Research Article

Investigation of *Ehrlichia canis* and *Anaplasma platys* from Dogs in Thailand, with Molecular Characterization and Haematological Profiles

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Abstract | *Ehrlichia canis* and *Anaplasma platys* are rickettsial pathogens that cause infections in the blood and have adverse health effects in animals, especially dogs and other canids. PCR targeting the 16S rRNA gene was performed to determine the prevalence of ehrlichiosis and anaplasmosis in 127 dogs collected from Rayong province, Thailand. To confirm the identification of these two pathogens, PCR was performed to detect the citrate synthase (*gltA*) gene followed by sequencing. In addition, the haematological responses of dogs infected with *E. canis* and *A. platys* were evaluated. By PCR, 22.8% (95%CI: 15.9-31.1) of the dog samples in this population were positive for one or both pathogens. Of these dogs, 18.1% were infected with *E. canis* while 7% were infected with *A. platys*. Mixed infections were found in 2.4%. Infection with *E. canis* was significantly related to hematocrit, haemoglobin, red blood cell (RBC), mean corpuscular volume (MCV), and platelet levels ($p < 0.05$). In addition, there were significant differences in white blood cell (WBC) and platelet levels between *A. platys*-infected and uninfected groups ($p < 0.05$). The results confirmed that *E. canis* and *A. platys* pathogens are circulating in dog populations in Thailand. This information will benefit veterinarians and dog owners by indicating the importance of regular ectoparasite control which is an effective strategy to control ehrlichiosis and anaplasmosis.

Keywords | Anaplasmosis, Citrate synthase gene, Ehrlichiosis, Epidemiology, Haematological

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INTRODUCTION

Canine ehrlichiosis and anaplasmosis are worldwide tick-borne diseases of dogs caused by rickettsial organisms called *Ehrlichia* and *Anaplasma* (Ogbu et al., 2018). These pathogens have a global distribution, especially in tropical and subtropical climates. In Thailand, the main species of canine blood parasites reported in dogs are *E. canis* and *A. platys* (Ahantarig et al., 2008; Pinyoowong et al., 2008). The route of infection for *E. canis* and *A. platys* is the bites of the brown dog tick, *Rhipicephalus sanguineus*. *E. canis* infects monocytes and lymphocytes (Harrus and Waner, 2011; Mylonakis and Theodorou, 2017) whereas *A. platys* infects platelets (Dyachenko et al., 2012) and can affect dogs and other members of the canids (Cardoso et al., 2015) and cats (Braga et al., 2014). In addition, human infections with *E. canis* (Bouza-Mora et al., 2017; Perez et al., 2006) and *A. platys* (Arraga-Alvarado et al., 2014)

are increasingly reported although the mode of transmission is unclear. The study of *E. canis* and *A. platys* infections demonstrated the public health importance of these canine diseases which can impact both canine and human health. Dogs infected with blood parasites often show similar clinical symptoms, such as fever, lethargy, anorexia, vomiting, weight loss, and pale mucous membranes. However, *Ehrlichia* sp. infection is often clinically manifested by high fever and fatigue. Haematological values usually show thrombocytopenia, anemia, leukopenia, hyperglobulinemia, and proteinuria (Gaunt et al., 2010). While *Anaplasma* sp. infection is regularly associated with thrombocytopenia, pale mucous membranes, petechial hemorrhage, nasal discharge, lymphadenomegaly, bilateral uveitis, and epistaxis have been noted in dogs (Sainz et al., 2015).

Blood parasite infection can range from asymptomatic to severe clinical symptoms. Currently, the diagnosis of blood parasite infections is based on clinical observation in combination with laboratory tests. Microscopic observation is the most common diagnostic method because it is simple and inexpensive. However, microscopic detection of *E. canis* and *A. platys* in blood smears is challenging which contributes to failure to detect because it requires trained, time-consuming techniques and has low sensitivity (Rucksaken et al., 2019). Serological tests that detect immunoglobulins against pathogens are also frequently used. However, these tests have low sensitivity and specificity due to cross-reactivity with other related parasites (Waner et al., 2001). For DNA detection, the polymerase chain reaction (PCR) test was developed to diagnose blood parasites and is commonly used to confirm infection with these blood parasites due to its high sensitivity and specificity (Sainz et al., 2015; Nair et al., 2016).

