

DOI: 10.14456/cmjv.2016.2

Chiang Mai Vet J 2016; 14(1): 13-22

Original Article

## Misdiagnose tick-borne pathogens in domestic dogs in Khon Kaen province, demonstrated using molecular identification

Amornrat Juasook<sup>1</sup>, Thidarut Boonmars<sup>2,3</sup>, Pranee Sriraj<sup>2,3,4</sup>, Ratchadawan Aukkanimart<sup>2,3</sup>,  
Pakkayanee Sudsan<sup>2,3</sup>, Natchanan Wonkchalee<sup>2,3</sup>, Sirintip Boonjaraspinyo<sup>3,5</sup>, Porntip Laummaunwai<sup>2,3</sup>,  
Wanchai Maleewong<sup>2,3</sup>, Trasida Ployngam<sup>6</sup>, Preenun Jitasombuti<sup>6</sup>, Panaratana Ratanasuwan<sup>7</sup>

<sup>1</sup>Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham 44000

<sup>2</sup>Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002

<sup>3</sup>Neglected zoonosis and vector borne disease group, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002

<sup>4</sup>Rajamangala University of Technology Isan Sakonnakhon Campus, Sakonnakhon 47160

<sup>5</sup>Department of Community Medicine, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002

<sup>6</sup>Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002

<sup>7</sup>Department of Anesthesiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002

**Abstract** The incidence of tick-borne blood disease in domestic dogs was determined using polymerase chain reaction (PCR) technology. A total of 280 left-over blood samples from domestic dogs were obtained from animal hospitals in Khon Kaen, Thailand, from May 2012–April 2013. Most dogs for which clinical data were available, exhibited loss of appetite and slight fever, but some appeared to be in normal condition and had no history of tick infection. All blood samples were negative for parasites when detected by Giemsa staining and microscopic examination. Eighty (28.57%) out of 280 samples were positive for tick-borne pathogen DNA. The most parasite that we detected was *Babesia canis* (48.75% of infected samples), followed by *Ehrlichia canis* (33.75%) and mixed infections of *B. canis* and *E. canis* (17.5%). All of the samples were negative to *Hepatozoon canis*. The present results indicate that PCR-based technology has high sensitivity and specificity for diagnosis of *B. canis* and *E. canis*. On the other hand, diagnosis of tick-borne pathogens by microscopic examination is not very sensitive, especially in cases of low infection or early stage of infection. Therefore, to ensure the accuracy of the results and to avoid misdiagnosis and incorrect treatment, detection at the molecular level may need to develop for diagnosis in animal clinics or animal hospitals.

**Keywords:** Tick, *Babesia*, *Hepatozoon*, *Ehrlichia*, Domestic dogs

\* **Corresponding author:** Thidarut Boonmars, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand. Tel. : 6689 777 9311, E-mail: bthida@kku.ac.th; boonmars@yahoo.com

**Article history;** received manuscript: 29 January 2016, accepted manuscript: 2 March 2016, published online: 3 March 2016



DOI: 10.14456/cmjv.2016.2

เชียงใหม่สัตวแพทยสาร 2559; 14(1): 13-22

บทความต้นฉบับ

## การวินิจฉัยเชื้อก่อโรคที่นำโดยเห็บที่ผิดพลาดในสุนัขบ้าน จังหวัดขอนแก่น แสดงให้เห็นด้วยการตรวจทางโมเลกุล

อมรรัตน์ เจือสุข<sup>1</sup> อิศารัตน์ บุญมาศ<sup>2,3</sup> ปราณิ ศรีราช<sup>2,3,4</sup> รัชฎาวรรณ อรรคนิมาตย์<sup>2,3</sup> ภัคญานี สุดสาร<sup>2,3</sup>  
ณัฐชานันท์ วงศ์ชาติ<sup>2,3</sup> ศรินทิพย์ บุญจรัสภิญโญ<sup>3,5</sup> พรทิพย์ เหลือหมื่นไวย<sup>2,3</sup> วันชัย มาลีวงศ์<sup>2</sup>  
พรรศิดา พลอยงาม<sup>6</sup> ปริณัน จิตะสมบัติ<sup>6</sup> พนารัตน์ รัตนสุวรรณ<sup>7</sup>

