Original Article

Molecular Characterization and Detection of *Babesia canis vogeli* in Asymptomatic Roaming Dogs in Chiang Mai, Thailand

Kittisak Buddhachat[1] Orachon Meesong[2] Korakot Nganvongpanit[2,3] Maslin Osathanunkul[1] Siriwadee Chomdej[1,3*]

Abstract

The purpose of this study is to detect *Babesia canis* by using 18S rDNA amplification in order to confirm the presence of the agents and to characterize molecularly the Thai *B. canis*. Three sets of primers, a *Babesia canis*-specific primer (BcSP), Piroplasm-specific primer (PSP) and *Babesia* species-specific primer (BsSP), were tested for sensitivity. The results showed that BcSP and PSP were 50 times more sensitive than BsSP. Both BcSP and PSP were specific enough to detect this parasite in asymptomatic dogs. Peripheral blood samples were collected from 102 asymptomatic dogs residing in Chiang Mai and assayed with a light microscope and PCR by using BcSP and PSP primers. As a result, fourteen (13.72%) and nine (8.82%) peripheral blood samples were positive by PCR using BcSP and PSP, respectively. No positive samples were found from blood smears. Moreover, Phylogenetic analysis demonstrated that Thai *B. canis* was subspecies *vogeli*. Homology sequencing of the partial 18S rDNA gene of Thai *B. canis vogeli* (accession number JF825145) compared to other sequences from different regions was identical to that found in China, Japan, Venezuela and Brazil with 99.86% homology. Our work represents the first molecular characterization of Thai *B. canis* by using the 18S rDNA gene.

Keywords: 18S rDNA gene, *Babesia canis vogeli*, blood parasite, Chiang Mai, Thailand

[1]*Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
[2]*Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand*
[3] *Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand*
***Corresponding author: E-mail:** siriwadee@yahoo.com*

บทคัดย่อ

การหาคุณลักษณะทางอณูชีววิทยา และตรวจหา *Babesia canis* ในสุนัขจรจัดที่ไม่มีการแสดงอาการของโรคในเมืองเชียงใหม่ ประเทศไทย

กิตติศักดิ์ พุทธชาติ[1] อรชร มีซอง[2] กรกฎ งานวงศ์พาณิชย์[2,3] มัสลิน โอสถานันต์กุล[1] สิริวดี ชมเดช[1,3]*

จุดประสงค์สำหรับการศึกษาครั้งนี้เพื่อตรวจหาเชื้อ *Babesia canis* โดยการเพิ่มปริมาณดีเอ็นเอส่วนยีน 18S rDNA เพื่อใช้ในการวินิจฉัยและตรวจสอบคุณลักษณะทางอณูชีววิทยาของ *B. canis* สายพันธุ์ไทย โดยใช้ไพรเมอร์ 3 ชุด คือ *Babesia canis*-specific primer (BcSP), Piroplasm-specific primer (PSP) และ *Babesia* species-specific primer (BsSP) และนำไพรเมอร์เหล่านี้มาหาความไว ผลการทดลองพบว่า ไพรเมอร์ BcSP และ PSP มีความไวสูงกว่า BsSP ถึง 50 เท่า และไพรเมอร์ทั้งสองมีความจำเพาะเจาะจงดีพอที่นำมาใช้ในการตรวจหาเชื้อนี้ในสุนัขที่ไม่มีอาการของโรค ดังนั้นสุนัข 102 ตัวในเมืองเชียงใหม่ที่ไม่แสดงอาการของโรค ถูกเก็บตัวอย่างเลือด และตรวจหาเชื้อภายใต้กล้องจุลทรรศน์และด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส โดยใช้ไพรเมอร์ BcSP and PSP ผลการศึกษาพบว่า 14 (ร้อยละ 13.72) ตัว และ 9 (ร้อยละ 8.82) ตัว ตรวจพบว่ามีเชื้อนี้อยู่ด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส แต่ตรวจไม่พบเชื้อนี้ด้วยวิธีฟิล์มเลือดบาง นอกจากนี้การวิเคราะห์ด้วย phylogenetic แสดงให้เห็นว่าเชื้อ *B. canis* สายพันธุ์ในประเทศไทยจัดอยู่ใน subspecies *B. canis vogeli* ความเหมือนของลำดับนิวคลีโอไทป์ของเชื้อ *B. canis* สายพันธุ์ไทย (accession number JF825145) เมื่อเทียบกับเชื้อนี้จากที่อื่นๆ มีความเหมือนกับสายพันธุ์ของ จีน ญี่ปุ่น เวเนซุเอลา และ บราซิล ถึงร้อยละ 99.86 การรายงานนี้เป็นการรายงานแรกที่แสดงถึงลักษณะทางอณูชีววิทยาของเชื้อ *B. canis* สายพันธุ์ไทย โดยการใช้บางส่วนของยีน 18S rDNA

