CLINICAL HISTORY AND HEMATOLOGICAL FINDINGS AMONG CANINES WITH MONOCYTIC EHRLICHIOSIS

Walasinee Moonarmart[1], Sivapong Sungpradit[2], Thanakorn Rawangchue[2], Karuna Suphaphiphat[2], Sineenart Suksusieng[2] and Charoonluk Jirapattharasate[2]

[1]Department of Clinical Science and Public Health, [2]Department of Pre-clinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Bangkok, Thailand

Abstract. Canine monocytic ehrlichiosis is a tick borne disease caused by *Ehrlichia canis*, an obligate intracellular rickettsial organism belonging to the family Anaplasmataceae. Canine ehrlichiosis causes hemaotological changes among infected animals which could be used as a potential predictor for diagnosing canine monocytic ehrlichiosis (CME). Ninety-four blood samples were obtained from canines that either presented for a routine health check-up or for clinical illness. A history, physical and laboratory test were conducted on each animal. All samples were examined for *E. canis* using a 16S rDNA polymerase chain reaction (PCR) amplification to confirm CME infection. Thirty-six of the samples were positive for *E. canis* using PCR and the rest were negative. The Mann-Whitney and chi-square test were used to compare the differences between the PCR-positive and negative animals. PCR-positive animals had a higher mean body temperature than PCR-negative animals. The following were significantly lower in PCR-positive animals: white blood cell count, eosinophil count, red blood cell count, hemoglobin, hematocrit, platelet count, and the random distribution of width (RDW) of the red blood cells. We evaluated complete blood cell count findings to determine factors associated with CME using multivariable logistic regression analysis and found thrombocytopenia was significantly associated with CME (OR=0.085; 95%CI: 0.78-0.92, $p<0.001$). For every decrease in the platelet count of 10,000 there was a 15% increase in the likelihood of having CME.

Keywords: canine monocytic ehrlichiosis, hematological profiles, thrombocytopenia, platelet count, predictor, 16S rDNA

INTRODUCTION

Canine ehrlichiosis is a parasitic disease endemic to Thailand (Pinyoowong *et al*, 2008; Foongladda *et al*, 2011). The causative agents are *Ehrlichia* spp, obligate intracellular rickettsia residing in leukocytes and transmitted through the bite of hard ticks (Groves *et al*, 1975). There are several species of *Ehrlichia* reported in Thailand: *E. canis*, *E. ewingii* and *E. chaffeensis* (Suksawat *et al*, 2001a; Parola *et al*, 2003; Pinyoowong *et al*, 2008).

Correspondence: Charoonluk Jirapattharasate, Department of Pre-clinic and Animal Science, Faculty of Veterinary Science, Mahidol University, 999 Putthamontol-4 Road, Salaya, Nakhon Pathom 73170, Thailand.
Tel/Fax: 66 (0) 2441 5242
E-mail: Charoonluk.jir@mahidol.ac.th

Ehrlichiosis can be classified into two groups based on the cells they infect: monocytic and granulocytic (Dumler, 2005). Dogs with canine monocytic ehrlichiosis (CME) have a variety of clinical signs ranging in severity from mild to fatal (Woody and Hoskins, 1991). CME has acute, subclinical and chronic forms; the clinical signs found during the acute stage include high fever, depression, lethargy, anorexia, lymphadenomegaly and splenomegaly (Skotarczak, 2003). Ophthalmological and neurological lesions can also be detected (Harrus and Waner, 2011). The clinical signs found in the chronic phase are similar to the acute phase but more severe (Waner *et al*, 1995). The signs of canine granulocytic ehrlichiosis (CGE) are nonspecific and include high fever, lethargy, anorexia, vomiting and diarrhea (Murphy *et al*, 1998). High fever and lethargy are the most prominent clinical signs in CGE (Skotarczak, 2003).

Canine ehrlichiosis may cause dramatic changes in complete blood counts (CME). During the acute stage, severe thrombocytopenia may be present and is a diagnostic finding and this result is still detectable in the chronic stage (Grindem *et al*, 2002). Abnormal CBC findings may serve as potential predictors of ehrlichiosis. There have been no published studies of CBC findings during the various stages of canine ehrichiosis in Thailand. Therefore, we conducted this study among canines in Thailand to determine the effects of CME on CBC results during the various stages of illness to study if any of these CBC changes might be associated with ehrlichiosis.

