# MOLECULAR DETECTION AND TREATMENT OF TICK-BORNE PATHOGENS IN DOMESTIC DOGS IN KHON KAEN, NORTHEASTERN THAILAND

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**Abstract.** We determined the prevalence of tick-borne pathogens in domestic dogs using microscopy and polymerase chain reaction (PCR) techniques. A total of 303 EDTA blood samples were collected from domestic dogs in Khon Kaen Province, Thailand, in May 2013. Microscopic observation of Giemsa-stained smears and molecular diagnosis using conventional PCR were performed. Infected dogs were treated with imidocarb dipropionate, a combination of imidocarb dipropionate and doxycycline, or doxycycline alone. Seventy-one (23.4%) out of 303 dogs were positive for DNA of tick-borne pathogens. Of the 303 animals, 13.2% and 1.3% were positive for a single infection with *Babesia* spp or *Ehrlichia canis*, respectively using microscopy; whereas 19.5% and 3.0% were positive using the PCR technique. Co-infection with *Babesia* spp and *E. canis* was observed in 0.7%, and co-infection with *Hepatozoon canis* and *E. canis* in 0.3%. Infected dogs were treated with the assigned drugs, and elimination of the pathogens was demonstrated by microscopy and PCR. The results indicated that while both microscopic and PCR diagnostic techniques were useful for tick-borne pathogen detection, PCR was more effective. Imidocarb dipropionate and doxycycline were found to be effective for treatment of babesiosis and ehrlichiosis, respectively. The present study suggests that the PCR technique has high sensitivity and specificity for *Babesia* and *Ehrlichia* diagnosis as well as for detection of *Babesia* spp, *E. canis* and *H. canis* DNA in EDTA blood specimens.

**Keywords:** ticks, blood pathogen, *Babesia*, *Hepatozoon*, *Ehrlichia*, domestic dogs, Thailand

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## INTRODUCTION

Tick-borne pathogens cause potentially fatal diseases in dogs worldwide, including Thailand. Among the common tick-borne diseases in dogs are babesiosis, ehrlichiosis and hepatozoonosis, which

are caused by *Babesia canis*, *Ehrlichia canis* and *Hepatozoon canis*, respectively (Jongejan and Uilenberg, 2004; Bratton and Corey, 2005; Foglia Manzillo *et al*, 2006; Jittapalapong *et al*, 2006; Torina and Caracappa, 2006; Torina *et al*, 2007; Williams *et al*, 2014).

In Thailand, there is less concern about the identification of tick-borne pathogens, so morphological observation under a microscope is the most commonly used diagnostic technique (Ahantarig *et al*, 2008). However, morphological identification has certain limitations such as the requirement for a lab technician with experience in canine blood parasites. Also, in cases of light infection, it may be difficult to observe the parasites by microscopy. Thus, in animal hospitals and/or animal clinics, tick-borne diseases, although commonly presenting, are frequently misdiagnosed leading to inadequate treatment and high fatality rates (Brett *et al*, 2014). In addition to misdiagnosis, ineffective tick control is another reason why blood parasites remain a health problem in domestic dogs (Grgic-Vitek *et al*, 2010).

Therefore, the aims of the present study are as follow: to develop an effective molecular technique with high sensitivity and specificity for diagnosis of canine blood parasites; to clarify the prevalence of tick-borne pathogens in Khon Kaen, northeastern Thailand; and to determine the most effective drug regimes for elimination of tick-borne pathogens. Improved methods of diagnosis and treatment could reduce the prevalence of tick infestation and tick-borne diseases.

## MATERIALS AND METHODS

### General characteristics of domestic dogs

In May 2013, 303 dogs were randomly selected from Pra Lab Sub-district, Mueang District, Khon Kaen Province, Thailand, for determination of the presence of blood parasites by microscopic examination and polymerase chain reaction (PCR) techniques. The study subjects included 159 (52.5%) males and 144 (47.5%) females. The canines were classified into six age groups: 1-12 months old, 13-24 months old, 25-36 months old, 37-48 months old, 49-60 months old, and >60 months old. Their body weights were classified into three groups: 0.0-5.0 kg, 5.1-10.0 kg and >10.0 kg (Table 1).