คำสำคัญ: ยีน 18S rDNA , *Babesia canis*, พยาธิในเลือด, เชียงใหม่, ประเทศไทย

[1]*ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ 50200*

[2]*ภาควิชาชีวศาสตร์ทางสัตวแพทย์และสัตวแพทย์สาธารณสุข คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ 50100*

[3]*ศูนย์วิจัยวัสดุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่ 50200*

***ผู้รับผิดชอบบทความ E-mail:** siriwadee@yahoo.com*

Introduction

Babesia is a protozoan genus residing in red blood cell (RBC) of humans and animals, and has a worldwide distribution (Uilenberg et al., 1989). Canine babesiosis is caused by *Babesia gibsoni* and *Babesia canis*, which are distinguished by the morphology of the intra-erythrocytic stage as large (*B. canis*; 2-4 μm) and small (*B. gibsoni*; 1.5-2.5 μm) which usually appears piriform (Walter et al., 2002). The abnormalities in hematology and blood biochemistry values for liver function usually found in canine babesiosis are microcytic hypochromic anemia and thrombocytopenia (Niwetpathomwat et al., 2006). *B. canis* is divided into three subspecies according to geographically specific ticks, whilst *B. canis vogeli* is widespread in only tropical and subtropical countries, including Thailand.

There are several detection methods for *B. canis* at the clinical level such as blood smears, ELISA, and IFA (Schetters et al., 1997; Schetters et al., 2001). Microscopic examination with stained blood smears has low sensitivity because the parasites are often not readily visible in smears. The PCR procedure is more specific and sensitive than conventional methods (Krause et al., 1996) as the 18S rDNA amplification by PCR allows clear detection of *B. canis* (Földvári et al., 2005; Martin et al., 2006; Santos et al., 2009).

In Thailand, there have been only a few reports on *B. canis* regarding its prevalence, for example, in stray dogs and cats in Bangkok (Kaewmongkol et al., 2004; Simking et al., 2010) as well as the clinical hematology and biochemistry of canine babesiosis in infected Thai dogs (Niwetpathomwat et al., 2006). Several studies on genetic diversity by using 18S rDNA gene revealed that *B. canis vogeli* had slight polymorphism from different continents (Zahler et al., 1998; Passos et al., 2005; Gülanber et al., 2006; Beck et al., 2009). Although *B. canis* has been reported in blood of Thai dogs, the exact genotype of Thai *B. canis* has never been characterized. The purposes of our study were to survey the distribution of *B. canis* in asymptomatic roaming dogs in order to detect the presence of *B. canis* and to compare Thai *B. canis* molecularly with *B. canis* from other countries in GenBank.

Materials and Methods

Dogs and blood collection: One hundred and two free-roaming dogs were sampled during May to September 2008 in Chiang Mai. Blood samples were collected from the cephalic or saphenous veins and preserved in EDTA as a blood anticoagulant for smear observations and PCR to detect *B. canis*.

Thin film blood smear: Each blood sample was fixed with methanol and then stained with Wright Giemsa solution. Blood smear slides were stained and observed under a light microscope. The parasites were blue, rod/piriform-shaped, in red blood cells. Blood smear detection was done by veterinary clinician.