In Thailand, a few studies have been conducted using PCR mainly based on the 16S rRNA gene (Juasook et al., 2016; Piratae et al., 2015; Piratae et al., 2020). However, these studies lacked adequate information on their genetic characterization which is essential for the control and prevention of these diseases. Therefore, this study was performed to determine the prevalence of *E. canis* and *A. platys* pathogens infecting dogs in Rayong, Thailand using molecular detection of the 16S rRNA gene. In addition, the positive samples were amplified, using the citrate synthase (*gltA*) to determine the phylogenetic relationship of *E. canis* and *A. platys* in Thailand with published sequences of *E. canis* and *A. platys* from other regions of the world.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU-04/2022).

Sample collection

From May 2021 to October 2021, a total of 127 blood samples were collected from owned dogs and stray dogs that were presented at an animal hospital in Rayong, Thailand. Blood samples of approximately 0.5-1 ml were collected from the cephalic vein and preserved in ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. The information of age, breed, sex, and haemotological values of the collected dogs were recorded.

DNA extraction and amplification

DNA was extracted from approximately 200 μL of whole blood using the GF-1 blood DNA extraction kit procedure (Vivatis, Malaysia) and stored at -20°C. Each extracted DNA sample was analyzed for *Ehrlichia* and *Anaplasma* infection by nested-PCR.

For the nested-PCR method, the first step of amplification used Anaplasmataceae 16s rRNA gene from previous studies (Anderson et al., 1992; Dawson et al., 1996) and the second step used *E. canis* specific primer or *A. platys* specific primer (Kordick et al., 1999; Little et al., 1998) (Table 1). Both amplification steps of nested-PCR reaction were performed in a 25 μL reaction volume consisting of 1x PCR buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 0.4 μM of each primer, 1 U of *Taq* polymerase (Vivatis, Malaysia), and DNA (1-50 ng of extracted DNA for the first PCR and 2 μL of PCR product of the first amplification for the second PCR). PCR was performed in 35 cycles, consisting of three steps: (i) denaturation at 95°C for 45 seconds, (ii) annealing for 45 seconds at 60°C and 62°C for the 1st and 2nd steps of *A. platys* and 60°C for both two steps of *E. canis*, (iii) extension for 90 seconds at 72°C, and a final extension at 72°C for 5 minutes. The PCR master mixes containing only the primers with no DNA template served as negative control. PCR amplification was performed using a thermal cycler (Biometra GmbH, Germany). The approximately 400 base pairs generated by nested-PCR were subsequently identified using 1% agarose gels. After gel electrophoresis, the agarose gel was stained with ViSafe Red Gel Stain (Vivantis, Malaysia), and visualized under ultraviolet light on a gel documentation system (Bio-Rad).

For genetic analysis, new primers were designed for the *gltA* genes of *E. canis* and *A. platys* using Primer3 (https://primer3.org/). The DNA sequences of the citrate synthase (*gltA*) gene of *E. canis* (AY647155.1), and the citrate synthase (*gltA*) gene of *A. platys* (EU516387.1) were retrieved from the GenBank nucleotide database. Then, the considered *gltA* sequences from database were multiple aligned by ClustalW in the BioEdit program to find suitable targets. The polymorphic regions were used as target DNA

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Table 1: Primers for DNA amplification

Blood parasites	Primers	Target genes	Sequences	Annealing temp	Product size (bp)	References
Ehrlichia sp.	ECC ECB	16s rRNA	5’ AGAACGAACGCTGGCGGCAAGCC 3’ 5’ CGTATTACCGCGGCTGCTGGCA 3’	60	478	(Dawson et al., 1996)
Ehrlichia canis	CANIS HE3	16s rRNA	5’ CAATTATTTATAGCCTCTGGCTATAGGA 3’ 5’TATAGGTACCGTCATTATCTTCCCTAT 3’	60	389	(Anderson et al., 1992; Dawson et al., 1996)
Ehrlichia canis	Ec-gltA-F Ec-gltA-R	gltA	5’ GCAGTATTGGAATTAGATGG 3’ 5’ CATGWGCTGGCCCCCATA 3’	58	769	This study
Anaplasma platys (former *Ehrlichia platys*)	PLATYS GA1UR	16s rRNA	5’TTTGTCGTAGCTTGCTATG 3’ 5’ GAGTTTGCCGGGACTTCTTCT 3’	62	402	(Kordick et al., 1999; Little et al., 1998)
Anaplasma platys	Ap-gltA-F Ap-gltA-R	gltA	5’ GATAAGAAAGTAAGCTTGCC 3’ 5’ CATGWGCTGGCCCCCATA 3’	58	746	This study

Table 2: Hematological values presentation of dogs mean (95% confidence interval)

