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ความชุกของเชื้อก่อโรคที่นำโดยเห็บในสุนัขที่ด่านกักกันสัตว์ จังหวัดนครพนม

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บทคัดย่อ: เพื่อเป็นการศึกษาความชุกของเชื้อก่อโรคที่นำโดยเห็บในสุนัข ณ ด่านกักกันสัตว์ จังหวัดนครพนม ช่วงเดือนกันยายน พ.ศ.2556 สุนัขเร่ร่อนและสุนัขบ้านจำนวน 5% (43 ตัว) ที่ด่านกักกันสัตว์ จังหวัดนครพนม ได้ถูกเก็บตัวอย่างเลือดในสาร EDTA และนำมาสกัดดีเอ็นเอเพื่อตรวจหาเชื้อ *Babesia* sp., *Erlichia* sp. และ *Hepatozoon* sp. ด้วยเทคนิคปฏิกิริยาลูกโซ่โพลิเมอร์เรส ผลการศึกษาพบว่า 29 ตัวอย่างจาก 43 ตัวอย่าง ติดเชื้อก่อโรคที่เกิดจากเห็บเพียงชนิดเดียวคือ *B. canis* (10.35%), *E. canis* (13.80%) และ *H. canis* (65.50%) อีกทั้งพบการติดเชื้อร่วมกันระหว่าง *E. canis* และ *B. canis* เป็น 3.45% และการติดเชื้อร่วมกันระหว่าง *E. canis* และ *H. canis* เป็น 3.45% การศึกษานี้แสดงให้เห็นว่าเชื้อก่อโรคที่นำโดยเห็บในสุนัขยังคงเป็นปัญหาด้านสุขภาพของสุนัขที่ด่านกักกันสัตว์ จังหวัดนครพนม ซึ่งเป็นสาเหตุให้เกิดการป่วยและเสียชีวิตได้ ดังนั้นจึงมีความจำเป็นต้องทำการรักษาเมื่อพบสัตว์ป่วยอีกทั้งต้องมีแนวทางการป้องกันโรคที่เกิดจากเห็บเป็นพาหะด้วย

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Prevalence of Tick-borne Pathogens in Quarantined Dogs at Nakornpranom Animal Quarantine Station, Thailand

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Abstract: To know the prevalence of tick-borne pathogens in quarantined dogs at Nakhonpranom animal quarantine station, Nakornpranom province, Thailand during September, 2013. About 5% (43 dogs) of EDTA-blood from stray and domestic dogs in Nakhonpranom animal quarantine station were collected and extracted DNA for detection of *Babesia* sp*., Erlichia* sp. and *Hepatozoon* sp. using conventional polymerase chain reaction (PCR). The result found that 29 out of 43 were infected with tick borne pathogens. There were single infection with *B.canis* (10.35%), *E.canis* (13.80%) and *H.canis* (65.50%). Co-infection with *B.canis*, *E.canis* and *H.canis* was 3.45%, co-infection with *E.canis* and *B.canis* was 3.45% and co-infection with *E.canis* and *H.canis* was 3.45%. This study suggests that tick-borne pathogens remain the health problem in dog which may cause of sick and death at Nakhonpranom animal quarantine station, Thailand. Therefore, treatment and prevention of tick-borne disease are extremely needed.

Keywords: Dogs, *Babesia*, *Hepatozoon*, *Ehrlichia*, Nakhonpranom

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Introduction

Ticks are the most important ectoparasites in temperate climates (Bryson *et al*., 2000; Gray *et al.*, 2013). These ectoparasites cause significant damages to the hosts during the feeding process, such as

