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Original paper

Associated risk factors and haematological presentation of *Ehrlichia canis* infected dogs in Phitsanulok, Thailand

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ABSTRACT. *Ehrlichia canis* is the common blood pathogen infected dogs in Thailand that significantly affect dog health and caused canine monocytic ehrlichiosis which leads to anaemia, high morbidity also mortality rates. This study was performed to analyse associated risk factors and evaluate the significance of haematological responses of dogs infected with *E. canis* in Phitsanulok province, the northern part of Thailand. Blood samples were collected from 94 dogs, 27 (28.7%) dogs have been confirmed *E. canis* infection by nested PCR method. Mostly of infected dogs had hypohemoglobinemia (<12.1 g/dl), leucocytosis (>15.5×10^3/µl), neutrophilia (>10.6×10^3/µl) and thrombocytopenia (<170×10^3µl). However, only thrombocytopenia was statistically different between *E. canis* infected and non-infected groups. Additionally, no significant statistical relationship between *E. canis* infection rate and sex, age or breed apparent. These data supported that infection with *E. canis* is endemic in dogs and thrombocytopenia may highlight during infection which reliability to use in the clinical diagnosis of *E. canis* infection.

Key words: clinical diagnosis, *Ehrlichia canis*, haematology, molecular detection, thrombocytopenia

Introduction

Ehrlichia canis is a rickettsial bacteria which enters the body of a host when an infected brown dog tick, *Rhipicephalus sanguineus* takes a blood meal. This blood pathogen infects mononuclear cells include monocytes and lymphocytes [1,2] which occurs primarily in dogs but can also affect humans [3]. *E. canis* multiplies within the target cell thereby causing canine monocytic ehrlichiosis which appearance haematological abnormality such as thrombocytopenia, decrease pack cell volume and mild anaemia [4–6]. Infected dogs may present fever, lethargy, anorexia, weakness, epistaxis, pale mucous membrane and petechial haemorrhage [7,8] occasionally, anterior uveitis and retinal haemorrhage will be found [9].

The routine technique for detection blood pathogens in laboratory is the simple blood smear which generally suitable in acute phase of infection but rarely seen any agents during subclinical or clinical phases [10]. However, simple blood smear still highly deficient especially for *E. canis* infection due to this rickettsia is very small, easy to confuse with dye residuals and infrequently detected in blood smear although acute stage of infection [11]. Moreover, this technique is time consuming and needs a high experience technician for analysis. The application of examining haematology responses in parallel for routine detection *E. canis* infection is needed to notice for diagnosis supporter.

Infection with *E. canis* in dogs has been described in northeast, central and south of Thailand with prevalence about 3.9–43.1% [12–16]. Therefore, we intended to study the infection rate of canine ehrlichiosis in the northern part of Thailand which

