05
γ -globulin	25.37 ± 4.46	22.99 ± 2.68	27.68 ± 4.90	n.s.
A/G ratio	0.65 ± 0.04	0.44 ± 0.042*	0.32 ± 0.06*	<0.05

Table 3 Results for the total protein and absolute values of protein fractions (g/l) in dogs with CVBD coinfection (mean ±SEM). The *P* value refers to the analysis of variance significance, while n.s. is not significant. *Difference in significance between normal and CVBD coinfection at *P*<0.05.

Parameters	Normal (n = 6)	Blood parasite coinfection (n = 16)	Blood parasite and filaria coinfection (n=6)	*P* value
Total protein	56.64 ± 9.35	81.49 ± 3.96*	86.30 ± 7.82*	<0.05
Albumin	22.50 ± 4.11	24.00 ± 1.426	19.88 ± 2.251	n.s.
α1-globulin	3.83 ± 1.14	4.813 ± 0.1875	4.750 ± 0.6021	n.s.
α2-globulin	4.33 ± 1.96	5.375 ± 1.179	7.900 ± 2.771	n.s.
β1-globulin	7.17 ± 1.99	9.125 ± 1.423	11.88 ± 4.068	n.s.
β2-globulin	6.17 ± 1.96	19.56 ± 2.714*	18.37 ± 5.178	<0.05
γ -globulin	13.00 ± 2.19	18.75 ± 2.454	23.65 ± 5.200	n.s.

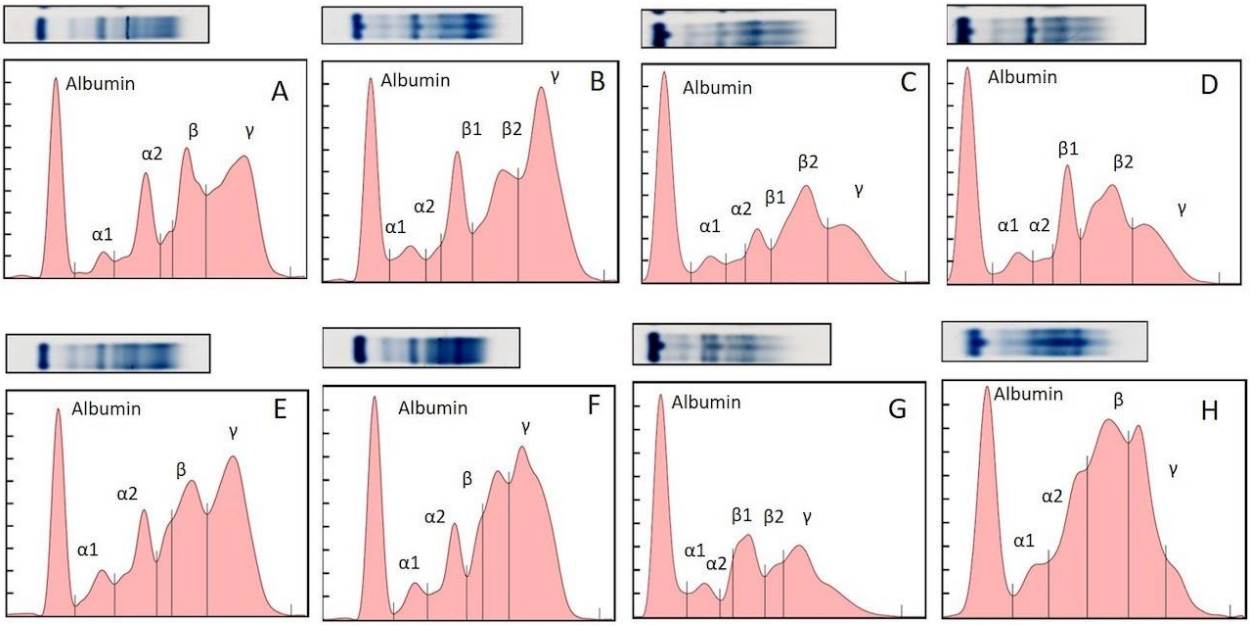

Figure 1 Representative serum electrophoresis profiles of dog samples with positive CVBD coinfection. (A) *B. canis* and *E. canis*; (B) *H. canis* and *E. canis*; (C) *H. canis, B. canis* and *E. canis*; (D) *A. platys* and *E. canis*; (E) *D. immitis* and *E. canis*; (F) *B. pahangi* and *E. canis*; (G) *D. immitis* and *H. canis*; (H) *B. pahangi* and *B. canis*.

Discussion

The hematological and blood chemistry profiles benefit the diagnosis, treatment monitoring, and checking of the animal's health status. All hematological profiles of both groups in this study contained anemia and thrombocytopenia abnormalities. In a previous report, anemia and thrombocytopenia could be typically found in *E. canis, B. canis,* and *H. canis* single infections. The hematological profiles of coinfection were compared with single blood parasite infections (Rucksaken *et al*., 2019; Thongsahuan *et al*., 2020). Lower RBC, Hb, and hematocrit levels were found in three cases: *H. canis, B. canis,* and *E. canis*; *D. immitis* and *E. canis;* and *B. pahangi* and *E. canis* coinfections. Blood transfusion should be considered in some coinfection cases because more severe anemia is likely to be found in single blood parasite infections. Blood parasite infection might lead to anemia because of antibody production against erythrocytes, immune-mediated hemolytic anemia (IMHA), and bone marrow destruction, especially in the case of *E. canis* infection. Thrombocytopenia might be caused by immunologic and inflammatory mechanisms and reduced platelet lifespan, while an elevated WBC could be due to the inflammatory response (Thongsahuan *et al*., 2020). In blood parasite and filaria coinfection, the average WBC and neutrophil counts were slightly increased and had high ALT levels. In this study, *H. canis, B. canis,* and *E. canis*; *A. platys* and *E. canis*; and *D. immitis* and *H. canis* coinfections were found to have leukocytosis, neutrophilia, and lymphocytosis. In the blood chemistry profiles, the ALT levels in *B. pahangi* and *E. canis* infections showed dramatic increases. The ALT marker provides good sensitivity for liver abnormalities in dogs. In *E. canis* coinfection with *B. pahangi,* the increase in the ALT level was caused by *E. canis* pathogenesis, potentially inducing hepatitis (Mylonakis *et al*., 2010).

