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Molecular detection of blood pathogens and their impacts on levels of packed cell volume in stray dogs from Thailand

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ABSTRACT

Objective: To evaluate the prevalence of blood parasite infection in stray dogs by PCR technique and the association between levels of packed cell volume (PCV) and blood parasitic infection in stray dogs.
Methods: A total of 65 blood samples were collected from stray dogs in animal quarantine station from Mahasarakham, Thailand to evaluate the levels of PCV before molecular screening for tick-borne pathogens infection.
Results: Stray dogs were positive with one or more pathogens in 44 (67.69%) out of 65 blood samples. *Ehrlichia canis* [43.1%, 95% confidence interval (*CI*): 38.1–48.1] was the most common blood pathogen found infecting in stray dogs in Mahasarakham Province, followed by *Anaplasma platys* (29.2%, 95% *CI*: 24.2–34.2), *Hepatozoon canis* (12.3%, 95% *CI*: 7.3–17.3) and *Babesia canis vogeli* (6.2%, 95% *CI*: 1.2–11.2), respectively. Moreover, co-infections with two pathogens were identified in 11 (16.9%) of dogs examined and two (2.9%) dogs were co-infections with three pathogens. Statistically significant relationship between the PCV levels and *Ehrlichia canis* infection was found ($P < 0.05$).
Conclusions: This study indicated that blood pathogens are spreading in stray dogs and they are potentially high risk of agent transmission to human via exposure with tick vectors. It was also the first report of *Anaplasma platys* infection in dogs in north-eastern part of Thailand.

1. Introduction

Canine tick-borne pathogens are recognized by the cause of morbidity and mortality in infected wild and domestic dogs and prevalent as zoonotic agents in human[1,2]. The brown dog tick, *Rhipicephalus sanguineus* which is worldwide distribution particularly in the tropical and subtropical countries, serves as the vector[3]. The significant blood pathogens related to *Rhipicephalus sanguineus* described for dogs in Thailand and neighboring countries are *Babesia canis vogeli* (*B. canis vogeli*), *Hepatozoon canis* (*H. canis*), *Anaplasma platys* (*A. platys*) and *Ehrlichia canis* (*E. canis*)[4,5]. Dogs who play the role as the reservoir, are infected principally by biting of infected ticks (*B. canis vogeli*, *A. platys* and *E. canis*) or ingestion of infected ticks (*H. canis*)[6]. Infections of these pathogens range in their effects from asymptomatic to severe illness. The compatibility of clinical signs includes fever, anorexia, lymphadenomegaly, pale mucous membranes, lethargy, scleral injection, anemia, weight loss, icterus and thrombocytopenia[7-9].

Several studies reported tick-borne diseases in dogs in Thailand and suggested that dogs were normally infected due to distribution of tick vectors. However, tick-borne diseases in human have increased[10,11] and should be concerned in this endemic region. Epidemiological data on prevalence of tick-borne diseases are not adequate, so studies of distribution and density of dog infections are necessary. Recently, routine diagnosis of canine blood parasitic infections can be defined based on the evidence of health status, clinical signs, serology and complete blood count or a platelet count, but most infected hosts are sub-clinical and non-specific signs. Moreover, laboratory diagnostic findings may include trend of low packed cell volume (PCV). We hypothesized that infected dogs may have a trend of PCV values lower than those of the non-infected. This study aims to evaluate the prevalence of blood parasite infection in stray dogs by PCR technique and the association between levels of PCV and blood parasitic infection in stray dogs.

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2. Materials and methods

2.1. Blood collection and measurement of PCV

This cross-sectional study collected 65 blood samples from Mahasarakham animal quarantine station, Thailand (16°25'50" N, 103°3'36.4" E) on 25–27th February, 2015. Blood was collected approximately 3 mL from the cephalic vein into sterile tubes with anticoagulant (ethylene diamine tetraacetic acid) and kept on ice during transport to the laboratory. For long term preservation, blood was stored at –20 ℃ until DNA extraction. All steps for animal handles and blood collections were conducted by veterinarians. Levels of the PCV was evaluated by filling blood directly into 2/3 of the heparinized microhematocrit tube. The tube was placed into a calibrated microhematocrit centrifuge and spined at 10000 r/min for 5 min. The height of the total blood column and the height of the red cell layer were measured within a minute after the centrifuge has stopped. The PCV levels were classified into four groups according to PCV (%) severity: level 1 was normal group (PCV $\geqslant$ 37%), level 2 was mild anemia group (PCV = 30%–37%), level 3 was moderate anemia group (PCV = 20%–29%) and level 4 was severe anemia group (PCV $\leqslant$ 20%).