Table 1. Dogs characteristics and *E. canis* detection

Characteristics	No. of dog	PCR results		*p*-value
		Positive (n=27)	Negative (n=67)	
Sex (n=94)				0.62
Female	45 (47.87%)	14 (14.89%)	31 (32.98%)	
Male	49 (52.13%)	13 (13.83%)	36 (38.30%)	
Age (n=94)				0.39
< 1 year	11 (11.70%)	4 (4.26%)	7 (7.45%)	
1 - 2 years	7 (7.45%)	4 (4.26%)	3 (3.19%)	
> 2 - 5 years	8 (8.51%)	3 (3.19%)	5 (5.31%)	
> 5 - 7 years	13 (13.83%)	3 (3.19%)	10 (10.64%)	
> 7 years	28 (29.79%)	5 (5.32%)	23 (24.47%)	
Others	27 (28.72%)	8 (8.31%)	19 (20.21%)	
Breeds (n=94)				0.88
Golden retriever	3 (3.19%)	1 (1.06%)	2 (2.13%)	
Pomerania	12 (12.76%)	2 (2.13%)	10 (10.64%)	
Poodle	9 (9.57%)	5 (5.32%)	4 (4.26%)	
Rottweiler	2 (2.13%)	0	2 (2.13%)	
Shih Tzu	6 (6.38%)	1 (1.06%)	5 (5.32%)	
Beijing	1 (1.06%)	0	1 (1.06%)	
Labrador retriever	2 (2.13%)	0	2 (2.13%)	
Fila brazilian	1 (1.06%)	0	1 (1.06%)	
French bulldog	3 (3.19%)	1 (1.06%)	2 (2.13%)	
Thai	8 (8.51%)	4 (4.26%)	4 (4.26%)	
Thai bangkaew	2 (2.13%)	0	2 (2.13%)	
Yorkshire terrier	2 (2.13%)	1 (1.06%)	1 (1.06%)	
Chihuahua	3 (3.19%)	1 (1.06%)	2 (2.13%)	
Bully	1 (1.06%)	0	1 (1.06%)	
Miniature	1 (1.06%)	0	1 (1.06%)	
Beagle	1 (1.06%)	0	1 (1.06%)	
Siberian husky	2 (2.13%)	1 (1.06%)	1 (1.06%)	
Thai ridgeback	1 (1.06%)	0	1 (1.06%)	
Cross breed	20 (21.28%)	6 (6.38%)	14 (14.89%)	
Others	14 (14.89%)	4 (4.26%)	10 (10.64%)	

has no data and compares the results to previous studies. This study involved determining the prevalence of *E. canis* infection in dogs, also evaluate the risk factors and association between *E. canis* infection in naturally infected dogs and the haematological alterations of 94 dogs treated in a veterinary hospital in Phitsanulok province, Thailand. The presence or exposure of *E. canis* was detected by nested PCR technique targeting 16s rRNA gene.

Materials and Methods

Sample collection. Blood samples were collected from 94 client-owned dogs attended to a Veterinary Hospital in Phitsanulok province, North, Thailand. All samples were taken from dogs that their owners notify for blood testing for either medical check up or clinical illness. Dogs involve in this study were both sexes, different ages and different breeds. Total of 94 blood samples, approximately 3 ml of blood were obtained each by venepuncture from the saphenous or cephalic vein, collected in sterile tubes with ethylenediamine-tetraacetic acid (EDTA). All experimental procedures involving animals were conducted in accordance to Animals for Scientific Purpose Act B.E. 2558 (A.D. 2015) (U1-01509-2558) and approved by the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU-048/2019).

Estimation of haematological parameter. Haematological parameters including haematocrit (%), haemoglobin (g/dl), white blood cell ($\times 10^3/\mu l$),

neutrophil ($\times 10^3$/µl), lymphocyte ($\times 10^3$/µl) and platelet count ($\times 10^3$/µl) were analysed by the commercial auto haematology analyser.

DNA extraction and examination blood pathogens infections by nested PCR. Blood of dogs were collected and transport on ice to laboratory in faculty of Veterinary Sciences of Mahasarakham University. Samples were kept at -20°C for subsequent DNA extraction. DNA was extracted from 200 µl of each blood samples using commercial kit (vivantis, Malaysia). DNA was eluted and stored at -20°C. Samples were examined for *E. canis* infection by nested PCR using specific primers targeting 16s rRNA gene as described previously [17,18]. The first step of amplification used primers for *Ehrlichia* genus specific (ECC 5'-AGA-ACG-AAC-GCT-GGC-GGC-AAG-CC-3', ECB 5'-CGT-ATT-ACC-GCG-GCT-GCT-GGC-A-3') and the second step used *E. canis* specific primer (CANIS 5'-CAA-TTA-TTT-ATA-GCC-TCT-GGC-TAT-AGG-A-3', HE3 5'-TAT-AGG-TAC-CGT-CAT-TAT-CTT-CCC-TAT-3'). Both 2 amplification of nested PCR reaction included 40 pmol of each primer in a final volume of 25 ul consisted of 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 1×PCR buffer, 0.2 U of *Taq* Polymerase (Vivantis) and 2 µl of template DNA (extracted DNA approximately 50–100 ng for the 1st step and PCR product from 1st amplification for the 2nd step). PCR conditions comprised of 35 cycles of denaturation for 30 sec at 95ºC, annealing for 30 s at 60ºC and extension for 1 min at 72ºC in both 2 round of amplifications using PCR machine (Biometra GmbH, Germany). PCR positive control were collected from dogs already confirmed for *E. canis* infection by Giemsa staining technique and PCR assay from previous studies [15,16]. PCR mixes containing only the primers with no DNA template served as negative control. Nested PCR was generation a 389 bp product which subsequently identified by 1% agarose gels stained with ViSafe Red Gel Stain (Vivantis) and visualized under ultraviolet light.