The serum protein electrophoresis technique is an easy and useful method for separating and analyzing albumin and each globulin fraction. It is used in clinical medicine to aid the monitoring and diagnosis of various clinicopathological conditions such as acute and chronic inflammation, monoclonal gammopathies, nephropathy, liver disease, etc. For single blood parasite and filaria infections such as babesiosis, ehrlichiosis, hepatozoonosis, and dirofilariasis, although there are many reports on SPEPs, a lack of data exists on CVBD coinfection. The results of this study indicate a higher total protein level while the A/G ratios in all CVBDs coinfection groups were lower than normal.

Both groups exhibited significantly lower than normal albumin concentrations (%) and A/G ratios at $p<0.05$. The A/G ratio decreased due to the low level of albumin and/or high level of globulin production. The low level of albumin might be caused by cachexia, inflammation, kidney disease, or liver damage (Jania and Andraszek, 2016), while the pathology of babesiosis, hepatozoonosis, ehrlichiosis, and anaplasmosis affected the liver and/or kidney function. In a previous report, the concurrence of dirofilariasis and blood parasites increased the serum alanine aminotransferase and serum alkaline phosphatase activities which are indicative of hepatocellular damage (Niwetpathomwat *et al*., 2006). According to the results, the β-γ bridging pattern could be found in *H. canis* and *E. canis* coinfections. The β-γ bridging pattern is usually found in liver disease (Camus *et al*., 2010).

The globulin fractions can be separated into five bands: α1-, α2-, β1-, β2-, and γ globulin. The α fraction is the most rapidly migrating protein of all globulins, and in most species, it migrates as α1 (fast) and α2 (slow). In group 2, *B. pahangi* and *E. canis* coinfection and *B. pahangi* and *B. canis* coinfection cases exhibited higher α2-globulin levels than normal. The α2 globulin fraction consists of various APPs such as haptoglobin, α2-microglobulin, α2-macroglobulin, and ceruloplasmin (Tóthová *et al*., 2016). The haptoglobin could bind the free hemoglobin released from destructive erythrocyte with high affinity (MacKellar and Vigerust, 2016). Blood parasite pathogenesis such as *Babesia* spp. could invade erythrocytes causing lysis and hemolytic anemia (Akel and Mobarakai, 2017). In a human patient infected with lymphatic filariasis, the levels of plasma haptoglobulin, serum amyloid protein A, and α2-macroglobulin increases, and is associated with disease pathogenesis (Anuradha *et al*., 2012). In addition, in the case of *E. canis* infection, the levels of ceruloplasmin and haptoglobin significantly increases on days 12 and 18, respectively (Munhoz *et al*., 2012). The significant increase in the ceruloplasmin and haptoglobin levels have been clearly identified in the canine babesiosis serum using the proteomic approach (Kuleš *et al*., 2014; Ulutas *et al*., 2005).

The β-globulin group of globular proteins is more mobile than α-globulins. Transferrin and complement are the main proteins in the β-globulin fraction. Other important proteins in this fraction are ferritin, haemopexin, and β2-microglobulin (Tóthová *et al*., 2016). At the β1-globulin level, *H.canis* and *E.canis*, *D.immitis* and *H.canis,* and *B.pahangi* and *B.canis* coinfection were found to be higher than normal. At the β2-globulin level, both groups has higher level than normal, but the blood parasite coinfection group exhibited significantly increased levels at $p<0.05$. In the case of *E. canis* infection, the ferritin and transferrin levels were higher in both acute and subclinical phases of the disease (Bottari *et al*., 2016).

The γ-globulin fraction consists of several immunoglobulins such as IgG and IgM. Immunoglobulin functions as part of the body's immune system, responding to stimulation by antigens (Tóthová *et al*., 2016). In the absolute γ-globulin concentration, most blood parasite coinfections and blood parasite and filaria coinfection showed higher levels than normal due to the immune system. In a previous report, *B. canis* and *D. immitis* coinfections exhibited increased levels of γ-globulin (Milanović *et al*., 2017). In addition, CRP in dogs forms part of the γ-globulin fraction (Jania and Andraszek, 2016). In canine filariasis and various blood parasite infections, the CRP (an APP) increases (Anuradha *et al*., 2012; Eichenberger *et al*., 2016; Ulutas *et al*., 2005). The CRP concentration in most blood parasite and/or filaria coinfection groups was found to be higher than normal. Increased immunoglobulin and CRP levels indicate a systemic infection, attempting to prevent infection and contributing toward resolution of the morbidity process.

In conclusion, coinfection in CVBD is identifiable through clinical pathological changes such as anemia, thrombocytopenia, hypoalbuminemia, increased β2 and γ globulin fractions, and an increase in CRP concentrations. The hematological profiles, serum protein profiles, and CRP level can provide a guideline for the clinical diagnosis and treatment of CVBD coinfection.

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