<sup>1</sup>คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหาสารคาม มหาสารคาม 44000

<sup>2</sup>ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002

<sup>3</sup>กลุ่มโรคติดเชื้อที่ถูกละเลย โรคติดเชื้อที่นำโดยสัตว์และโรคติดเชื้อที่นำโดยพาหะ คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น  
ขอนแก่น 40002

<sup>4</sup>มหาวิทยาลัยเทคโนโลยีราชมงคลอีสาน วิทยาเขตสกลนคร สกลนคร 47160

<sup>5</sup>ภาควิชาเวชศาสตร์ชุมชน คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002

<sup>6</sup>คณะสัตวแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002

<sup>7</sup>ภาควิชาวิสัญญีวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002

**บทคัดย่อ** อุบัติการณ์ของโรคที่นำโดยเห็บในสุนัขบ้านได้มีการศึกษาด้วยการใช้เทคนิคปฏิกิริยาลูกโซ่โพลีเมอร์เรส โดยใช้เลือดของสุนัขบ้านที่เหลือจากโรงพยาบาลสัตว์ในจังหวัดขอนแก่น จำนวน 280 ตัว ตั้งแต่เดือนพฤษภาคม พ.ศ. 2555 – เดือนเมษายน พ.ศ. 2556 สุนัขทั้งหมดที่มีข้อมูลทางคลินิกพบลักษณะอาการเบื่ออาหารและมีไข้เล็กน้อย แต่สุนัขบางตัวพบว่าอาการค่อนข้างปกติและไม่มีประวัติการติดเห็บมาก่อน ตัวอย่างเลือดสุนัขทั้งหมดไม่พบการติดเชื้อปรสิตในเลือดด้วยการย้อมสี Giemsa และส่องตรวจภายใต้กล้องจุลทรรศน์ชนิดส่องสว่าง พบตัวอย่างเลือดจำนวน 80 ตัวอย่างจากทั้งหมด 280 ตัวอย่าง (28.57%) ให้ผลบวกต่อการตรวจหาดีเอ็นเอของเชื้อก่อโรคที่นำโดยเห็บ ความชุกของการติดเชื้อสูงสุดคือ *Babesia canis* (48.75% ของตัวอย่างที่ติดเชื้อ) ตามมาด้วย *Ehrlichia canis* (33.75%) และการติดเชื้อร่วมกันระหว่าง *B. canis* และ *E. canis* (17.5%) จากตัวอย่างทั้งหมดนี้ไม่พบการติดเชื้อ *Hepatozoon canis* ผลการศึกษานี้แสดงให้เห็นว่าการใช้เทคนิคปฏิกิริยาลูกโซ่โพลีเมอร์เรสมีความไวสูงและมีความจำเพาะต่อการตรวจวินิจฉัยเชื้อ *B. canis* และ *E. canis* ในทางตรงกันข้ามการใช้กล้องจุลทรรศน์นั้นมีความไวน้อยสำหรับการตรวจวินิจฉัยเชื้อก่อโรคที่นำโดยเห็บ โดยเฉพาะในรายที่มีการติดเชื้ออย่างอ่อนหรือในระยะแรกของการติดเชื้อ ดังนั้น เพื่อให้ได้ผลที่แม่นยำ หลีกเลี่ยงการวินิจฉัยผิดพลาดและการรักษาที่ไม่ถูกต้อง การตรวจในระดับโมเลกุลน่าจะต้องมีการพัฒนาเพื่อนำมาใช้วินิจฉัยโรคในคลินิกหรือโรงพยาบาลสัตว์ต่อไป