### Blood collection

One ml of blood in EDTA was taken from each domestic dog and the sample divided for use in two separate protocols: 20 µl for thin blood films for morphological identification, and the rest for DNA extraction for molecular diagnosis. All protocols were approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 64/2555).

### Thin blood films

A thin blood smear staining method was used, following a routine laboratory model. To prepare each thin blood smear, 20 µl of blood was placed on an alcohol-cleaned glass slide. Blood was spread across the slide and then left to dry at room temperature. Dried blood smear slides were fixed with absolute methanol for 1 minute and then stained with Giemsa stain. Infected red blood cells and white blood cells were observed under microscopy at 1,000×. Blood parasites were identified on the basis of morphology.

### DNA extraction from EDTA blood

DNA was extracted from EDTA blood samples using the phenol-chloroform technique. In brief, each sample was initially incubated in 50 mM phosphate buffered saline buffer pH 7.4 (1:10) for 1 hour at 4°C then subjected to centrifuga-

Table 1
General characteristics of domestic dogs in this study.

| Factors | Average Mean ± SD | Category | No. (%) |
|---|---|---|---|
| Sex | | Male | 159 (52.5) |
| | | Female | 144 (47.5) |
| | | Total | 303 (100.0) |
| Age (months) | 37.26 ± 41.38 | 1 - 12 | 90 (29.7) |
| | | 13 - 24 | 77 (25.4) |
| | | 25 - 36 | 41 (13.5) |
| | | 37 - 48 | 24 (7.9) |
| | | 49 - 60 | 25 (8.3) |
| | | > 60 | 46 (15.2) |
| Body weight (kg) | 10.91 ± 8.25 | 0.0 - 5.0 | 81 (26.7) |
| | | 5.1 - 10.0 | 103 (34.0) |
| | | > 10.0 | 119 (39.3) |

tion (1,164*g* for 10 minutes at 20°C). 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 $MgCl_2$, and 1%v/v Triton X-100). The tube was inverted a few times to mix the contents and then centrifuged at 1,164*g* for 10 minutes at 20°C. The supernatant was discarded and the pellet was placed in a new tube together with fresh lysis buffer; this mixture was centrifuged at 13,684*g* for 10 minutes at 8°C. The supernatant was again discarded and 400 µl of proteinase K solution added (20 mg/ml in 10 mM Tris-HCl and 1% SDS, pH 8.0), followed by incubation for 2 hours in a water bath at 56°C. DNA was extracted by adding 400 µl of phenol:chloroform (1:1) mixture and centrifuging at 13,684*g* for 10 minutes at 8°C. The supernatant was transferred to a new tube together with 3 M sodium acetate (1/10 v/v) and cold absolute ethanol (2.5 V). This was held for 30 minutes at -20°C and then centrifuged at 13,684*g* for 10 minutes at 4°C. After discarding the supernatant, the DNA pellet was washed with 75% ethanol and centrifuged at 13,684*g* 4°C for 10 minutes. The pellet was air-dried and then dissolved in 15 µl deionized water. One µl of extracted DNA was used for PCR amplification.

**PCR assay and DNA sequencing**

Primers for *Ehrlichia canis* were designed based on *virB9* gene sequences (GenBank accession no.: AY205342.1): Ecanis, 5′-CCATAAGCATAGCTGATAACCCTGTTACAA-3′ and 5′-TGGATAATAAAACCGTACTATGTATGCTAG-3′; resulting in an amplicon size of 380 bp. Primers for *Babesia* spp and *Hepatozoon canis* were designed based on published 18S ribosomal RNA gene sequences, as follows: Bab, 5′-CAGGGCTAATGTCTTGTAATTGG-3′ and 5′-ATTTCTCTCAAGCTCCTGAAGG-3′ (based on GenBank accession no. JQ613105; resulting in an amplicon size of 557 bp); Hepcanis, 5′-TTAACGGGGGATTAGGGTTC-3′ and 5′-CGGCCTGCTAGAAACACTCT-3′ (based on GenBank accession no. AF176835.1; resulting in an amplicon size of 437 bp). The DNA was used in conventional PCR. The PCR mixture contained