blood loss and dilacerations of tissues due to mechanical action of mouthparts of ticks, in addition to being vectors of several pathogens, which cause serious harms to animals and public health. The recognized tick-borne diseases for dogs that transmitted by the main vector, *Rhipicephalus sangiuneus* are babesiosis, hepatozoonosis and ehrlichiosis (Coutinho *et al.*, 2005; Demma *et al.*, 2005; Dantas-Torres, 2008). The majority of patients with these diseases cause a mild sickness or asymptomatic case but some develop severe sickness that may result in death. Therefore, rapid diagnostic technique with more sensitivity, specificity and reproducibility are needed for early treatment (Krause *et al.*, 1996). In general, laboratory method for tick-borne diseases diagnosis is microscopic examination of stained blood smears but it has very limited sensitivity and cannot be distinguished by visual identification. Thus, molecular techniques have become the preferred method for detection of tick-borne blood parasites in vertebrates (Matjila *et al.*, 2008; Irwin, 2009; Abd Rani *et al.*, 2011; Laummaunwai *et al.*, 2014). This technique can use for diagnose and differentiate various tick-borne parasites and has a sensitivity tool in asymptomatic case for early treatment (Moraes *et al.*, 2014).

The aim of this study was to investigate the prevalence of canine tick-borne pathogens (*Babesia canis*, *Hepatozoon canis* and *Ehrlichia canis*) in domestic dogs from Nakhonpranom animal quarantine station, Nakornpranom province, Thailand by using microscopic examination and conventional polymerase chain reaction (PCR).

Materials and Methods

Specimen collection

EDTA-blood of 43 domestic dogs from Nakornpranom province at latitude 17° 23’7.34”, 104° 45’56.15” were collected and then processed for Giemsa’s staining and DNA extraction.

Fig. 1 The Nakornpranom quarantine unit (A) domestic and stray dogs in quarantine unit (B) blood collection (C).

Microscopic examination

To perform Giemsa's straining slides, fresh bloods were thin smear and fixed with absolute methanol for 1 minute and subsequently stained with 2.5% Giemsa in phosphate buffer, pH 7.2, for 30-60 minutes, and then rinse with tap water. Tick-borne pathogens were examined on the thin blood film under a light microscope. The morphology of the blood parasite was identified as shown in Fig. 2.

Conventional polymerase chain reaction (PCR)

0.5 ML of EDTA blood of each sample was used for DNA extraction. In brief, hemolytic buffer was added to the EDTA blood and then centrifuged at 12,000 rpm for 10 min. One hundred µl. of 0.5% PK and 0.1% SDS in TE buffer was added and incubated at 60 ^{0}C for 1 hr. After that, 100 µl. of 1:1 of chloroform and phenol was added and then precipitated with 10% of 2.5M sodium acetate and 2.5X ethyl alcohol. The reaction was incubated at -20^{0}C overnight and then centrifuged at 12,000 rpm for 10 min. and then washed with 75% ethyl alcohol. Twenty µl. of distilled water was added and the DNA concentration was measured with Nano-drop technology (Nanodrop2000®, USA).

All samples were confirmed the result using conventional PCR for genus and species diagnosis. The analysis was carried out by the Department of Parasitology, Faculty of Medicine, Khon Kaen University using a modified method previously (Moraes *et al.,* 2014). The PCR mixture contained 10 µl of PCR master mix 1 µl of 5mM dNTP, 1 µl of $MgCl_2$, 0.2 µl of *Taq* polymerase (Fermentus® Germany) 1 µl of primer pair mix consisting of 5 pmol/µl each primer, and 6 µl of distilled water. PCR condition was 95°C for 10 min, 1 cycle at 95 °C for 1 min,

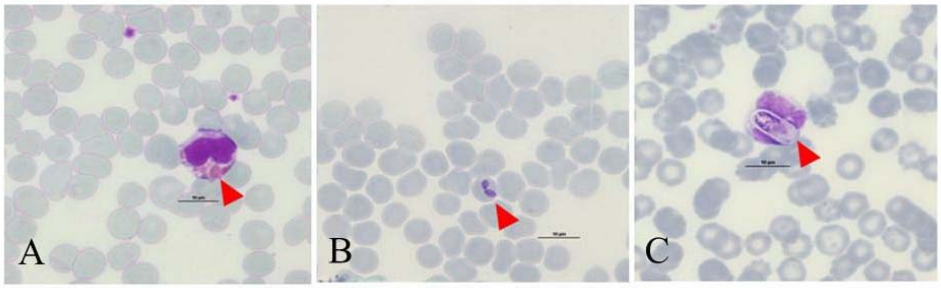

Fig. 2 The morphology of tick-borne pathogens from microscopic observation. The representative picture of *E. canis* reside in the membrane-lined cytoplasmic vacuoles of infected leukocytes in dog. Within the cytoplasmic vacuoles *Ehrlichia* form morulae (A) merozoite form of *Babesia* spp. in red blood cell (B) and ellipsoidal-shaped gamont of *H. canis* in the cytoplasm of a leukocyte (C).