**คำสำคัญ** เห็บ บาบีเซีย เฮปาโตซูน เออร์ลิเชีย สุนัขบ้าน

\*ผู้รับผิดชอบบทความ อิศารัตน์ บุญมาศ ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น ขอนแก่น 40002 โทรศัพท์: 6689

777 9311 อีเมลล์: bthida@kku.ac.th; boonmars@yahoo.com

**ข้อมูลบทความ** วันที่ได้รับบทความ 29 มกราคม พ.ศ.2559 วันที่ได้รับการตีพิมพ์ 2 มีนาคม พ.ศ.2559 วันที่ตีพิมพ์ออนไลน์ 3 มีนาคม พ.ศ.

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## Introduction

Tick-borne pathogens cause significant disease in domestic dogs worldwide and potentially are of public health significance. There are many tick-borne diseases in dogs, such as babesiosis, ehrlichiosis and hepatozoonosis, which are caused by *Babesia canis*, *Ehrlichia canis* and *Hepatozoon canis*, respectively (Ahantarig et al., 2008; Jittapalapong et al., 2006; Jongejan and Uilenberg, 2004; Niwetpathomwat et al., 2007; Parola et al., 2005). There have been a few reports of canine tick-borne blood parasite infections in Thailand, mostly from small-animal hospitals in Bangkok, and most of the reports used microscopic examination for diagnosis.

The diagnosis of tick-borne pathogens is usually based on the microscopic examination of pathogens in peripheral blood smears using Wright's or Giemsa staining. Serology is sometimes employed, but molecular methods are rarely used (Gal et al., 2008; Harrus et al., 2002; Krause et al., 1996; Shaw et al., 2001; Waner et al., 2001). Microscopic examination may lack sensitivity and is time-consuming; while serology usually indicates exposure rather than active infection, and the results might be misleading due to serological cross-reactions with other closely related organisms (Harrus et al., 2002; Mylonakis et al., 2004; Waner et al., 2001). Diagnosis based on microscopic examination has other limitations: this technique requires a lab technician with extensive experience in identification of canine blood parasites. Furthermore, in cases of mild infection it may be difficult to find any parasites using

microscopic examination. Conversely, molecular detection based on PCR or real-time PCR techniques is more sensitive and specific (Adaszek and Winiarczyk, 2008; Breitschwerdt et al., 2002; Jittapalapong et al., 2006; Rubini et al., 2008; Sacchini et al., 2007; Spolidorio et al., 2011).

Thus, in small-animal hospitals and/or clinics where tick-borne diseases are quite often found, these diseases can be fatal because of misdiagnosis and subsequent incorrect treatment. Misdiagnosis is not the only problem; tick control is also largely ineffective, which is blood parasites remain a health problem in domestic dogs.

Therefore, the present study was conducted to confirm that microscopic examination of tick-borne pathogens in Thailand is indeed very insensitive. Domestic dog blood samples with negative results from microscopic examination were obtained from small-animal hospitals in Khon Kaen, Thailand, between February 2012 and March 2013. The samples were subjected to PCR using primers for detection of DNAs of *B. canis*, *E. canis* and *H. canis*. Species' identities were confirmed by DNA sequencing.

## Materials and Methods

### Clinical data

Left-over diagnostic blood samples from a total of 280 dogs were examined during this study. Clinical data were found for only 100 of the dogs. Of these; 43 (15.35%) were males and 57 (20.35%) were females. No information on sex was available for the other 180 (64.28%) samples analyzed. For



dogs with clinical data (35.72%) the age range was 73-115 months (Table 1).

### Blood collection

Left-over blood samples that were microscopically negative for tick-borne diseases were provided by animal hospitals in Khon Kaen Province. Two hundred eighty samples of domestic dog blood were used to confirm the results of thin and thick blood-films from routine laboratory tests using multiplex PCR to detect pathogen DNA. Positive specimens from PCR were used for re-diagnosis and the results used to indicate what treatment should be applied. Physical and blood examinations including clinical data were analyzed when available. All protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 64/2555).