DNA extraction, PCR, and sequencing: Total DNA was extracted from each blood sample with phenol-chloroform-isoamyl alcohol (25:24:1) solution. The 200 µl-whole blood was separated from white blood cells by 500 µl Tris-EDTA (TE) and then centrifuged at 7,000 rpm for 5 min. The DNA from white blood cells was extracted by our laboratory using phenol: chloroform procedure (adapted from Sambrook and Russell, 2001). Subsequently, the DNA was precipitated by absolute ethanol. The DNA samples were stored in TE (10mM Tris-HCl, pH 8.0 and 1 mM EDTA) buffer at -20^{0}C until use. For the screening of *B. canis*, PCR amplification was performed in a 25 µl reaction mixture containing 2 µl of each DNA template with a primer set (Table 1) 1 U of Taq DNA polymerase (Vivantis, Malaysia), 200 mM of each dNTP, 0.2 µmol of each primer, 1x PCR buffer (10mM Tris HCl pH 8.8, 50mM KCl, and 0.1% Triton X-100), and 2 mM of $MgCl_2$. Touchdown PCR amplification was performed using MyCycler (BioRAD, Applied Biosystems, USA). An initial at 94^{0}C for 3 min was followed by 10 cycles of denaturation at 94^{0}C for 15 sec, annealing at 65^{0}C for 30 sec with a decrease in temperature at 0.5^{0}C/cycle and extension at 72^{0}C for 30 sec, and then followed by 40 cycles of denaturation at 94^{0}C for 15 sec, annealing at 55^{0}C for 30 sec, and extension at 72^{0}C for 30 sec. Final extension was done at 72^{0}C for 7 min, followed by a hold step at 4^{0}C. Amplified DNA was subjected to electrophoresis in a 1.5% agarose gel (75 V, 30 minutes) pre-stained with ethidium-bromide, and viewed under ultra-violet light by a UV transmitter (BioRAD, Applied Biosystems, USA). Positive control of PCR reaction from three pairs of primers were randomly chosen to construct plasmid (TA cloning vector; RBC, Taiwan) for sequencing at 1st base Company Limited (Singapore) which pGEM was used as positive.

Sensitivity of sets of primers: DNA sample from *B. canis vogeli* was diluted in distilled water as 1:10, 1:50, 1:100 1:500 and 1:1,000. These were used as templates for PCR. The sensitivity of individual primers was expressed as fold sensitivity of the primer that had the lowest sensitivity.

Phylogenetic analysis: Multiple sequence alignments and the variation of nucleotide position for amplicon of 18S rDNA gene sequences from different regions were obtained from GenBank and analyzed by using ClustalX2 version 2.0 (Larkin et al., 2007). A phylogenetic tree was constructed using neighbor-joining (NJ) analysis with a bootstrap resampling technique of 1000 replications by MEGA software version 4.0 (Tamura et al., 2007).

Results

Specificity and sensitivity of primers: To examine whether dogs were infected by *B. canis*, three sets of primers, i.e. *Babesia canis*-specific primer (BcSP), Piroplasm-specific primer (PSP), and *Babesia* species-specific primer (BsSP) were used. These primer sets were determined for their specificity on *B. canis*. As a result, these primers could amplify the PCR product from the positive blood smear samples and were not found in the PCR product from the negative blood smear samples (Fig 1), excluding BsSP since a sequence derived from a negative blood smear showed 99% similarity to *Hepatozoon canis* (GenBank accession number EU28922). This evidence indicates that BsSP may be not only specific to *B. canis*, but also amplified DNA from other pathogens. The partial 18S rDNA gene sequences amplified by three primer sets were combined and deposited in GenBank database accession number JF825145. For the sensitivity of the three primers, the lowest DNA dilution that BcSP and PSP could amplify the PCR product was 1/50, whereas BsSP was positive PCR for the stock DNA sample only. These results revealed that BcSP and PSP were 50 times as sensitive as BsSP (Fig 2).

Table 1 Oligonucleotide sequences of primer sets amplified 18S rRNA gene of Babesia spp. to determine agents in the present study.

Primer	Oligonucleotide sequence (5′→3′)	product size (bp)
Babesia canis-specific primer (BcSP) (Martin et al., 2006)		
Bab-f	: AAG-TAC-AAG-CTT –TTT-ACG-GTG	
Bab-c	: CCT-GTA-TTG-TTA-TTT-CTT-GTC-ACT-ACC-TC	394
Piroplasm-specific primer (PSP) (Földvári et al., 2005)		
PIRO-A1	: AGG-GAG-CCT-GAG-AGA-CGG-CTA-CC	
PIRO-B	: TTA-AAT-ACG-AAT-GCC-CCC-AAC	450
Babesia species-specific primer (BsSP) (Santos et al., 2009)		
Babgen-81	: GAA-GCT-GCG-AAT-GGC-TCA-TTA	
Babgen-722	: CCA-TGC-TGA-AGT-ATT-CAA-GAC	642