Table 2
The prevalence of tick-borne pathogens in domestic dogs in Khon Kaen Province.

| Pathogens | Pre-treatment | | Post-treatment | |
|---|---|---|---|---|
| | Microscopic examination % (*n*) | PCR technique % (*n*) | Microscopic examination % (*n*) | PCR technique % (*n*) |
| Positive *Babesia* spp | 13.2 (40) | 19.5 (59)[a] | 0 (0)[b] | 0 (0)[c] |
| Positive *Ehrlichia canis* | 1.3 (4) | 3.0 (9) | 0 (0) | 0 (0) |
| Positive *Hepatozoon canis* | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Positive *Babesia* spp and *E. canis* | 0 (0) | 0.7 (2) | 0 (0) | 0 (0) |
| Positive *Babesia* spp and *H. canis* | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Positive *E. canis* and *H. canis* | 0.3 (1) | 0.3 (1) | 0 (0) | 0 (0) |
| Negative for all three pathogens | 85.2 (258) | 76.6 (232) | 100 (303) | 100 (303) |
| Total | 303 | 303 | 303 | 303 |

[a]$p \leq 0.01$ (comparison of percentage of infected individuals detected using PCR relative to microscopic examination pre-treatment).
[b]$p < 0.05$ (percentage of pathogen detection using microscopic examination pre- and post-treatment).
[c]$p < 0.001$ (percentage of pathogen detection using PCR technique in pre- and post-treatment).

1 μl of 5 μM of each primer, 1 μl of 25 mM $MgCl_2$, 1 μl of 5 mM dNTP, and 1 μl of DNA extract, 1 μl of 10× buffer, 0.04 μl of 5 U/μl of DNA taq polymerase (RBC Bioscience, Taipei, Taiwan), and then nuclease-free water added to obtain a final volume of 10 μl. Reactions were performed in a GeneAmp PCR System C1000 thermal cycler (Bio-Rad, Hercules, CA). The cycling conditions consisted of an initial denaturing phase at 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, annealing at 63°C for 1 minute, and then extension at 72°C for 1 minute. The primer extension phase was prolonged for 10 minutes at 72°C in the last cycle. The PCR products were visualized by electrophoresis on 2% agarose gel stained with ethidium bromide. Each set of experiments included negative and positive controls. Nuclease-free water replaced DNA templates for negative controls. Template DNA from reference tick-borne pathogen species of *B. canis*, *E. canis* and *H. canis* were used as positive controls. A specific band of each DNA sample was cut from agarose gel and submitted to DNA sequencing for species confirmation. DNA sequencing was performed at Gifu University, Japan. PCR products were sequenced using a BigDye® Terminator Cycle Sequencing Kit [Applied Biosystems (ABI), Foster City, CA]. The sequenced products were analyzed using an ABI PRISM® 3100 Genetic Analyzer (ABI). The results were compared with the GenBank sequence database using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/).

**Treatment of tick-borne pathogens**

Treatment programs for tick-borne pathogens were modified and assigned to individual infected dogs based on previously published data (Eddlestone *et al*, 2006; Vial and Gorenflot, 2006; Sasanelli *et al*, 2010; Pasa *et al*, 2011; Mosqueda

Table 3
The correlation of age with infection in dogs with blood parasites.

| Age of dogs (months) | % (*n*) | Positive by PCR technique; % (*n*) | | | |
|---|---|---|---|---|---|
| | | *Babesia* spp | *E. canis* | *Babesia* spp and *E. canis* | *E. canis* and *H. canis* |
| 1-12 | 29.7 (90) | 15.6 (14) | 8.9 (8) | 2.2 (2) | 0 (0) |
| 13-24 | 25.4 (77) | 24.7 (19) | 1.3 (1) | 0 (0) | 1.3 (1) |
| 25-36 | 13.5 (41) | 19.5 (8) | 0 (0) | 0 (0) | 0 (0) |
| 37-48 | 7.9 (24) | 16.7 (4) | 0 (0) | 0 (0) | 0 (0) |
| 49-60 | 8.3 (25) | 20.0 (5) | 0 (0) | 0 (0) | 0 (0) |
| >60 | 15.2 (46) | 19.6 (9) | 0 (0) | 0 (0) | 0 (0) |

Each age group was not correlated with infection status ($p>0.05$).