MATERIALS AND METHODS

Ninety-four blood samples collected in EDTA treated tubes were obtained from canines coming in for either a health check-up or clinical illness to Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University. The study was approved by the Faculty of Veterinary Science Animal Care and Use Committee, no. MUVS-2010-23. All samples were examined for the presence of molurae in monocytes using a Giemsa stain and observed under a light microscope. The samples were divided into 2 groups: molurae positive (59 samples) and molurae negative (35 samples). To prevent false positive results with microscopy, all the samples were again tested for CME using a polymerase chain reaction as described below.

DNA was extracted from 200 l of blood using the QIAamp® DNA blood Mini Kit (QIAGEN, Hilden, Germany) and the samples were stored at -20ºC until further processing. The DNA from each sample was amplified with a PCR using primers specific for the 16S rDNA gene as previously described (Murphy *et al*, 1998). The primer sequences specific for *E. canis* 16S rDNA used were HE3 (3′-5′) ATAGGTACCGTCATTATCTTCCCTAT and ECAN5 (3′-5′) CAATTATTTATAGCCTCTGGCTATAGGA.

Amplification was performed in a total volume of 25 l containing 2 l of template DNA, 2 l of 2.5 mM dNTP, 2 l of 25 mM $MgCl_2$, 5 pmol of each primer (Bio Basic, Kaohsiung, Taiwan), 2.5 l of 10X PCR buffer, 15.125 l of water and 2.5 U of *Taq*-polymerase (i-Tag® DNA polymerase, Intron Biotechnology, Gyeonggido, Korea). Thermocycling consisted of 30 cycles of 94ºC for 45 seconds and 59ºC for 30 seconds. PCR amplicons (396 bp) were eletrophoresed in 2.0% agarose gel, stained with GelRed™ (Biotium, Hayward, CA) and visualized under a UV light (Gene Genius, Cambridge, UK).

A history, physical examination and complete blood count (CBC) were performed on each of the 94 canines included in the study. Statistical analysis was performed using SPSS version 17.0 for Windows (SPSS, Chicago, IL). The results obtained for each group were tested for normality with the Shapiro-Wilk and Kolmonogorov-Smirnov tests. Comparisons between the canines with and without a positive test for CME in body temperature, white blood cell (WBC) count, eosinophil count, red blood cell (RBC) count, hemoglobin, hematocrit, platelet count, red cell distribution width, corrected WBC count and alanine aminotransferase were performed with the Mann-Whitney *U* test. The chi-square test was used to compare gender, appetite, water intake, general appearance, attitude, tick infestation and platelet counts between the two groups. Univariable logistic regression was used to evaluate age, gender, body weight, body temperature, heart rate, white blood cell count, monocyte count, neutrophil count, lymphocyte count, eosinophil count, red blood cell count, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW, plasma protein, corrected nRBC, ALT and creatinine to identify their relationship with a positive PCR result for *Ehrlichia* spp. Odds ratios and 95% confidence intervals (CI) were calculated. Variables with a p-value ≤ 0.05 on univariable analysis were evaluated with a backward elimination multivariable logistic regression model. Model fit was assessed using the Hosmer-Lemeshow test. Results are reported as medians with interquartile ranges where appropriate.

RESULTS

Ninety-four blood samples were examined for CME by thin blood smear and PCR. The thin blood smear results showed 59 samples were positive and 35 samples were negative but the PCR results showed 36 samples were positive and 58 samples were negative. The difference between the PCR and standard parasitological methods did not reach statistical significance (p=0.17). We then divided the subjects into 2 groups based on the PCR results: PCR positive (PP) and PCR negative (PN).

The data obtained from the medical records are summarized in Table 1. Of the 36 PP canines, 17 were males and 19 females. Eleven of the PP canines were mixed breeds, 5 were Thai breeds, 4 were poodles, 4 were Shih tzus, 3 were Pomeranians, 2 were Bangkeows and there were 1 each of the following: beagle, Franch bull dog, golden retriever, Labrador retriever, pug, Rottweiler and unknown. Of the 58 PN negative canines, 30 were males and 28 were females. Thirteen of the PN canines were mixed breeds, 8 were poodles, 6 were golden retrievers, 4 were Shih tzus, 4 were Thai breeds, 3 were Labrador retrievers, 2 were pomeranians, 2 were Bangkeows and there were 1 each of following breeds: akita, beagle, basset hound, chihuahua, dachshund, German shepherd, penkingese, pug, Rottweiler, St. Bernard, spitz, terrier and unknown.

