RESEARCH NOTE

GENETIC AND IMMUNOLOGICAL EVIDENCES OF *BORRELIA BURGDORFERI* IN DOG IN THAILAND

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Abstract. Lyme disease is a tick-borne zoonotic disease caused by spirochete *Borrelia burgdorferi*. It is transmitted from animals to humans by the bite of infected ticks of the genus *Ixodes*. Although Lyme disease has been reported in China and Japan, the disease has never been reported in Thailand. Blood samples and ticks were collected from 402 dogs from 7 and 3 animal clinics in Chiang Mai and Phuket Provinces, Thailand, respectively. Blood samples were tested for antibodies against *B. burgdorferi*, *Anaplasma* spp, *Ehrlichia* spp and *Dirofilaria immitis* using a commercial kit, and positive blood samples were subjected to nested PCR assay for *B. burgdorferi fla*, *ospA* and *ospC*, amplicons of which also were sequenced. Only one dog (from Chiang Mai) was positive for *B. burgdorferi*, with 97% to 100% genetic identity, depending on the sequences used for comparison, with strains from United State of America. All 376 ticks collected were *Rhipicephalus sanguineus*, but no tick was found on the infected dog. Further investigations of the infection source and vector are needed to understand potential risks of Lyme disease to dogs and humans in Thailand.

Keywords: *Borrelia burgdorferi*, Lyme disease, dog, tick, Thailand

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the US Centers for Disease Control and Prevention.

INTRODUCTION

Lyme disease is a tick-borne infection caused by spirochete *Borrelia burgdorferi*, which can be transmitted from animals to humans by the bite of infected ticks of the genus *Ixodes*, *viz*. *I. scapularis* and *I. ricinus* (Bartosik *et al*, 2011; Rudenko *et al*, 2011). Symptoms of Lyme disease in humans include erythema migrans originating from the tick-bite and flu-like symptoms (Aguero-Rosenfeld *et al*, 2005). Chronic

disease leads to neurological problems and arthritis. Dogs are one of the most susceptible domestic animals to Lyme disease, with clinical signs that include fever, general malaise, lameness, and swelling of lymph nodes near the site of the tick-bite (Krupka and Straubinger, 2010).

Lyme disease in humans and dogs has been reported in the temperate zones, *viz*. China, Egypt, Europe, Japan, and North America, where *Ixodes* ticks are widely distributed (Helmy, 2000; Muzasawa, 2004; Bartosik *et al*, 2011; Rudenko *et al*, 2011). However, emerging tick-borne diseases in Thailand have been reported, including chronic Q fever in 2003 (Suputtamongkol *et al*, 2003) and *Francisella novicida* infection in 2007, which suggest that other tick-borne diseases may emerge or re-emerge in the country (Leelaporn *et al*, 2008). To date, no evidence of Lyme disease in humans or dogs has been found in Thailand, although there is evidence of the presence of *Ixodes* tick (Ahantarig *et al*, 2008; Krupka and Straubinger, 2010). Chiang Mai (in the north) and Phuket (in the south) Provinces of Thailand are popular places of residence of expatriates, who commonly bring into the country pet dogs. As testing for tick-borne diseases is not required under the Thai Animal Health Act 2015, there exists a risk that these dog could be carriers of tick-borne diseases.

Previous study in 2011 have demonstrated that antibodies (Abs) to *B. burgdorferi* is present in dogs in Chiang Mai and Phuket Provinces (Jinawan *et al*, 2013). However, there has been no isolation or genetic evidence of *B. burgdorferi* in Thailand or elsewhere in Southeast Asia (Ahantarig *et al*, 2008). This study employed PCR-based technique to investigate the presence of *B. burgdorferi* in dogs in Chiang Mai and Phuket Provinces.