*et al*, 2012). *Babesia*-positive dogs were divided into three treatment groups: (i) intramuscular injection with a single dose of imidocarb dipropionate (6 mg/kg of body weight); (ii) intramuscular injection with a single dose of imidocarb dipropionate (6 mg/kg of body weight) plus oral administration of doxycycline (10 mg/kg of body weight); and (iii) intramuscular injection with a single dose of imidocarb dipropionate (6 mg/kg of body weight) plus oral administration of doxycycline for 30 days (at dosages of 10 mg/kg of body weight). Dogs that tested positive for *Ehrlichia canis* and /or *Hepatozoon canis* were treated with oral administration of doxycycline (at dosages of 10 mg/kg of body weight) for at least one month.

**Statistical analysis**

Statistical correlations between body weight, sex, age, and infection were performed by ANOVA (Tables 3-5), chi-square (Table 6) and independent sample *t*-test (Table 2) using SPSS version 19.0 (IBM, Armonk, NY). The effect of infection was summarized using Cox proportional hazards regression analysis with 95%CI using Stata statistical software version 11 (Collage Station, TX) (Table 6). The results were considered statistically significant when $p$-value<0.05.

## RESULTS

**Tick-borne pathogen prevalence**

DNA of tick-borne pathogens was detected in blood from 303 domestic dogs (pre-treatment), as shown in Table 2. From microscopic observation, *Babesia* spp and *E. canis* infections were found in 13.2% ($n = 40$) and 1.3% ($n = 4$) of dogs, respectively. Mixed infection with *H. canis* and *E. canis* was detected in 1 dog (0.3%). However, PCR proved more sensitive and found a significantly ($p \leq 0.01$) higher prevalence of infection. *Babesia* spp and *E. canis* infections were observed in 19.5% ($n = 59$) and 3.0% ($n = 9$), respectively. Mixed infection with *Babesia* spp and *E. canis* was found in 0.7% ($n = 2$), and mixed infection with *H. canis* and *E. canis* in 0.3% ($n = 1$). All the dogs found positive by microscopy were also positive by PCR.

Ages and sex of dogs were not correlated with infection status (Tables 3, 4), whereas dogs with a body weight of

Table 4
The correlation of sex with infection in dogs harboring blood parasites.

| Sex | % (*n*) | Positive by PCR technique; % (*n*) | | | |
|---|---|---|---|---|---|
| | | *Babesia* spp | *E. canis* | *Babesia* spp and *E. canis* | *E. canis* and *H. canis* |
| Male | 52.5 (159) | 17.6 (28) | 3.8 (6) | 0.6 (1) | 0.6 (1) |
| Female | 47.5 (144) | 20.8 (30) | 2.1 (3) | 0.7 (1) | 0 (0) |

Number of infected males was similar to that of females ($p>0.05$).

Table 5
The correlation of body weight with infection in dogs harboring blood parasites.

| Body weight (kg) | % (*n*) | Positive by PCR technique; % (*n*) | | | |
|---|---|---|---|---|---|
| | | *Babesia* spp | *E. canis* | *Babesia* spp and *E. canis* | *E. canis* and *H. canis* |
| 0.0-5.0 | 26.7 (81) | 29.6 (24)[a] | 1.2 (1) | 0 (0) | 1.2 (1) |
| 5.1-10.0 | 34.0 (103) | 18.5 (19) | 4.9 (5) | 0 (0) | 0 (0) |
| >10.0 | 39.3 (119) | 12.6 (15) | 2.5 (3) | 1.7 (2) | 0 (0) |

Dogs weighing 0.0-5.0 kg were most likely to be infected with *Babesia* spp.
[a]Significant result with $p = 0.007$.