2.2. DNA extraction and amplification of tick-borne pathogens

Total DNA was extracted from blood samples using GF-1 blood DNA extraction kit (Vivantis, Selangor Ehsan, Malaysia). Concentrations of total DNA were determined by exposing the DNA to ultraviolet light at a wavelength of 260 nm with UV-vis spectrophotometer (Mecasys, Korea). *B. canis vogeli* and *H. canis* 18S rRNA gene were amplified by single PCR. *E. canis* and *A. platys* 16S rRNA gene were amplified by nested PCR. In amplification steps, primers used in PCR for detection of DNA of *B. canis vogeli*, *H. canis*, *A. platys* and *E. canis* were selected based on previously description[12-17] (Table 1). The PCR reactions contained 10–50 ng of extracting DNA, 10 pmol of each primer, 200 μmol/L of each dNTPs, 1.5 mmol/L of $MgCl_2$ and 1 U *Taq* polymerase (Vivantis, Selangor Darul Ehsan, Malaysia). The PCR protocols were done with 35 cycles of denaturation at 95 ℃ for 1 min, annealing at 55 ℃ for *B. canis vogeli*, 57 ℃ for *H. canis*, 60 ℃ and 62 ℃ for first and second steps of *A. platys* and 60 ℃ for both 2 steps of *E. canis* for 1 min, extension at 72 ℃ for 2 min and a final extension at 72 ℃ for 5 min. PCR amplification was performed using Biometra GmbH thermocycle (Germany). PCR products were identified by 1% agarose gels stained with ethidium bromide and visualized under ultraviolet light.

2.3. Statistic analysis

The association between level of PCV and blood parasite infections was compared with Pearson's *Chi*-squared test. The prevalence (%) and 95% confidence intervals (*CI*) were calculated.

3. Results

3.1 Detection of B. canis vogeli, H. canis, E. canis and A. platys in stray dogs from Mahasarakham animal quarantine station, Thailand

A total of 65 stray dogs samples collected from the animal quarantine station, Mahasarakham included 32 (49.2%) males and 33 (58.8%) females. Blood parasitic detection showed 44 (67.7%) samples were positive for at least one species of blood pathogens. Overall 31 (47.7%) were single infection, and 11 (16.9%) were double infection and 2 (3.1%) were multiple infection (Table 2). PCR detection was positive at 6.2% (95% *CI*: 1.2–11.2) and 12.3% (95%

Table 1
Primers for PCR amplification.

Blood parasites	Primers	Sequences	Product size (bp)	References
B. canis vogeli	BAB1	5' GTG-AAC-CTT-ATC-ACT-TAA-AGG 3'	602	[17]
	BAB4	5' CAA-CTC-CTC-CAC-GCA-ATC-G 3'		
H. canis	HepF	5' ATA-CAT-GAG-CAA-AAT-CTC-AAC 3'	665	[16]
	HepR	5' CTT-ATT-ATT-CCA-TGC-TGC-AG 3'		
E. canis	ECC	5' AGA-ACG-AAC-GCT-GGC-GGC-AAG-CC 3'	478	[13]
	ECB	5' CGT-ATT-ACC-GCG-GCT-GCT-GGC-A 3'		
	CANIS	5' CAA-TTA-TTT-ATA-GCC-TCT-GGC-TAT-AGG-A 3'	389	[13]
	HE3	5' TAT-AGG-TAC-CGT-CAT-TAT-CTT-CCC-TAT 3'		[12]
A. platys	ECC	5' AGA-ACG-AAC-GCT-GGC-GGC-AAG-CC 3'	473	[13]
	ECB	5' CGT-ATT-ACC-GCG-GCT-GCT-GGC-A 3'		
	PLATYS	5' TTT-GTC-GTA-GCT-TGC-TAT-G 3'	402	[15]
	GA1UR	5' GAG-TTT-GCC-GGG-ACT-TCT-TCT 3'		[14]

Table 2
Blood pathogens among stray dogs (No.).