Statistical analysis. The presence of *E. canis* antigen was determined and the percentage of infection was calculate. The association between *E. canis* infection with haematology response was compared with Pearson's Chi-squared test. The haematological values between infected and non-infected groups were compared by independent sample T test. Statistical differences were considered when *p*-value was less than 0.05.

Results

Dogs characteristics and *E. canis* infection

A total of 94 samples were selected from the dogs in various regions of Phitsanulok province which attended to the Veterinary Hospital, during July 2018–July 2019. Cases were age range from 1 month to 20 years old (11.70% were <1 year and 88.30% were >1 year old), 45 (47.87%) were female and 49 (52.13%) were male. For breeding, 60 (63.83%) were pure breed, 21.28% were cross breed and 14 (14.89%) were data missing.

For nested PCR detection, 27 dogs were infected with *E. canis* (28.72%); 14 were female and 13 were male. Dogs of any age and sexes can get infection with no variations. Cross breed dogs show the most infection rate (22.2%) followed by poodle (18.5%) but with no statistical difference (p=0.88) (Table 1).

Haematological analysis of *E. canis* infected dogs

Haematology responses in 27 dogs with natural infection with *E. canis* were compared and interpreted with 67 dogs with *E. canis* non-infection dogs. From haematological parameters, there were more decreased haematocrit, haemoglobin and lymphocyte values in *E. canis* infected group than non-infected group but no statistical difference. The only haematological parameter that was significantly different between positive and negative dogs was platelet count (p=0.020) which was significantly lower in *E. canis* positive dogs. Infection with *E. canis* showed no significant effect on neutrophil and white blood cell (Table 2).

Discussion

E. canis is the blood borne rickettsia which commonly in Thailand and tropical regions. This pathogen can be spread to other dogs via tick, *Rhipicephalus sanguineus*, biting. Additionally, infected tick also transmitted *E. canis* pathogens to human causing human monocytic ehrlichiosis [3]. In this study, the rate of *E. canis* infected dogs in Phitsanulok province was 28.7% which higher than the reported of *E. canis* infection in dogs in Songkhla 3.9% [12], Bangkok 9.88% [14] and Kalasin 25% [16] but lower than the reported from Khonkaen 31.75% [13] and Maha Sarakham 43.1% [15] of Thailand. The differences in the infection rate may occur due to the geographical, the climate which effects spreading of tick carriers, health management program, tick eliminating program and

Table 2. Hematological values presentation of dogs (Mean ± SD)

	E. canis infected dogs (n=27)	*E. canis* non-infected dogs (n=67)	*p*-value	Reference data [17]	
				Mean	Reference range
Haematocrit (%)	29.8 ± 12.90	33.8 ± 10.50	0.119	45	37.0-55.0
Haemoglobin (g/dl)	10.08 ± 4.70	11.24 ± 3.86	0.220	15.0	12.0-18.0
White blood cell count ($\times 10^3/\mu l$)	17.12 ± 7.84	17.61 ± 10.62	0.828	11.5	6-17
Neutrophil count ($\times 10^3/\mu l$)	14.65 ± 8.35	14.62 ± 9.54	0.992	7	3-11.5
Lymphocyte count ($\times 10^3/\mu l$)	2.09 ± 1.33	2.97 ± 2.25	0.06	2.8	1-4.8
Platelet count ($\times 10^3/\mu l$)	190.85 ± 172.856	231.21 ± 132.62	0.020*	300	200.0-500.0

the raising of sampling dogs from their owners. Moreover, it is highly possible that the high temperature for most of the year and humid tropical climate in Phitsanulok favor tick vector breeding in the environment which effect on prevalent infection rate in this region.