Table 6
The correlations between gender, age, body weight and infection in dogs harboring blood parasites.

| Parameters | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | OR | *p*-value | 95% CI | OR | *p*-value | 95% CI |
| Gender | | | | | | |
| Male *vs* female | 1.095 | 0.74 | 0.639-1.873 | 1.091 | 0.757 | 0.627-1.899 |
| Age | | | | | | |
| <24 months *vs* 24-48 months | 0.589 | 0.206 | 0.259-1.337 | 0.559 | 0.174 | 0.242-1.292 |
| <24 months *vs* >48 months | 0.754 | 0.488 | 0.340-1.671 | 0.858 | 0.713 | 0.379-1.938 |
| Body weight | | | | | | |
| <5 kg *vs* 5-10 kg | 2.295 | 0.016[a] | 1.170-4.501 | 2.284 | 0.018[a] | 1.150-4.535 |
| <5 kg *vs* >10 kg | 1.553 | 0.193 | 0.800-3.015 | 1.581 | 0.183 | 0.805-3.105 |

[a]Dogs weighing 0.0 - <5.0 kg were more likely to be infected with pathogens than dogs weighing 5-10 kg; CI; Confidence interval, OR; Odds ratio.

0.0-5.0 kg had the highest infection rates ($p = 0.007$; Table 5). To determine which factors, *ie*, age, sex, and body weight, were independent predictors of tick-borne pathogen infection, odds ratio was used. Table 6 shows the results of univariate and multivariate analyses of overall infection rates. Among the potential infection factors, body weight demonstrated a significantly increased risk for tick-borne pathogen infection, with odds ratios of 2.295 (95% CI: 1.170-4.501, $p = 0.016$) and 2.284 (95% CI: 1.150-4.535, $p = 0.018$) by univariate and multivariate analysis, respectively. However, the effects of age and sex were not significant.

**Tick-borne pathogen treatment**

After 30 days of treatment, among 61 *Babesia*-positive dogs in group (i), 19 dogs treated by intramuscular injection of a single dose of imidocarb dipropionate, were found to be negative in both microscopy and PCR for tick-borne pathogens. Similar results were observed for dogs in group (ii), 24 dogs were treated by intramuscular injection with a single dose of imidocarb dipropionate plus oral administration of doxycycline, and group (iii), 18 dogs were treated by intramuscular injection with a single dose of imidocarb dipropionate plus oral administration of doxycycline for 30 days. Nine dogs that tested positive for *E. canis* or 1 dogs that tested positive for *H. canis* and co-infected with *E. canis* were treated with oral administration of doxycycline were negative for tick borne pathogens using microscopy and PCR.

All groups had 100% cure rates (as determined by PCR) by the end of the treatment regimes. In pre- treatment, the percentage of pathogens detection using PCR technique was statistically higher than those of detection using microscopic examination ($p < 0.05$).

## DISCUSSION

Tick-borne pathogens are a widespread health problem in mammals, including dogs. The present study is the first report on tick-borne pathogens in Khon Kaen, northeastern Thailand. The results showed that *Babesia* spp had a higher prevalence than *E. canis* and *H. canis*. Moreover, imidocarb dipropionate was found to have high efficacy for treatment of *Babesia* infection, while doxycycline was effective for treatment of *E. canis* and *H. canis* infections.

In Thailand, there are three main tick-borne diseases: babesiosis, ehrlichiosis and hepatozoonosis. Few previous studies have investigated canine tick-borne blood parasite infection in Thailand (Jittapalapong *et al*, 2006; Ahantarig *et al*, 2008; Kaewkong *et al*, 2014). Jittapalapong *et al* (2006) found not only the tick-borne diseases babesiosis, ehrlichiosis and hepatozoonosis, but also dirofilariasis, a mosquito-borne disease in domestic and stray dogs in Bangkok. Most cases were mixed infections and hepatozoonosis was uncommon, similar to the findings in the present study.