MATERIALS AND METHODS

Collection of blood samples

Blood [3 ml in ethylenediamine tetraacetic acid (EDTA)-coated tube] were taken from dogs at 10 small animal hospitals: 7 hospitals at Chiang Mai Province (n = 295) and 3 at Phuket Province (n = 107), Thailand and tested for Abs against *B. burgdorferi*, *Anaplasma* spp, *Ehrlichia* spp and *Dirofilaria immitis* using a commercial test kit (Snap 4Dx, IDEXX Laboratories, Westbrook, MN). Remaining blood samples were sent to the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai for DNA analysis. Any ticks present on a dog were stored in 70% alcohol and also sent to the Faculty of Veterinary Medicine, Chiang Mai University for identification (species, life cycle stage and sex) (Wall and Shearer, 1997). The study protocol was approved by the Animal Welfare Committee of the Faculty of Veterinary Medicine, Chiang Mai University (approval no. S10/2556).

Nested PCR-based assay

DNA was extracted from canine whole blood samples using a DNA extraction kit (QIAGEN, Valencia, CA). Primers specific for *B. burgdorferi fla* (encoding flagella protein) (Wallich *et al*, 1992), 2 different regions of *ospA* (encoding OspA protein) (Motor *et al*, 1994; Demaerschalck *et al*, 1995; Yang *et al*, 2012), and *ospC* (encoding OspC protein) (Qiu *et al*, 2002) are listed in Table 1. PCR was performed with 20 ng of genomic DNA, 0.6 μM each of primers (Table 1), 2.0X *Pfx* amplification buffer, 0.3 mM dNTPs, 1.5 mM $MgSO_4$ and 1.0 U Platinum *Pfx*® DNA polymerase (Invitrogen, Carlsbad, CA) in a total volume 50 μl of reaction solution. Thermocycling was performed in GeneAmp PCR System 9700 (AB Applied Biosystems, Foster City, CA) under following conditions: 94°C for

Table 1
Borrelia burgdorferi-specific primers used in the study.

Target gene	Primer name	Sequence (5′→3′)	Annealing temperature (°C)	Amplicon size (bp)
ospA	BSL-F	AATAGGTCTAATAATAGCCTTAATAGC	58	309
	BSL-R	CTAGTGTTTTGCCATCTTCTTTGAAAA		
ospC	OC6(+)	AAAGAATACATTAAGTGCGATATT	54	597
	OC602(-)	GGGCTTGTAAGCTCTTTAACTG		
ospC	OC6(+)	AAAGAATACATTAAGTGCGATATT	54	635
	OC623(-)	TTAAGGTTTTTTTTGGACTTTCTGC		
ospA	Outer primer 1	GGGAATAGGTCTAATATTAGCC	60	665
	Outer primer 2	CACTAATTGTTAAAGTGGAAGT		
	Inner primer 1	GCAAAATGTTAGCAGCCTTGAT	60	392
	Inner primer 2	CTGTGTATTCAAGTCTGGTTCC		
fla	Outer primer 1	CTGCTGGCATGGGAGTTTCT	55	792
	Outer primer 2	TCAATTGCATACTCAGTACT		
	Inner primer 1	GCAGTTCAATCAGGTAACGGC	55	410
	Inner primer 2	AGAAGGTGCTGTAGCAGGTG		

5 minutes; then 35 cycles of 94°C for 30 seconds, annealing for 1 minute at a temperature deepening on the primers (Table 1), and 68°C for 1 minute; with a final heating at 68°C for 10 minutes. Positive and negative control contained *B. burgdorferi* strain B31 DNA (ATCC®35210™) was used as a positive control, and sterile water was used as the blank control. Amplicons were analyzed by 1% agarose gel-electrophoresis and ethidium bromide staining. Then amplicons were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Purified amplicons were directly sequenced with BigDye® Terminator v1.1 Cycle Sequencing Kit (AB Applied Biosystems, Foster City, CA) and displayed with ABI Prism® 310 Genetic Analyzer (AB Applied Biosystems). Sequence analysis was conducted with Applied Biosystems DNA Sequencing Analysis Software Version5.1 (AB Applied Biosystems). Sequence similarity searches were performed at the National Center for Biotechnology Information (NCBI) with the BLAST network service. Sequences were reported to NCBI and deposited at GenBank [accession nos. KJ676826 (for *fla*), KM056342 and KP734208 (*OspA*), and KP734209 (*OspC*)]. In addition, *ospA* and *ospC* were amplified at 2 different regions to determine similarity to other strains.