Parameters	*E. canis* positive group (n=23)	*A. platys* positive group (n=9)	*E. canis/ A. platys* positive group (n=3)	Negative group (n=96)	Reference range
Hematocrit (%)	33.62* (28.31-38.93)	36.9 (26.06-47.73)	19.9*	43.98 (41.55-46.41)	37-55
Hemoglobin (g/dL)	11.64* (9.86-13.41)	12.73 (8.92-16.54)	6.87*	15.10 (14.26-15.94)	12-18
RBC (10^6 cells/µL)	4.93* (4.18-5.68)	5.04 (3.52-6.57)	3.16*	6.18 (5.84-6.53)	5.5-8.5
MCHC (%)	34.29 (33.31-35.27)	33.89 (31.95-35.82)	34.87	34.29 (33.49-34.56)	32-36
MCV (fL)	66.25* (61.16-71.37)	67.58 (48.93-86.24)	66.49	71.03 (69.63-72.42)	66-77
WBC (10^3 cell/µL)	10.17 (7.98-12.36)	15.56* (4.93-26.19)	23.39*	11.38 (10.64-12.13)	6-17
Neutrophil (10^3 cell/µL)	7.83 (5.94-9.971)	9.50 (2.12-16.89)	11.59	10.77 (8.45-13.08)	3-11.5
Eosinophil (10^3 cell/µL)	0.23 (0.11-0.35)	0.33 (0.11-0.56)	0.35	0.36 (0.28-0.44)	0.1-1.25
Platelet (10^3 cell/µL)	137.4* (69.25-205.55)	97.51* (19.36-175.66)	170.67	302.48 (266.83-338.12)	200-500

RBC=Red blood cell; MCHC=Mean corpuscular hemoglobin concentration; MCV=Mean corpuscular volume; WBC=White blood cell (Latimer, 2011)

*statistical significant as $p < 0.05$

sites. All positive samples with the 16s rRNA gene were amplified by partial citrate synthase (*gltA*) genes with an amplicon size of 749-769 bp using the primers designed in this study. The annealing temperatures for the *gltA* gene from *E. canis* and *A. platys* were optimized at 58°C.

Nucleotide sequencing and analysis

The PCR products of the *gltA* genes were purified and sequenced at the ATGC sequencing company Thailand using the primer set for *gltA* genes developed in this study. ClustalW multiple sequences alignment of BioEdit (Hall, 1999) was used to analyze the obtained sequences of *E. canis* and *A. platys*. Moreover, the achieved sequences were

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compared for similarity using BLAST program (https://www.ncbi.nlm.nih.gov/). The partial *gltA* sequences of *E. canis* and *A. platys* from dogs in this study were submitted in the GenBank database. The phylogenetic tree was constructed based on the neighbor-joining distance method using MEGA X program (Kumar et al., 2018). The confidence of the branching patterns of the trees was estimated by bootstrap analysis through 1000 replications. The nucleotide sequence of *Rickettsia monacensis* (KU961970.1) was used as an outgroup.

Haematological profile investigation

Haematological parameters including hematocrit, haemoglobin, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), neutrophils, eosinophils, white blood cells (WBC), and platelets were analyzed using a commercial auto haematology analyzer. The haematological values of infected and uninfected groups were compared by an independent sample t-test. The *p*-value lower than 0.05 (< 0.05) is considered statistically significant.

RESULTS

Characteristics of dogs and their blood parasites infections

Of the 127 blood samples, 75 (59%) were male and 52 (41%) were female. Cases of blood parasite infections were found in dogs aged 2 months to 16 years, with 16 (12.6%) from young dogs (≤12 months), 107 (84.3%) from adult dogs, and 4 (3.2%) missing data. Seventy-one dogs (55.9%) were purebred, 29 (22.8%) were crossbred and 27 (21.3%) had missing breed data. Based on the nested PCR test, 29 of 127 dogs (22.8%; 95%CI: 15.9-31.1) were positive for one or two pathogens. Of the positive dogs, 23 (18.1%; 95% CI: 11.2-25.9) were infected with *E. canis* while 9 (7%; 95%CI: 3.3-13) samples were infected with *A. platys*. Double infections were also found in 3 (2.4%; 95% CI: 0.5-6.8) samples.