60°C for 30 sec, and at 72 °C for 1 min, 35 cycles. Primers for *B. canis* , *H. canis* and *E. canis* were designed as follows: *B. canis* (GenBank accession no. JQ613105) 5'-CAGGG CTAATGTCTTGTAATTGG-3' and 5'-ATTTCTCT CAAGCTCCTGAAGG-3'; 557 bp, *H. canis* (GenBank accession no. AF176835.1) 5'-TTAA CGGGGGATTAGGGTTC-3' and 5'-CGGCCTGCTA GAAACACTCT-3'; 437 bp, *E. canis* (GenBank accession no. AY205342.1) 5'-CCATAAGCATAG CTGATAACCCTGTTACAA-3' and 5'-TGGATAATA AAACCGTACTATGTATGCTAG-3'; 380 bp (Laummaunwai *et al*., 2014). Specific band of each primer was observed and photographed as shown in Fig. 3.

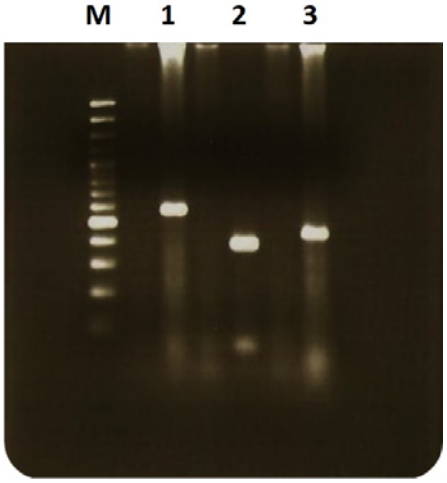

Fig. 3 The representative specific bands of *B. canis* (lane 1), *E. canis* (lane 2) and *H. canis* (lane 3). (M; marker)

Results

Detection of tick-borne pathogens by microscopic examination

Out of 43 samples, 19 were detected positive for blood parasites by microscopic examination. The highest positive rate was *H. canis* (32.56%), *E. canis* (4.56%), *B. canis* (2.33%), *B. canis* co-infection with *E. canis* (2.33%) and *E. canis* co-infection with *H. canis* (2.33%) respectively. There were no found *B. canis* co-infection with *H. canis* and co-infection with all of three blood protozoon.

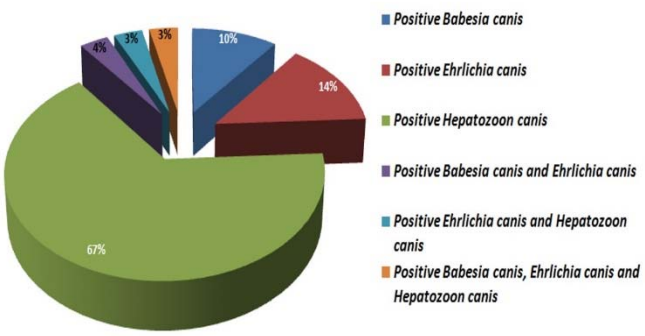

Fig. 4 The percentage of tick borne pathogen DNAs in EDTA blood using PCR technique.

Detection of tick-borne pathogens by PCR technique

Tick blood parasites were found in 29 samples by PCR technique, the positive samples were 19 samples of *H. canis* (67%), 4 samples of *E. canis* (14%), 3 samples of *B. canis* (10%), 1 sample of *B. canis* co-infection with *E. canis* (4%), 1 sample of *E. canis* co-infection with *H. canis* (3%) and 1 sample was co-infection with all of these parasites (3%) respectively (Fig. 4). The diagnostic of tick-borne pathogen using PCR technique showed higher positive rate (67.44%) when compared with microscopic examination (44.19%) (Table 1 and 2) resulting to PCR method showed

negative rate less than blood smear technique (32.55% and 55.8% respectively).