RESULTS

Immunoassay showed that only 1/402 (0.3%) blood samples was positive for *B. burgdorferi* and *Anaplasma* spp. This was from a 5-month-old male German shepherd puppy in Chiang Mai. The puppy was born in Thailand, but pedigree record indicated that the dam and sire were imported from the United States. The puppy was admitted to an animal hospital for lameness but also presented signs of pale

Table 2
Comparison of three *Borrelia burgdorferi* gene fragments.

Gene	*B. burgdorferi* strain (origin)	GenBank accession No.	Identity (%)
ospA	CMU-057 (Thailand)	KP734208	-
	B31 (USA)	CP009657.1	98
	03021331 (USA)	KC954743.1	99
	N40 (USA)	JN413096.1	99
ospC	CMU-057 (Thailand)	KP734209	-
	B31 (USA)	U01894.1	99
	HII (USA)	EF537418.1	99
	BTW11 (USA)	JQ308218.1	99
ospA	CMU-057 (Thailand)	KM056342	-
	B31 (USA)	CP009656.1	100
	CA382 (USA)	CP005925.1	99
	N40 (USA)	CP002228.1	97
fla	CMU-057 (Thailand)	KJ676826	-
	B31 (USA)	CP009656.1	98
	CA382 (USA)	CP005925.1	98
	N40 (USA)	CP002228.1	98

mucous membrane and listlessness. Blood samples collected from the other 18 dogs boarded in the same animal during the same period were all negative for antibodies to *B. burgdorferi*. The puppy was treated with doxycycline and discharged after two weeks.

Nested PCR assays conducted on the blood sample were positive for *B. burgdorferi fla, ospA* and *ospC* (Fig 1). Nucleotide sequences of three gene fragments of this putative *B. burgdorferi* isolate (designated CMU-057) were 97%-100% identical to those of the positive control strain and other strains retrieved from GenBank (Table 2).

A total of 376 ticks, identified as *Rhipicephalus sanguineus* (128 males and 248 females) were collected from dogs in Chiang Mai ($n = 298$) and Phuket ($n = 78$) Provinces. However, no tick was present on the puppy as it had been treated with acaricide prior to admittance to the animal hospital.

DISCUSSION

Presence of genetic material specific to *B. burgdorferi*, the cause of Lyme disease, and its antibodies were found in a puppy at an animal clinic, Chiang Mai, Thailand. Clinical signs of the infected puppy were consistent with Lyme disease in dogs even though *Ixodes* tick vector was not observed. The origin of the infection was uncertain. The puppy lived in close proximity to a zoo where a group of deers usually graze at the front gate on a main road to the city.

Borrelia spp causing Lyme disease and *Ixodes* ticks are unlikely to be present in Thailand or elsewhere in Southeast Asian countries (Ahantarig *et al*, 2008). In Thailand, *R. sanguineus* is an indigenous tick (Sangvaranond, 1990), but this tick has never been identified as a vector of Lyme borreliosis. However, *Borrelia* DNA has been detected in *R. sanguineus* tick in China (Yang *et al*, 2012). On the other hand,