Phylogenetic analysis

After screening positive samples using the 16s rRNA gene, the *gltA* gene of *E. canis* and *A. platys* were amplified to obtain longer fragments for phylogenetic analysis. Concurrently, three positive samples of *E. canis* and *A. platys* which showed a band of the *gltA* partial sequences of approximately 678 and 709 base pairs in agarose gel, were purified and sequenced. After analysis, the *gltA* sequences of *E. canis* and *A. platys* were submitted to GenBank under accession numbers OL549103- OL549105 for *E. canis* and OL549106- OL549108 for *A. platys*. Phylogenetic relationships were established between the *gltA* sequences of *E. canis* and *A. platys* in this study and 19 related sequences from GenBank database. Phylogenetic analysis showed that *E. canis* and *A. platys* in this report were clustered together with other *E. canis* and *A. platys* from the database (Figure 1). *E. canis* was divided into two subgroups. *E. canis* from Thailand was found in subgroup one with others from China (KX987357.1), Myanmar (LC545962.1), Thailand (KU765198.1), Philippines (LC428206.1), Spain (AY615901.1) and Malawi (LC556380.1). Moreover, *A. platys* in Thailand and others in the database were highly conserved and clustered into one clade.

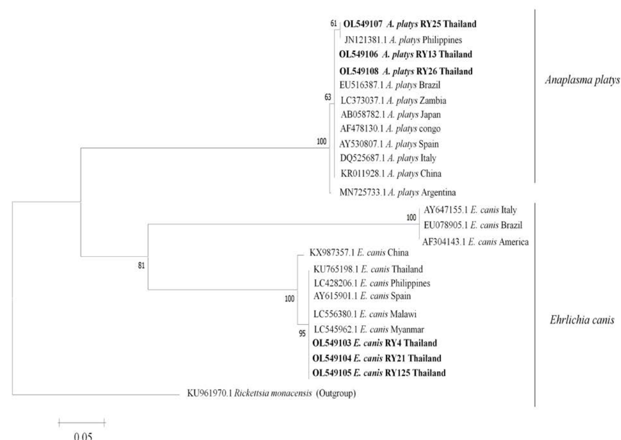

Figure 1: Phylogenetic tree based on gltA gene of *E. canis* and *A. platys* sequences from Rayong, Thailand (indicated in bold typeface) together with related sequences in GenBank. Sequences were compared with the neighbor-joining method operated by MEGA X.

Haematological analysis of *E. canis* and *A. platys* infected dogs

Hematology responses between dogs naturally infected with *E. canis* or *A. platys* were compared with those of the uninfected groups. In the group infected with *E. canis*, hematocrit, haemoglobin, erythrocytes, MCV, and platelet count were significantly lower than in the uninfected group ($p < 0.05$). In *A. platys* infection, platelet counts were statistically significantly lower in the infected group, but WBC was higher than in the uninfected group ($p < 0.05$). Co-infected with *E. canis* and *A. platys* showed anemia and had significantly lower hematocrit, haemoglobin, and RBC values, but higher WBC values compared to the uninfected group ($p < 0.05$) (Table 2).

DISCUSSION

Canine tick-borne disease is a worldwide veterinary health problem, especially in Thailand and other tropical regions. Infections with *E. canis* and *A. platy* are endemic in Thailand; however, insufficient studies have been conducted to determine the prevalence of these pathogens, especially in the eastern part of the country. Therefore, for the first time, we investigated *E. canis* and *A. platys* infections in

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dogs in the Rayong province, Thailand using PCR. All blood samples were randomly collected from dogs registered for blood testing by their owners. In addition, samples from asymptomatic individuals (healthy animals) or with various typical symptoms were included in the study. In Thailand, the prevalence of *E. canis* and *A. platys* was approximately 3.9-43% for *E. canis* infection and 4.4-29% for *A. platys* infection. For *E. canis*, the prevalence was 14% in Nakornpranom Animal Quarantine Station (Juasook et al., 2016), 3.9% in Songkhla province (Liu et al., 2016), 43.08% in Maha Sarakham province (Piratae et al., 2017), 25% in Kalasin province (Piratae et al., 2019), and 28.7% in Phitsanulok province (Piratae et al., 2020). For *A. platys*, the prevalence was 4.4% in Songkhla province (Liu et al., 2016), 29.2% in Maha Sarakham province (Piratae et al., 2017), and 29.4% in Kalasin province (Piratae et al., 2019). In different countries, the prevalence of *E. canis* infection in dogs varies; with 0.75% in Myanmar (Hmoon et al., 2021); 9.7% in Egypt (Selim et al., 2020); 10.41% in Paraguay (Pérez-Macchi et al., 2019); 15.3% in northern Colombia (Pesapane et al., 2019); 16.8% in the Caribbean (Alhassan et al., 2021); 28% in Pakistan (Malik et al., 2018) and 29.26% in Mexico (Ojeda-Chi et al., 2019). For *A. platys* infections in dogs, the figures are 0.25% in Myanmar (Hmoon et al., 2021); 6.4% in Egypt (Selim et al., 2021); 10.67% in Paraguay (Pérez-Macchi et al., 2019); 18.7 % in the Caribbean (Alhassan et al., 2021) and 32.9% in Brazil (Ribeiro et al., 2017).