The course of ehrlichiosis present as acute, subclinical and chronic stages, respectively. The acute stage begins after 8–20 days after infection by tick biting. The subclinical stage occurred follows the acute phase and might last 40–120 days or years which dogs may continue persistently infected and remain asymptomatic. The final is the chronic stage with severe and life threatening clinical signs influenced by recurrent infection, infection with other organisms or illness from other diseases [19]. However, nested PCR is specific and highly sensitive in detecting in every stages as early as day 4 postinoculation [20].

In this study, we examined associated factors for *E. canis* infection and the results showed gender, age and breeding were not different in each group which indicated that infection with *E. canis* was not probably related with these factors and also supported by the study from *E. canis* infected dogs in three districts in Punjab of Pakistan [21]. Similarly, a study also in agreement with gender and age were not associated with the risk of *E. canis* infection among dogs population [22]. However, a study was reported that only breed and gender were not significantly associated with the occurrence of ehrlichiosis but dogs younger than six-month-old showed higher prevalence [23].

We compared various haematological values between *E. canis* infected and non-infected dogs and found only platelet count was affected by *E. canis* infection and presented significantly lower, nevertheless, haematocrit, haemoglobin, white blood cell, neutrophil and lymphocyte were no statistically differences. The finding of significant lower platelet count in *E. canis* infected dogs is also supported by previous reported of ehrlichiosis [11,24-28]. We recommended that these evidences strongly reinforced that platelet count might be used in diagnosis supporter or screening test. However, many studies showed not only platelet count but also white blood cell and haemoglobin have been reported in association with canine ehrlichiosis [25,27] which was not found relationship in our study. This finding suggested that non-infected group in this study may be afflicted with other diseases associated with circulatory disorders or get external factors that affect haematology parameters such as dehydration [29]. Interestingly, it was known that mostly dogs infected with *E. canis* in subclinical stage may be presented in non-alteration on blood parameter value [30].

The abnormal haematological values are all related to the pathogenesis of *E. canis* infection, after these agents entry into dog's blood circulation, they multiplied in their target cell which are monocytes and lymphocytes then destroyed the nest cell before spreading to other mononuclear lymphocytes including endothelial cells which resulting in vasculitis. Therefore, the immune system was triggered to responds the inflammation by increasing platelet consumption, resulting in thrombocytopenia. Consequently, it is often found that the first haematological parameters that are changed are platelet counts. The changes in haematological response can result in clinical manifestations of the disease, including depression,

shortness of breath and pale mucous membranes from anaemia until developing into non-responsive anaemia in the bone marrow. For acute infection, infected dogs can cause nose-bleed due to decreased platelet aggregation. In case of chronic infection, infected dogs showed more severe clinical symptoms and all types of blood cells can be reduced in production from suppression of bone marrow.

Of the total 94 dog blood samples, 27 (28.72%) were nested PCR positive for 16s rRNA of *E. canis*. Infection with *E. canis* showed the statistical relationship with lower platelet count ($p<0.05$). These findings are consistent with previous studies that support thrombocytopenia in *E. canis* infected dogs and the use of platelet counts as a screening test for ehrlichiosis. This study is the first report of *E. canis* infection in dogs in Phitsanulok Province, the northern part of Thailand.

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Authors' contributions. SP design of the experiments and analysis of data, wrote the manuscript, reviewed, and edited the manuscript. KD, BI and SD conceived the project, collected the samples, performed the examinations, and analysis of data. AP collected the samples. All authors read and approved the final manuscript.

Conflicts of interest. The authors declared no conflicts of interest.

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