Table 1. Comparison of tick-borne pathogens positive samples examined by microscopic examination and PCR technique (N=43)

Positive samples	Microscopic examination	PCR technique
n	19	29
%	44.19	67.44

Discussion

In present study, microscopic examination and PCR technique are useful to diagnostic the epidemiology of canine tick-borne pathogens. The identification of thin blood film is often used for detection of blood parasite morphology in practical laboratory because of it is a rapid diagnostic method. However, the accuracy of this method relies on the training and skill of technician and parasites are not revealed in early infection (Krause *et al.*, 1996). PCR method is a sensitivity tool for blood diagnosis in current study which showed higher positive rate than traditional identification. Therefore, molecular assay could be advantage as an alternative way and a sensitivity method in asymptomatic case for early treatment, prevention and control in prevalence region.

From this result, the highest prevalence rate of tick-borne pathogens at Nakhon-pranom animal quarantine station, Thailand was canine hapatozoonosis, ehrlichiosis and

Table 2. Examination of tick-borne pathogens by microscopic examination and PCR technique (N=43)

Pathogens	Microscopic examination	PCR technique
	% (n)	% (n)
Positive *B. canis*	2.33 (1)	6.97 (3)
Positive *E. canis*	4.65 (2)	9.30 (4)
Positive *H. canis*	32.56 (14)	44.19 (19)
Positive *B. canis* and *E. canis*	2.33 (1)	2.33 (1)
Positive *B. canis* and *H. canis*	0.00 (0)	0.00 (0)
Positive *E. canis and H. canis*	2.33 (1)	2.33 (1)
Positive *B. canis*, *E. canis and H. canis*	0.00 (0)	2.33 (1)
Negative *B. canis*, *E. canis and H. canis*	55.8 (24)	32.55 (14)

babesiosis respectively. The highest prevalence of hepatozoonosis in this area confirmed that *H. canis* is innate tick-borne parasite to Thailand (Jittapalapong *et al*., 2006). Hepatozoonosis transmit to dogs by the ingestion of tick containing *Hepatozoon canis* mature oocyst (Baneth, 2011; O'Dwyer, 2011). *H. canis* infects leukocytes and parenchymal tissues of its host and affects the haematological system organs such as spleen, lymph nodes and bone marrow, and it causes the abnormality such as increased white blood cell number, stiffness, pain, weight loss, lethargy, anemia and fever; although generally chronic or mild disease, the infection can be life-threatening in severe clinical manifestations associated with high parasitemia level (Baneth and Weigler, 1997). The diagnosis of *H. canis* infection is usually performed by cytology of blood but this method may not be sensitive (Otranto *et al*., 2011). Babesiosis, the disease caused by the *Babesia* parasite, is most common among vertebrates (dogs, cattle, horses, rodents) including (though rarely) humans (Homer *et al.*, 2000). Canine babesiosis is characterized by fever, anemia, hemoglobinuria, thrombo-cytopenia, jaundice, and functional disorders of organs (Solano-Gallego and Baneth, 2011). Moreover, an obligate intracellular bacterium of the family Anaplasmataceae and gram-negative coccobacilli are best known for their etiological agency in the transmission of a group of tick-borne illnesses known as ehrlichiosis. All members of the genus *Ehrlichia* are pathogenic, causing mild to severe symptoms, specifically in humans and canines. The most commonly clinical signs in dog are weight loss, anorexia, pale mucous membranes, high fever, lethargy, lymphadenopathy and splenomegaly (Skotarczak, 2003; Harrus and Waner, 2011; De Tommasi *et al*., 2013; Aktas *et al*., 2015).

From stained blood smear and PCR technique results indicated that canine tick-borne disease is remaining the health problem in dog which may cause of sick and death at Nakhonpranom animal quarantine station, Thailand. Therefore, a sensitivity diagnostic method for early treatment and prevention of tick-borne disease are needed. Moreover, tick population control should be conducted in dog and environment in this region also.

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