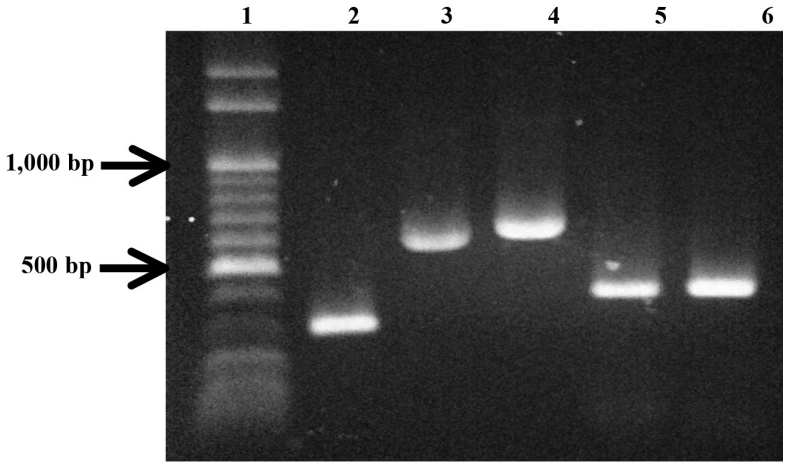

Fig 1–Electrophoresis profile of *Borrelia burgdorferi*-specific amplicons from puppy blood sample. Nested PCR primers, amplification protocols and gel-electrophoresis conditions are described in Materials and Methods. Lane 1, 100 bp DNA size markers (Invitrogen); lane 2, *ospA*; lane 3, *ospC*; lane 4, *ospC*; lane 5, *ospA*; lane 6, *fla*. *OspA* and *ospC* were amplified at 2 different regions.

Ixodes granulatus is widely distributed in China, India, Japan, Korea, Taiwan and Southeast Asian countries (Kollars *et al*, 2001; Muzasawa, 2004; Durden *et al*, 2008; Chao *et al*, 2009, 2012; Hou *et al*, 2014). *I. granulatus* has been collected from *Rattus rattus* in Pak Thong Chai District, Nakhon Ratchasima Province, northeastern Thailand (Kollars *et al*, 2001) but there is no report of the presence of *B. burgdorferi*. *I. granulatus* in Thailand is the most reasonable explanation *B. burgdorferi* infection in dogs. Ticks biting dogs together with the presence of a local rodent population will allow complete *I. granulatus* transmission cycle.

Various laboratory techniques have been developed to identify *B. burgdorferi* (Aguero-Rosenfeld *et al*, 2005). *B. burgdorferi* culture in a selective medium offers the best confirmation during infection. PCR is an acceptable method to identify infection using various types of samples (Aguero-Rosenfeld *et al*, 2005). The target genes of interest, namely, *fla*, *ospA*, 16S rDNA, and rDNA intergenic region (Hojgaard *et al*, 2014), have been successfully employed to identify spirochetes from a variety of human samples (Aguero-Rosenfeld *et al*, 2005). However, the sensitivity of PCR-based method for detecting *B. burgdorferi* DNA in human blood, plasma and serum samples from humans with Lyme disease is low because the appropriate time to collect blood sample is during spirochetemia (Aguero-Rosenfeld *et al*, 2005). Accordingly, the puppy was likely to be in a spirochetemia phase of infection during the blood collection.

The infected puppy was positive for Abs to both *Anaplasma* spp and *B. burgdorferi*. Co-infection of spirochete and other blood parasites have been detected in dogs. For example, *A. phagocytophilum* is often found in *Ixodes* ticks that also carry *B. burgdorferi* (Greig and Armstrong, 2006). In a Lyme disease endemic area such as North America, dogs that concurrently carry antibodies against *B. burgdorferi* and *A. phagocytophilum* are more prone to show clinical signs than dogs with single infections (Bowman *et al*, 2009). Dogs with thrombocytopenia, fatigue, and lameness might be more likely to be co-infected with *A. phagocytophilum* (Beall *et al*, 2006).

In summary, this study has identified the presence of *B. burgdorferi* DNA in blood sample collected from a puppy suspected of Lyme disease in Chiang Mai, Thailand. Although no tick was found on the puppy, *I. granulatus* is a possible vector. This finding underscores the value of testing animals, including domestic animals, to identify possible emerging and re-emerging pathogens that could pose a zoonotic risk in Thailand. Appropriate quarantine of imported dogs will help prevent future introduction and spread of Lyme disease. Further investigation of the infection source is needed to understand the potential risks to domestic animals and their human companions, along with tick diversity in domestic and wild animals in Thailand.

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