### DNA extraction from EDTA blood

DNA was extracted from EDTA blood samples using the phenol-chloroform technique. The EDTA blood was lysed by 1XPBS buffer (1:10) at 4°C for 1 hr, then centrifuged at 1,164g, 20°C for 10 min. The supernatant was discarded and 10 ml of lysis buffer was added (0.32 M glucose, 10 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 1% v/v Triton X-10). This was mixed and centrifuged at 1,164g, 20°C for 10 min. The supernatant was discarded and the pellet was removed and placed in a new tube; lysis buffer was again added, followed by centrifuging at 13,684g, 8°C for 10 min. The supernatant was discarded and 400 µl of proteinase K solution was added (20 mg/ml in 10 mM Tris-HCl and 1% SDS, pH 8.0); this was

incubated at 56°C in a water bath for 2 hr. DNA was then extracted by adding 400 µl of phenol:chloroform (1:1) mixture and the tube centrifuged at 13,684g, 8°C for 10 min. The supernatant was transferred to a new tube, and 3 M sodium acetate (1/10, v/v) and cold absolute ethanol (2.5 V/V) were added; this was incubated at -20 °C for 30 min and then centrifuged at 13,684g, 4 °C for 10 min. The supernatant was discarded and the pellet was washed with 75% ethanol and centrifuged at 13,684g, 4 °C for 10 min. The DNA pellet was air-dried and then dissolved with 15 µl deionized water. One µl of extracted DNA was used for PCR amplification.

### Performing PCR assay in the study population

Primers for *Babesia* sp. and *H. canis* were designed based on the published 18S ribosomal RNA gene sequences in GenBank as follows:

Bab, 5'-CAGGGCTAATGTCTTGTAAATTGG-3' and 5'-ATTTCTCTCAAGCTCCTGAAGG -3'; 557 bp (GenBank accession no. JQ613105),

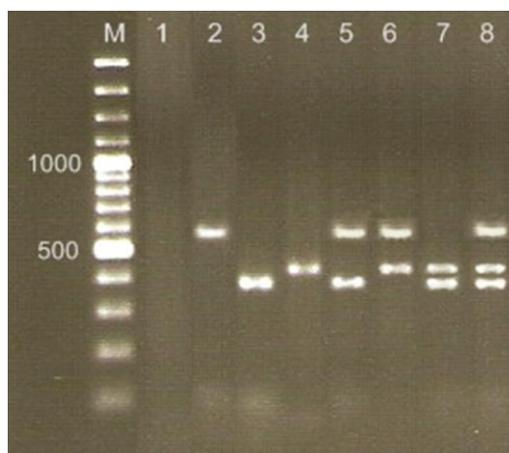
Hep, 5'-TTAACGGGGGATTAGGGTTC-3' and 5'-CGGCCTGCTAGAAACTCT-3'; 437 bp (GenBank accession no. AF176835.1).

Primers for *E. canis* were based on the published virB9 gene sequences: *E. canis*, 5'-CCATAAGCATAGCTGATAACCCTGTTACAA-3' and 5'-TGGATAATAAAACCGTACTATGTATGCTAG -3'; 380 bp (GenBank accession no. AY205342.1) (Laummaunwai et al., 2014). The DNA was used in multiplex PCRs containing primers for all three species. The different amplicon size expected for each species makes it easy to distinguish between



them on a gel. The PCR mixture contained 0.5  $\mu$ M of each primer, 2.5 mM MgCl<sub>2</sub>, 0.5 mM dNTP and 1  $\mu$ l of extracted DNA, with nuclease-free water added to a final volume of 10  $\mu$ l. Each set of experiments included negative and positive controls. Nuclease-free water replaced DNA

templates for negative controls. Template DNAs from reference species of *Babesia* sp., *E. canis* and *H. canis* from proven positive dogs were used as positive controls and photographed as shown in Figure 1.