In this study, the prevalence of *E. canis* and *A. platys* in dogs was 17.3% and 5.5%, respectively, which is within the previously described range. Infections with *E. canis* and *A. platys* in dogs are quite common in Thailand and could be explained by the presence of *Rhipicephalus sanguineus*, the brown dog tick, which is a common vector of these pathogens in Thailand. Subsequently, dogs were naturally infected by the bite of infected ticks (Ogbu et al., 2018; Ahantarig et al., 2008). In addition, differences in prevalence rates across countries can be described as a result of climatic conditions affecting tick vector dispersal, host susceptibility, geography, canine health management programs, vector prevention, and diagnostic protocols.

In this work, we found that dogs infected with *E. canis* had statistically significantly decreased hematocrit, haemoglobin, RBC, MCV and platelet counts compared to the uninfected group ($p < 0.05$). Dogs infected with *A. platys* had significantly higher WBC but lower platelet counts than in the uninfected group ($p < 0.05$). This finding may be due to the fact that these blood parasites cause excessive destruction of blood cells and blood cell components. Previous reports showed a decrease in hematocrit, haemoglobin, erythrocytes, MCV, and platelets in dogs infected with *E. canis* (Bai et al., 2017; Bhadesiya and Raval, 2015; Parashar et al., 2016). In addition, several studies have shown a decrease in platelets in dogs infected with *A. platys* (Bouzouraa et al., 2016; Brown et al., 2006). However, there are relatively small sample sizes of groups infected with *A. platys* that are biased and may provide statistically significant results.

The citrate synthase (*gltA*) derived sequences have been widely used to study the phylogenetic analysis and genetic diversity of rickettsial organisms (De la Fuente et al., 2006; Inokuma et al., 2001; Loftis et al., 2008; Shih et al., 2021). In Thailand, there have been a few studies that used PCR mainly based on the 16S rRNA gene to construct a phylogenetic tree; however, there is a lack of studies using the *gltA* gene, which has more variation than the 16s rRNA gene. In this study, we constructed a phylogenetic tree using *Rickettsia monacensis* (KU961970.1) as an outgroup. The results showed that *E. canis* and *A. platys* from Thailand clustered together and closely related with other *E. canis* (99.6 - 100% similarities) and *A. platys* (99.6 - 100% similarities) sequences that had been downloaded from the database. The results of our study confirmed that *gltA* sequences can be used to determine the evolutionary relationships of these blood parasites at the species level.

The prevalence of *E. canis* and *A. platys* infections in dogs in Rayong was investigated using a nested PCR assay. The genetic diversity of parasites and blood profiles of infected dogs were also evaluated. The results showed that the prevalence of *E. canis*, *A. platys*, and co-infections were 17.3%, 5.5%, and 2.4%, respectively. Infection with *E. canis* was associated with anemia as evidenced by decreased hematocrit, haemoglobin, erythrocyte, MCV, and platelet levels ($p < 0.05$). In addition, the group infected with *E. canis* showed a downward trend in MCV (microcytic) and MCHC, suggesting that the infected animals exhibited hemolysis due to regenerative anemia. Infection with *A. platys* resulted in thrombocytopenia, as confirmed by lower platelet counts ($p < 0.05$). Sequences generated from the citrate synthase (*gltA*) gene confirmed the presence of *E. canis* and *A. platys*. The prevalence of these pathogens necessitates further studies to investigate the presence of these pathogens in their tick vectors. In addition, eliminating ticks breeding sites and regularly applying an effective tick prevention product to prevent these tick-borne diseases in dogs are recommended.

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CONFLICT OF INTEREST

The authors declared no conflicts of interest.

NOVELTY STATEMENT

There is inadequate data on the epidemiology of canine tick-borne disease in Thailand especially in the eastern part of the country. This study investigated E. canis and A. platys infections in dogs in this region by molecular detection. The study also provided the phylogenetic relationship of E. canis and A. platys in Thailand with published sequences and assessed the haematological alterations associated with the infection.

AUTHOR'S CONTRIBUTION

SP designed of the experiments and analysis of data, writing the manuscript, reviewed and revised the manuscript. NK, NM and CT conceived the project, collected the samples, performed the examinations, and analysis of data. TS collected the samples. ST and LP reviewed and revised the manuscript. All authors read and approved the final manuscript.

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