PCV (%)	Samples	Infected samples	Single infection				Double infection					Multiple infection
			B	H	A	E	B + A	B + E	H + A	H + E	A + E	H + A + E
> 37%	39	22	1	3	7	6	-	-	1	-	3	1
30%–37%	18	16	1	-	-	8	1	1	1	1	2	1
20%–29%	6	5	-	-	1	3	-	-	-	-	1	-
< 20%	2	1	-	-	-	1	-	-	-	-	-	-
Total	65 (100.0%)	44 (67.7%)	2 (3.1%)	3 (4.6%)	8 (12.3%)	18 (27.7%)	1 (1.5%)	1 (1.5%)	2 (3.1%)	1 (1.5%)	6 (9.2%)	2 (3.1%)
Total	65 (100.0%)	44 (67.7%)	31 (47.7%)				11 (16.9%)					2 (3.1%)

B = *B. canis vogeli*; H = *H.canis*; A = *A. platys*; E = *E. canis*.

Table 3
Blood pathogens infection and determination on the levels of PCV.

Pathogens	Status	Levels of PCV (%)				infection [*n* (%)]	95% *CI*[a]	*P*[b]	*df*
		> 37%	30%–37%	20%–29%	< 20%				
B. canis vogeli	Positive	1 (1.5%)	3 (4.6%)	-	-	4 (6.2%)	1.2–11.2	0.184	3
	Negative	38 (58.5%)	15 (23.1%)	6 (9.2%)	2 (3.1%)	61 (93.8%)	-	-	-
H. canis	Positive	5 (7.7%)	3 (4.6%)	-	-	8 (12.3%)	7.3–17.3	0.694	3
	Negative	34 (52.3%)	15 (23.1%)	6 (9.2%)	2 (3.1%)	57 (87.7%)	-	-	-
A. platys	Positive	12 (18.5%)	5 (7.7%)	2 (3.1%)	-	19 (29.2%)	24.2–34.2	0.816	3
	Negative	27 (41.5%)	13 (20.0%)	4 (6.2%)	2 (3.1%)	46 (70.8%)	-	-	-
E. canis	Positive	10 (15.4%)	13 (20.0%)	4 (6.2%)	1 (1.5%)	28 (43.1%)	38.1–48.1	0.006*	3
	Negative	29 (44.6%)	5 (7.7%)	2 (7.7%)	1 (1.5%)	37 (56.9%)	-	-	-
Total		39	18	6	2	-	-	-	-

[a]: 95% *CI*; [b]: Pearson's *Chi*-squared test; *: Statistically significant ($P < 0.05$).

CI: 7.3–17.3) for DNA of *B. canis vogeli* and *H. canis*. Nested PCR detection was positive at 43.1% (95% *CI*: 38.1–48.1) and 29.2% (95% *CI*: 24.2–34.2) for DNA of *E. canis* and *A. platys* (Table 3).

3.2. Evaluation infections on levels of PCV

The PCV values of these dogs were classified into 4 levels: level 1 was normal group (PCV (%) $\geqslant$ 37%); level 2 was mild anemia group (PCV (%) = 30%–37%); level 3 was moderate anemia group (PCV (%) = 20%–29%) and level 4 was severe anemia group (PCV (%) $\leqslant$ 20%). Among 65 dogs in this study, 39, 18, 6 and 2 were classified by PCV values into level 1, 2, 3 and 4, respectively. There were overall 39 in level 1, 22 dogs infection, 17 for single infection, 4 for double infections, 1 for multiple infections and 17 for non-infection. Of the 18 dogs in level 2, 16 dogs were infected, 9, 6 and 1 dogs were single, double and multiple infections, respectively, and 2 dogs were negative for blood pathogens. At level 3, 4 and 1 of the dogs were positive with one and two pathogens, respectively, and 1 dog was negative tested. Two dogs in level 4 were single infection and non-infection, respectively (Table 2). Stray dogs which were positive for blood pathogens showed a low tendency of PCV values. The highest rate of tick-borne infection was the PCV level 2 (mild anemia), followed by PCV level 3 (moderate anemia), PCV level 1 (normal) and PCV level 4 (severe anemia) (Figure 1). Results from *Chi*-square testing showed a statistically significant relationship between levels of PCV and *E. canis* positive dogs ($P < 0.05$). However, other pathogen had no significant effect between blood parasitic infection and PCV levels (Table 3).