**Figure 1** The multiplex PCR result for specific primer analysis. Non infection was shown in lane 1 as negative sample and tick-borne pathogen infections were shown in lane 2-8 as positive samples (only *B. canis* infection; lane 2, only *E. canis* infection; lane 3, only *H. canis* infection; lane 4, both *B. canis* and *E. canis* infection; lane 5, both *B. canis* and *H. canis* infection; lane 6, both *E. canis* and *H. canis* infection; lane 7 and mix *B. canis*, *E. canis* and *H. canis* infection; lane 8). (M; marker, 500; molecular weight 500 bp., 1000; molecular weight 1000 bp.)

### DNA sequencing

A specific band amplified from each species was cut out of an agarose gel and sequenced for species confirmation. For genus and species diagnosis, amplicons from 10% of samples positive for each pathogen were sequenced. To obtain DNA for sequencing, a PCR was carried out using only one pair of species-specific primers and the product purified using a PCR product purification kit (MP Biomedicals, OH). DNA sequencing was performed at Gifu University, Japan, using a BigDye<sup>®</sup> Terminator Cycle

Sequencing Kit (Applied Biosystems [ABI], CA) and an ABI PRISM<sup>®</sup> 3100 Genetic Analyzer (ABI). The results were compared with the GenBank sequence database using BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## Results

### Clinical data

Most of dogs had been brought to the hospitals with histories of weakness, lethargy, pale



gums and tongue, and a few with fever. They had no specific signs and symptoms for tick-borne disease. The signs and symptoms, complete blood count (e.g. hematocrit, hemoglobin, platelet, RBC size, WBC) and liver function tests had no correlation with the pathogens identified, as shown in Table 1.

### Prevalence of tick-borne pathogens in blood circulation

Partial 18S rRNA and virB9 gene primers were used to detect tick-borne pathogens. DNAs of *B. canis* and *E. canis* with amplicon sizes of 557 and 380 bp respectively, were detected in EDTA blood from cases that had proved negative by microscopic examination. Eighty (28.57%) out of 280 cases were positive for DNA of tick-borne pathogens. Single infections with *B. canis* were most common (48.75%), followed by *E. canis* (33.75%) and mixed infections of *B. canis* and *E. canis* (17.5%). All of samples were negative to *H. canis* infection. Positive samples underwent DNA sequencing to confirm the tick-borne pathogen species, which showed 100% identity with *B. canis* or *E. canis*.

### Discussion

The most common method for blood parasite diagnosis is microscopic examination but this technique requires a highly skilled microscopist. In addition, in case of light infection it may be hard to discover any pathogens using microscopic examination. There are several reports have demonstrated that microscopic examination

can cause misdiagnosis and lack the ability to parasite identification (Llewellyn et al., 2016; Repetto et al., 2013; Verweij et al., 2007). This is the first molecular diagnostic investigation in this region of vector-borne pathogens in domestic dogs using blood samples which had been proven as negative by microscopic examination. Specific primers for each of *B. canis*, *E. canis* and *H. canis* were used in multiplex PCR.

In this study, high prevalences of babesiosis and ehrlichiosis were found using the PCR technique. This is consistent with previous studies which have shown that molecular detection based on PCR or real-time PCR techniques are more sensitive and specific than microscopic examination (Adaszek and Winiarczyk, 2008; Jittapalapong et al., 2006; Rubini et al., 2008; Sacchini et al., 2007; Spolidorio et al., 2011). For example, the PCR technique could be used to detect mild or asymptomatic *Giardia* infections and malarial infections in low endemic areas (Berry et al., 2008; Prasertbun et al., 2012; Steenkeste et al., 2009). This is supported by a previous report that studied canine hepatozoonosis in stray dogs using microscopic examination compared with DNA detection using 18S rRNA *Hepatozoon* primers.