The chief symptoms in the PP canines were: depression and anorexia (8 dogs), anorexia (5 dogs), epistaxis (4 dogs), depression (1 dog), fever (1 dog), anorexia, depression and vomiting (1 dog), anorexia, depression and fever (1 dog), depression, anorexia and constipation (1 dog), anorexia, depression and coughing (1 dog), epistaxis and coughing (1 dog), anorexia, depression and panting (1 dog), anorexia, depression and tick infestation (1 dog), anorexia and blood from the mouth (1 dog), anorexia, vomiting and diarrhea (1 dog), fever and bloody diar-

rhea (1 dog), health check (1 dog), hind limb paresis (1 dog), annual vaccination (1 dog), skin disease (1 dog), dyspnea (1 dog), seizures (1 dog) and unknown (1 dog). The chief symptoms in the PN group were: depression and anorexia (5 dogs), anorexia (4 dogs), epistaxis (4 dogs), general health check (4 dogs), hit by a car (4 dogs), skin disease (3 dogs), general health check prior to performing ovariohysterectomy (3 dogs), unknown (3 dogs), anorexia and lateral recumbency (2 dogs), general health check prior to perform tartar scraping (2 dogs), anorexia and vomiting (2 dogs), left hind limb lameness (2 dogs), stiffness and ataxia (1 dog), anorexia and fever (1 dog), annual vaccination (1 dog), pulurent vaginal discharge (1 dog), panting (1 dog), petechial hemorrhages (1 dog), biting (1 dog), seizures (1 dog), hematuria (1 dog), coughing (1 dog), pain of the left hind limb (1 dog), upper canine problem (1 dog), mass in the thorax (1 dog), vaginal discharge (1 dog), depression (1 dog), depression and fever (1 dog), hematemesis (1 dog), anorexia, depression and vomiting (1 dog), leptospirosis and post-operative management of a urinary bladder rupture (1 dog) and pallor and panting (1 dog).

The mean body temperature of the dogs in the PP group was higher than in the PN group ($p = 0.02$). The mean WBC count of the dogs in the PP group was lower than in the PN group ($p = 0.02$). The mean eosinophil count of the dogs in the PP group was lower than in the PN group ($p<0.001$). The mean RBC count of the dogs in the PP group was lower than in the PN group ($p = 0.008$). The mean hemoglobin of the dog in the PP group was lower than in the PN group ($p = 0.03$). The mean hematocrit of the dogs in the PP group was lower than in the PN group ($p = 0.02$). The mean platelet count of the dogs in the PP group was lower than in the PN group ($p <0.001$). The mean RDW of the dogs in the PP group was higher than in the PN group ($p = 0.03$). The mean corrected nucleated RBC count of the dogs in the PP group was lower than the in PN group ($p = 0.01$). The mean ALT level of the dogs in the PP group was higher than in the PN group ($p = 0.01$). There were no differences in the mean age, body weight, heart rate, monocytes, neutrophils, lymphocytes, MCV, MCH, MCHC, plasma protein or creatinine levels between the PP and PN groups.

The appetites of the dogs in the PP group were generally poorer than in the PN group ($p = 0.03$). Tick infestations in the PP and PN groups were significantly different ($p = 0.03$). There were no differences in attitude between the canines in the PP and PN ($p = 0.15$). The general appearance of the skin and coats of the dogs in the PP and PN groups were not significantly different ($p = 0.17$). There were no differences in water intake between the dogs in the PP and PN groups ($p = 0.45$) (Table 2).

The univariable logistic regression analysis of each variable is shown in Table 3. On multivariable logistic regression, eight variables were significantly associated with having CME: poor appetite, elevated body temperature, lower eosinophil count, lower red blood cell count, lower hemoglobin, lower hematocrit, lower platelet count. The Hosmer-Lameshow goodness-of-fit test showed the model had a good fit ($p>0.05$). Six variables were tested by multivariable logistic regression with the Hosmer-Lameshow goodness-of-fit test showing the model had a good fit ($p>0.05$). Of these, the platelet count had the best association with CME (OR=0.85; 95%CI: 0.77-0.92, $p<0.001$) (Fig 1).

Table 1
Comparison of age, physical examination data and laboratory findings between PCR positive and PCR negative canines (Mann-Whitney *U* test).