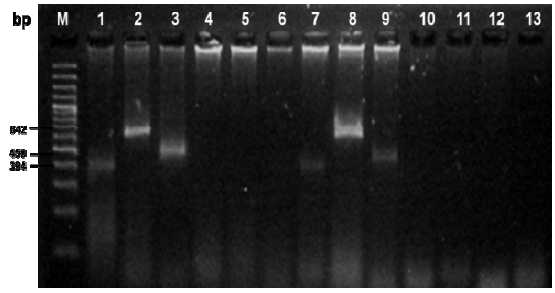

Figure 1 Specificity of individual sets of primers used to determine the presence of *B. canis* by using PCR. Lane 1-3 and 7-8 were positive blood smear samples. Lane 4-6 and 10-12 were negative blood smear samples. Lane 13 was negative control for PCR condition. Lane 1, 4, 7 and 10 were detected by using a *Babesia canis* specific primer amplifying a 394-bp product. Lane 2, 5, 8 and 11 were detected by using a *Babesia* species specific primer amplifying a 642-bp product. Lane 3, 6, 9 and 12 were detected by using a piroplasm-specific primer amplifying a 450-bp product.

The examination of B. canis vogeli in dog blood samples: Blood samples of 102 stray dogs were examined for the presence of *B. canis* by using microscopic evaluation and 18S rDNA gene amplification by PCR. From the results of specific and sensitivity of these primers, both BcSP and PSP were used to detect *B. canis* by PCR. Consequently, microscopic evaluation of the 102 blood samples showed that none were positive, whereas with 18S rDNA gene amplification, 14 (13.72%) and 9 (8.82%) were positive for PCR using BcSP and PSP, respectively.

Nucleotide polymorphism of 18S rDNA gene from B. canis vogeli: The 18S rDNA gene sequence of Thai *B. canis vogeli*, sequenced from three primers (703 bp), was compared to other *B. canis vogeli* strains from different regions (Table 2), The results revealed that the Thai strain is closely related to strains from China, Japan, Venezuela, and Brazil with 99.86% homology and the remaining *B. canis vogeli* is identical ranging from 99.72-99.43%. Homology analysis revealed four groups with polymorphism at 8 (1.13%) positions which included 7 nucleotide substitutions and an insertion. Comparisons between Thai *B. canis vogeli* and (i) China, Japan, Venezuela, and Brazil had a different nucleotide position as an insertion; (ii) USA and France had a nucleotide substitution (a transversions) and an insertion; (iii) Spain and Egypt had two nucleotide substitutions (two transversions for Spain and two transitions for Egypt) and an insertion and (iv) Taiwan had three transitions and an insertion (Table 2).

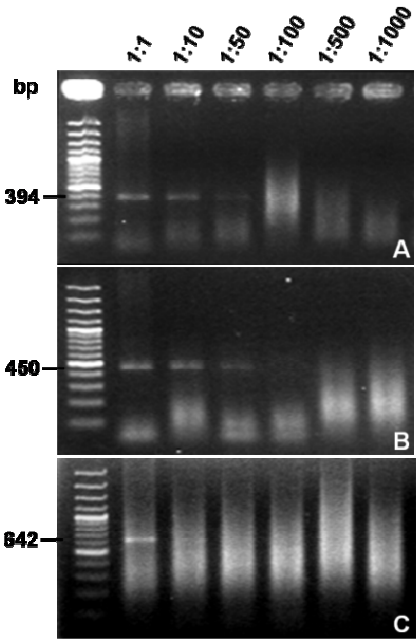

Figure 2. Sensitivity of individual sets of primers used to examine the presence of B. canis by using PCR. Lane 1 was GeneRuler^{M} 100 bp DNA ladder plus marker, Lane 2-6 product was obtained at 1:1, 1:10, 1:50, 1:100, 1:500 and 1:1000, respectively. (A) A 394-bp product amplified using a *Babesia canis* specific primer. (B) A 450-bp product amplified using a piroplasm-specific primer. (C) A 642-bp product amplified using a *Babesia* species specific primer

Table 2 Genetic variation in 704 bp of 18S rDNA gene sequences geographically from some countries with *Babesia canis vogeli* isolates from dogs. Positions are on the full-length 18S rDNA gene sequence (GenBank Accession No. AY072925)