It is well known that diagnosis of tick-borne pathogens using microscopy, has low sensitivity in cases of light infection or low parasitemia. Misdiagnosis and hence inappropriate treatment is frequent. Therefore, detection at a molecular level may need to be used in small animal hospitals and clinics, as well as for follow-up of mass treatment or control programs in the field. Moreover, detection of DNA from tick-borne pathogens in ticks is more convenient than blood collection from mammals. The routine laboratory diagnosis of blood parasite infections

is by microscopy. However, a definitive diagnosis of blood parasite infection requires molecular techniques. Recent studies have successfully used molecular techniques for the diagnosis of infections: conventional PCR and real-time PCR using specific primers for *Ehrlichia* species (Vinasco *et al*, 2007); analysis of 16S rRNA gene sequences of *Ehrlichia* spp using nested PCR (Jirapattharasate *et al*, 2012); identification of *Babesia* spp. using nested PCR to amplify the 18S rRNA gene (Majláthová *et al*, 2011); and identification of *H. canis* by amplifying a variable region of the 18S rRNA gene using nested PCR (Allen *et al*, 2008; Li *et al*, 2008).

In the present study there was a 100% cure rate for babesiosis using any of the three treatment protocols: a single dose of imidocarb dipropionate (6 mg/kg of body weight); intramuscular injection with a single dose of imidocarb dipropionate (6 mg/kg of body weight) plus oral administration of doxycycline (10 mg/kg of body weight); and intramuscular injection with a single dose of imidocarb dipropionate (6 mg/kg of body weight) plus oral administration of doxycycline for 30 days (at dosages of 10 mg/kg of body weight). Therefore, a single dose of imidocarb dipropionate can be used for effective treatment of *Babesia*-infected dogs.

A 100% cure rate was achieved for ehrlichiosis by administration of doxycycline; this is in accordance with previous reports indicating that it is the drug of choice for treatment of ehrlichiosis, and frequently is used alone (Breitschwerdt *et al*, 1998; Eddlestone *et al*, 2007).

For hepatozoonosis, the currently recommended therapy is to administer trimethoprim-sulfadiazine, clindamycin and pyrimethamine (TCP) for two weeks to destroy the pathogens (Krampitz and Haberkorn, 1988; Douglass, 2001; Macintire *et al*, 2001). The single *H. canis*-infected dogs found in our study were cured by administering a combination of imidocarb dipropionate and doxycycline. In contrast, previous studies reported that imidocarb dipropionate at a regular dose of 6 mg/kg was not effective in eliminating *H. canis* from dogs treated repeatedly over 8 months (Torina and Caracappa, 2006; Torina *et al*, 2007). Similarly, other reports found that imidocarb dipropionate and/or toltrazuril treatment could not eliminate *H. canis* infection. Sasanelli *et al* (2010) reported that imidocarb dipropionate alone (5 mg/kg, administered once subcutaneously) could not eliminate *H. canis* in naturally infected dogs, based on parasitological and molecular evaluation methods. Moreover, Pasa *et al* (2011) found that a combination of imidocarb dipropionate (6 mg/kg, subcutaneously, every 14 days) and toltrazuril (5-10 mg/kg, subcutaneously or orally, once a day for 3-5 days; or 5 mg/kg, orally, twice a day for 4 days) failed to clear *H. canis* infection in dogs. However, Elias and Homans (1988) showed that the combined administration of tetracycline hydrochloride (22 mg/kg, 3 times daily) and imidocarb dipropionate (6 mg/kg, by subcutaneous injection, repeated after 14 days) was accompanied by clinical improvement and suppression of gametocyte parasitemia.

An effective diagnostic technique is of great importance. Microscopic detection alone is not sufficient for evaluating the responses to treatment of tick-borne infections, so follow-up using molecular techniques is recommended (Eddlestone *et al*, 2006; Sasanelli *et al*, 2010; Pasa *et al*, 2011). Treatment of tick-borne diseases is difficult because of the complicated life cycle of ticks. Problems may also arise due to

differences in drug resistance in different regions. However, this study shows that recommended treatment regimes for these diseases in Thailand are effective, with no evidence of drug resistance. Given the prevalence levels of tick-borne diseases in the Khon Kaen area, we encourage dogs owners and others to increase tick control measures for the reduction and prevention of tick-borne diseases in this region.

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