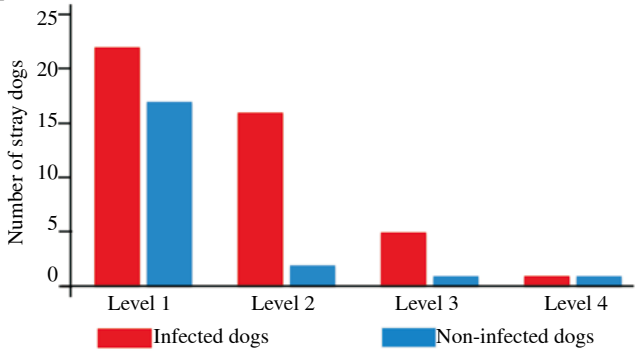

Figure 1. Comparison number of infected and non-infected dogs in different PCV (%). Of 65 dogs in this study, 39, 18, 6 and 2 were classified by PCV (%) in level 1 (> 37%), level 2 (30%–37%), level 3 (20%–29%) and level 4 (< 20%), respectively. Level 1: 22 dogs were infections; Level 2: 16 dogs were infections; level 3: 5 dogs were infections; level 4: one of dog was infected with at least a pathogen.

4. Discussion

This study observed tick-borne pathogen in blood of stray dogs and evaluated the association between parasitic infections and the levels of PCV. We discovered the elevated infection rate status of stray dogs and reported the first study on the prevalence of *A. platys* in dogs in Northeast Thailand. The prevalence of *E. canis* was the highest with 43.08%, followed by *A. platys* (29.2%), *H. canis* (12.3%) and *B. canis vogeli* (6.2%), respectively. The results of this study supported the previous report regarding the presence of the highest prevalence of *E. canis* (21.5%) then *H. canis* (10.1%) and *B. canis vogeli* (6.3%) in stray dogs in Mahasarakham, Thailand[18]. In the southern region of Thailand, occurrence of *E. canis* infection in stray dogs was the lowest at 3.9%[19]. The variations of all tick-borne pathogen infection rate may have been caused by differences in sampling location, distribution of the tick vector, season and examination technique. Moreover, accumulation of the stray dogs is an indispensable factor, because they act as potential reservoir hosts in the transmission cycle. A study conducted in Turkey found stray dogs had a higher prevalence of tick-borne infections (7.4%) when compared to pet dogs (1.2%)[20]. The high prevalence of *E. canis* and *A. platys* in stray dogs may be remarkable for the increasing possibility of disease transmission to human.

Several studies reported blood pathogen infections can affect the PCV values[21]. From this study, we found the frequency of tick-borne infection in stray dogs was higher in mild anemia cases than normal group but there was no statistically significance. Moreover, only *E. canis* positive dogs displayed significant relationship with levels of PCV. Likewise, tick-borne pathogen infection is not necessarily effected by PCV values in shelter dogs in Mauritius also[22]. On the other hand, lower PCV indicated a significant positive correlation with infection with blood pathogens. Moreover, co-infected dogs had lower PCV values compared to non-infected and single-infected dogs from Costa Rica[23]. However, the discrepancy of PCV values may be occurred by many factors such as dehydration, lack of nutrition, intestinal parasitic infection and blood parasitic infection, *etc*. Therefore, evaluation of the PCV values can be used in combining for diagnosis tick-borne pathogen infections but necessarily standardize other confounding factors.

This cross-sectional study examined the prevalence of *B. canis vogeli*, *H. canis*, *A. platys* and *E. canis* and evaluated the association between parasitic infections and the levels of PCV. Sixty-five blood

samples of stray dogs were collected from the animal quarantine station of Mahasarakham, Thailand. The results indicated that blood pathogens were abundantly spreading in stray dogs and they were potentially high risk of agent transmission to human via exposure with tick vectors. On the volume percentage of red blood cell, the PCV values of all stray dogs were ranged from 16% to 57%. *Chi*-square testing revealed a statistically significant relationship between the PCV levels and *E. canis* infection, but other pathogens had no significant effect. Moreover, this is the first report of *A. platys* infection in dogs in north-eastern part of Thailand.

Conflict of interest statement

We declare that we have no conflict of interest.

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