B. canis strains	GenBank acession numbers	Identity (%)[a]	Nucleotide differences at position							
			125	458	461	488	599	632	669	760
Thailand-CM	JF825145	100	T	A	T	A	T	T	T	-
China	HM590440	99.86	●	●	●	●	●	●	●	T
Japan	AY077719	99.86	●	●	●	●	●	●	●	T
Venezuela	DQ297390	99.86	●	●	●	●	●	●	●	T
Brazil	AY371195	99.86	●	●	●	●	●	●	●	T
USA	AY371198	99.72	●	●	●	C	●	●	●	T
France	AY072925	99.72	●	●	●	C	●	●	●	T
Spain	DQ439545	99.57	●	●	●	C	●	●	A	T
Egypt	AY371197	99.57	●	●	●	●	C	C	●	T
Taiwan	HQ148663	99.43	C	G	C	●	●	●	●	T

[a]The values are percentage of nucleotides identities for 704 bp calculated from pairwise alignment.

Discussion

Canine babesiosis is tick-borne disease that is one of the most remarkable diseases worldwide. Although *B. canis* could be found throughout many regions in Thailand, there is no genotype study of Thai *B. canis*. This is the first report for molecular characterization of the Thai *B. canis* by using 18S rDNA gene.

The partial 18S rDNA sequence amplified by BcSP showed 98% homology to *B. canis vogeli* (GenBank accession number FJ213774), which had specificity to this primer. PSP and BsSP revealed 99% similarity to *B. canis vogeli* (GenBank accession number DQ297390). Carret et al. (1999) and Flöldávri et al. (2005) reported that PSP are nonspecific, positive products that do not provide information on the subspecies or species level without a restriction fragment length polymorphism (RFLP) or sequencing.

From the examination of the parasite in blood dog using PCR and microscope, it is clear that diagnosis by microscope of blood smears does not provide a definitive result to determine whether dogs are infected because of low parasitemia in blood samples (Krause et al., 1996; Földvári et al., 2005). Hence, the molecular technique is more exact. Kaewmongkol et al. (2004) reported that the prevalence of *B. canis* in stray dogs in Bangkok using PCR-based assay was 12.3%. Additionally, *B. canis vogeli* was also found among stray cats in metropolitan Bangkok by molecular methods with a prevalence of 1.4% (Simking et al., 2010). Asymptomatic roaming dogs were parasite positive by using PCR, indicating that dogs are reservoirs of more agents due to the fact that *B. canis vogeli* is less pathogenic than *B. canis canis*. Moreover, infection with *B. canis vogeli* is believed to cause a pretty mild disease with thrombocytopenia (Brown et al., 2006). Consequently, there are still babesiosis outbreaks (Cacciò et al., 2002; Brown et al., 2006). Our findings are consistent with those of Kaewmongkol et al. (2004), Beck et al. (2009) and Simking et al. (2010) and suggest that dogs and cats could have a risk opportunity to expose with tick-borne parasites.

Based on the sequence of the 18S rDNA gene (703 bases) of Thai *B. canis vogeli* and other subspecies, a phylogenetic tree was constructed by using neighbor joining (Figure 3). As expected, *B. canis* from Chiang Mai was determined as *B. canis vogeli* and was more closely related to those subspecies from China, Japan, Venezuela, and Brazil than the other countries noted. Even though *B. canis* strains are from different geographic areas of tropical and subtropical countries, multiple alignments among *B. canis vogeli* indicate that there is little genetic diversity. These results are consistent with other studies and suggest that *B. canis vogeli* strains are not geographically segregated (Passos et al., 2005; Gülanber et al., 2006; Beck et al., 2009). One possible explanation may be because of a slow mutation rate of 18S rDNA, which is used for diagnosis and making phylogenetic inferences (Waters, 1994). Therefore, other genes that have greater genetic variation such as cytochrome *b* gene

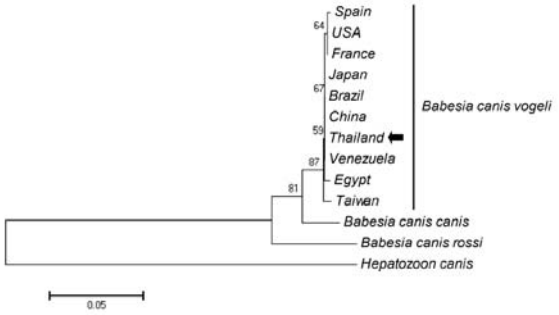

Figure 3 Neighbor-joining phylogram calculated using the MEGA4 program, based on 704 nucleotides of 18S rRNA gene introduced GenBank (*B. canis vogeli*; Venezuala (DQ297390), Japan (Okinawa) (AY077719), Brazil (AY371196), China (HM590440), Thailand (Chiang Mai) (JF825145), France (AY072925), USA (AY371198), Spain (DQ439545), Egypt (AY371197), Taiwan (HQ148663), *B. canis canis* (AY072926), *B. canis rossi* (DQ111760) and *Hepatozoon canis* (EU289222)) Numbers are bootstrap values (in percent 1000 replications) of neighbor joining. The arrow marks the *Babesia canis vogeli* investigated in our study. *Hepatozoon canis* was used as an outgroup.