They found more *Hepatozoon*-positive samples by PCR (11.4%) than by microscopic examination (2.6%) (Jittapalapong et al., 2006). In 2012, Buddhachat *et al.* had been detected *B. canis* in blood samples of 102 asymptomatic dogs residing in Chaing Mai, Thailand by using PCR and microscopic examination. This work reported that had no positive samples by microscopic examination but 14 samples were positive for *B. canis* by PCR.



Table 1 Summary of clinical data relating to the 100 dogs for which this was available

Pathogen	% (N)	Sex		Age (Months)	Fever % (N)	No- appetite % (N)	Anemia % (N)	Leukocytosis		Platelets		High creatinine % (N)	High ALT % (N)
		Male	Female					Neutrophilia % (N)	Eosinophilia % (N)	Low % (N)	High % (N)		
		% (N)	% (N)										
Positive <i>B. canis</i>	19	27.92	12.28	115.27	33.33	36.00	12.90	0.00	0.00	14.29	28.57	20.00	28.57
	(19)	(12)	(7)		(9)	(9)	(4)	(0)	(0)	(3)	(4)	(1)	(4)
Positive <i>E. canis</i>	11	6.97	14.04	73.86	3.70	8.00	9.68	0.00	0.00	19.05	0.00	20.00	14.29
	(11)	(3)	(8)		(1)	(2)	(3)	(0)	(0)	(4)	(0)	(1)	(2)
Positive <i>B. canis</i> and <i>E. canis</i>	3	2.32	3.51	86.5	7.41	0	3.23	0.00	0.00	9.53	0.00	0.00	7.14
	(3)	(1)	(2)		(2)	(0)	(1)	(0)	(0)	(2)	(0)	(0)	(1)
Negative <i>B. canis</i> and <i>E. canis</i>	67	62.79	70.17	98.67	55.56	56.00	74.19	100	100	57.14	71.43	60.00	50.00
	(67)	(27)	(40)		(15)	(14)	(23)	(10)	(12)	(12)	(10)	(3)	(7)
Total	100	100	100	-	100	100	100	100	100	100	100	100	100
	(100)	(43)	(57)		(27)	(25)	(31)	(10)	(12)	(21)	(14)	(5)	(14)



Moreover, Duengai *et al* (2013) found that for cases of opisthorchiasis, PCR is suitable for early diagnosis, evaluation of drug efficacy, and detection of *Opisthorchis* re-infection post-treatment. Umeha *et al* (2008) found that 75 microscopically positive fecal specimens were positive for opisthorchiasis by PCR, while 23 of 30 (76.6%) microscopically negative samples were also PCR-positive. The findings described above support this present study, which shows that the high sensitivity of the PCR technique could detect tick-borne DNA pathogens in cases of light infection with no specific signs and symptoms.

The data from the current study indicated that a high percentage of dogs were exposed to, or infected with, *B. canis* and *E. canis*. This may be due to several factors, e.g.: i) many dogs may receive an incomplete vaccination or inadequate tick/parasite controls; ii) there are many stray dogs in Thailand, so they can be easily infested with ticks and act as reservoir hosts for tick-borne pathogens; and iii) the environment is hot and humid, conditions which are optimal for tick development and for maintaining the tick life cycle. Moreover, tick-borne pathogens such as *Babesia* are the most common blood protozoa that can be found during the rainy season in Thailand. Babesiosis is transmitted by the brown dog tick; dog-to-dog transmission via bite wounds which is thought to be another mode of transmission, as well as blood transfusion and transplacental transmission. The techniques for diagnosis in animal clinics or hospitals are limited. The use of PCR to detect the presence of canine *B. canis* and *E. canis* confirms that this technique is a highly sensitive and specific method for parasite

detection. The advantage of using a species-specific PCR is that drug treatment may differ according to the pathogen(s) identified.

## Acknowledgements

This work was supported by the Thailand Research Fund (TRF Senior Research Scholar Grant no. RTA5580004). We thank the Department of Research Affairs (AS56203) and the Department of Parasitology, Faculty of Medicine, Khon Kaen University, for their support.

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