Variables	*n*	Range	Quartiles			*p*-value
			25th percentile	Median	75th percentile	
Age						0.58
PCR positive	35	0.17-13.00	1.08	4.17	8.17	
PCR negative	58	0.25-16.00	1.88	5.17	8.00	
Body weight						0.72
PCR positive	31	2.00-41.20	4.50	12.50	24.20	
PCR negative	49	2.20-43.80	5.20	11.60	24.50	
Temperature						0.02
PCR positive	28	100.00-105.00	101.53	102.60	103.60	
PCR negative	43	96.80-105.00	101.00	101.60	102.40	
Heart rate						0.70
PCR positive	11	96.00-144.00	100.00	108.00	120.00	
PCR negative	19	0.00-144.00	100.00	120.00	120.00	
WBC						
PCR positive	36	2,500.00-41,300.00	5,575.00	6,950.00	9,550.00	0.02
PCR negative	58	9.00-49,400.00	6,900.00	8,500.00	12,600.00	
Monocyte counts						0.30
PCR positive	34	0.00-5,782.00	0.00	141.00	306.25	
PCR negative	56	0.00-1,887.00	0.00	0.09	310.00	
Neutrophil counts						0.07
PCR positive	34	1,120.00-33,040.00	3,799.50	4,469.00	7,209.00	
PCR negative	56	5.00-45,448.00	4,626.00	5,735.50	8,505.75	
Lymphocyte counts						0.09
PCR positive	34	2.50-12,150.00	927.75	1,778.00	2,408.25	
PCR negative	56	1.00-9,028.00	1,451.75	2,100.00	3,542.50	
Eosinophil counts						<0.001
PCR positive	34	0.00-568.00	0.00	0.00	14.50	
PCR negative	56	0.00-3,420.00	0.00	144.50	481.50	
RBC x 1000						0.01
PCR-Positive	36	1.00-7.00	3.55	4.65	5.59	
PCR-Negative	58	2.00-9.00	4.22	5.35	6.80	
Hemoglobin						0.03
PCR-Positive	36	2.00-18.80	7.48	10.55	12.38	
PCR-Negative	58	4.10-19.30	9.15	11.55	15.10	
HCT						0.02
PCR-Positive	36	7.00-45.00	21.53	30.80	36.93	
PCR-Negative	58	12.60-57.00	28.00	34.95	47.85	
MCV						0.52
PCR-Positive	35	53.00-75.00	62.30	66.00	68.00	
PCR-Negative	58	14.00-77.00	61.75	66.00	71.00	

Table 1 (Continued).

Variables	*n*	Range	Quartiles			*p*-value
			25th percentile	Median	75th percentile	
MCH						0.82
PCR-Positive	35	12.30-24.70	20.10	21.80	23.00	
PCR-Negative	58	13.10-41.70	20.60	22.00	23.00	
MCHC						0.67
PCR-Positive	35	28.10-332.80	31.00	33.10	35.20	
PCR-Negative	58	23.00-46.00	31.68	33.00	34.03	
Platelets						<0.001
PCR-Positive	36	18.00-174.00	32.25	49.5	89.50	
PCR-Negative	58	17.50-717.00	78.75	200.00	290.25	
RDW						0.03
PCR-Positive	34	12.10-20.10	13.88	14.65	15.65	
PCR-Negative	58	7.40-19.90	14.50	15.55	17.13	
Plasma protein						0.67
PCR-Positive	34	6.60-12.00	8.20	9.00	9.75	
PCR-Negative	57	5.20-12.00	8.50	9.00	9.80	
Corrected nRBC						0.01
PCR-Positive	36	2,500.00-41,300.00	5,575.00	6,950.00	9,950.00	
PCR-Negative	58	9.30-49,400.00	6,900.00	8,500.00	12,625.00	
ALT						0.01
PCR-Positive	31	26.00-5,360.00	40.00	70.30	141.00	
PCR-Negative	52	6.70-581.00	29.50	42.50	76.68	
Creatinine						0.10
PCR-Positive	32	0.50-4.45	0.73	1.17	1.71	
PCR-Negative	52	0.40-7.20	0.80	0.90	1.19	

DISCUSSION

In this study, the difference between the PCR and the conventional methods for detecting CMG was not statistically significant (p=0.17). This may be due to the difficulty of finding morulae on Giemsa stain. The chances of finding *E. canis* morulae may be as low as 4%, particularly in the subclinical stage (Woody and Hoskins, 1991; Mylonakis *et al*, 2010; Harrus and Waner, 2011).

A history and physical examination were performed in the out-patient department, PP canines had poorer appetites, higher body temperatures, more depression, anorexia, epistaxis and tick infestation than PN canines. These findings are similar to previous reports of CME, the most frequent symptoms consist of high fever, anorexia, depression, lethargy (McQuiston *et al*, 2003). Anorexia and depression are frequently found in dogs with CME due to parasitic infestation (Das and Konar, 2013). Epistixis was seen in a PP dog in our study. This phenomena are frequently seen in ehrlichiosis; bleeding may occur due to thrombocytopenia (Shekhar *et al*, 2011). Tick infestation or a history of tick infestation was more

Table 2
Comparison of signs and platelet smear findings between PCR positive and negative canines (chi-square test).

Variables	PCR positive	PCR negative	*p*-value
Appetite			0.03
Normal	8	21	
Decreased/anorexia	24	21	
Water intake			0.45
Normal	16	22	
Decreased	9	9	
Not drinking at all	0	1	
Increased	0	2	
General appearance			0.17
Alert	16	37	
Depressed	13	13	
Stuporous	0	1	
Attitude			0.15
Normal	13	25	
Abnormal	16	15	
Tick infestation			0.03
Yes	23	21	
No	3	10	
Platelet smear			<0.001
Adequate	2	30	
Decrease	34	26	

Table 3
Univariable logistic regression analysis of significant factors associated with PCR positive blood samples.

Variable	N	Odds ratio (95% CI)	*p*-value
Low eosinophil count (total/ l)	90	0.63 (0.46-0.86)	0.004
Low red blood cell count (x10^6/ l)	94	0.68 (0.51-0.90)	0.008
Low hemoglobin (g/dl)	94	0.87 (0.77-0.98)	0.02
Low hematocrit	94	0.59 (0.40-0.88)	0.01
Low platelet count (x10^3/ l)	94	0.85 (0.78-0.92)	<0.001

likely to be found in PP dogs in our study. The transmission of CME occurrs by the brown dog tick (*Rhipicephalus sanguineus*) (Groves *et al*, 1975). This vector can transmit various blood parasites to dogs and cats worldwide, such as *Ehrlichia* spp (Dixit *et al*, 2012), *Babesia* spp (Shortt, 1973) and *Hepatozoon* spp (Kumar *et al*, 2012).

We compared various hematological and serological results between canines with and without CME and found white blood cell count, neutrophil count, lym-

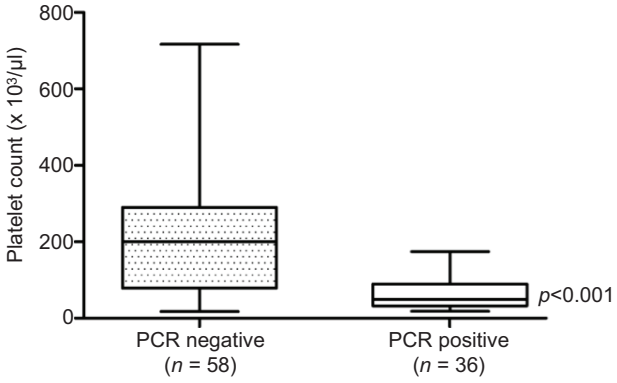

Fig 1–Platelet count in canines with positive and negative PCR results for CME.

phocyte count, eosinophil count, red blood cell count, hemoglobin, hematocrit, platelet count, RDW, corrected nRBC and alanine aminotransferase, were significantly lower in dogs with CME. The anemia, leukopenia and thrombocytopenia are commonly found in CME (Harrus *et al*, 1997a,b, 1998; Macieira *et al*, 2005; Niwetpathomwat *et al*, 2006; Harrus *et al*, 2011). The pathology of anemia and leukopenia in CMG may be due to suppression of bone marrow activity (Waner *et al*, 1997). Blood chemistry results were compared between groups. In the PCR positive group, we found ALT (alanine aminotransferase) levels were significantly elevated. This finding has been seen in many studies, showing the liver is affected with canines ehrlichiosis (Waner *et al*, 1995; McQuiston *et al*, 2003; Rungsipipat *et al*, 2009).

Thrombocytopenia has been reported in canine ehrlichiosis (Suksawat *et al*, 2001a,b; Dangnone *et al*, 2003) and was found in our study too. We found the platelet count was the best predictor for ehrlichiosis in dogs. This might be used as a screening test before performing a direct diagnostic test. More dogs with platelet counts < 200,000 platelet/ l had *E. canis* than dogs with higher platelet counts (Bulla *et al*, 2004). In our study for every decrease in platelets of 10,000 cells the likelihood of having chrlichiosis increased by 15% (Fig 1). However, there are a number of diseases that can cause thrombocytopenia, including immune-mediated thrombocytopenia, neoplasia-associated thrombocytopenia, inflammatory diseases and infectious diseases (Grindem *et al*, 2002). In Thailand, anaplasmosis is considered a tick-borne disease and a cause of thrombocytopenia in dogs as well as ehrlichiosis (McQuiston *et al*, 2003; Pinyoowong *et al*, 2008). Our study focused on ehrlichia infection; some canines with thrombocytopenia may also have co-infection with anaplasmosis.

In summary, we evaluated clinical, hematological and serological findings among canines with CME. Multivariable logistic regression analysis showed thrombocytopenia was associated with CME. The lower the platelet count the greater the chance of having CME.

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