(Criado et al., 2006) should be used to study the genetic diversity of this parasite. Besides, alternative idea may be the fact that these parasites need to have high genetic stability for host specificity. Comparisons of the partial 18S rDNA gene sequences showed an insertion in Thai *B. canis vogeli* which is different from other strains. This indicates slight genetic variation of Thai *B. canis vogeli* differing from other strains. Most of the partial 18S rDNA sequences among *B. canis vogeli* from different countries share nucleotides with more than 99% homology (Passos et al., 2005; Gülanber et al., 2006; Beck et al., 2009).

In conclusion, BcSP and PSP used for 18S rDNA gene amplification were more sensitive than BsSP. The 18S rDNA-based PCR assay was considerably more sensitive than light microscopy for detection of *Babesia* infections in dogs. The molecular prevalence was reported as 13.72% (used BcSP) among asymptomatic dogs in Chiang Mai, Thailand. Thai strain has a slight difference in genotype of 18S rDNA gene from others. It is necessary to detect this parasite in order to control and prevent parasitic infections, including making clinical diagnosis.

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References

Beck, R., Vojta, L., Mrljak, V., Marinculić, A., Beck A., Živičnjak T. and Cacciò, S.M. 2009. Diversity of *Babesia* and *Theileria* species in symptomatic and asymptomatic dogs in Croatia. Int J Parasitol. 39(7): 843-848.

Brown, G.K., Canfield, P.J., Dunstan, R.H., Roberts, T.K., Martin, A.R., Brown, C.S. and Irving, R. 2006. Detection of *Anaplasma platys* and *Babesia canis vogeli* and their impact on platelet numbers in free-roaming dogs associated with remote Aboriginal communities in Australia. Aust Vet J. 84(9): 321-325.

Cacciò, S.M., Antunovic, B., Moretti, A., Mangili, V., Marinculić, A., Bari, R.R., Slemenda, S.B. and Pieniazek, N.J. 2002. Molecular characterization of *Babesia canis canis* and *Babesia canis vogeli* from naturally infected European dogs. Vet Parasitol. 106(4): 285-292.

Carret, C., Walas, F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters, T.P. and Gorenflot, A. 1999. *Babesia canis canis*, *Babesia canis vogeli*, *Babesia canis rossi*: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. J Euk Microbiol. 46(3): 298-303.

Criado, A., Martinez, J., Buling, A., Barba, J.C., Merino, S., Jefferies, R. and Irwin, P.J. 2006. New data on epizootiology and genetics of piroplasms based on sequences of small ribosomal subunit and cytochrome *b* genes. Vet Parasitol. 142(3-4): 238-247.

Földvári, G., Hell, E. and Farkas, R. 2005. *Babesia canis* in dogs from Hungary: Detection by PCR and sequencing. Vet Parasitol. 127(3-4): 221-226.

Gülanber, T., Gorenflot, A., Schetters, P.M. and Carcy, B. 2006. First molecular diagnosis of *Babesia vogeli* in domestic dogs from Turkey. Vet Parasitol. 139(1-3): 224-230.

Kaewmongkol, G., Rukkwarmsuk, T., Sirinarumitr, T., Songserm, T., Tipsawake, S. and Jittapalapong, S. 2004. A prevalence of *Babesia canis* in stray dogs in Bangkok using PCR technique. J Kasetsart Vet. 14(1): 24-33.

Krause, P.J., Telford, S., Spielman, A., Ryan, R., Magera, J., Rajan, T.V., Christianson, D., Alberghini, T.V., Bow, L. and Persing, D. 1996. Comparison of PCR with blood smear and inoculation of small animals for diagnosis of *Babesia microti* parasitemia. J Clin Microb. 34(11): 2791-2794.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23(21): 2947-2948.

Martin, A.R., Dunstan, R.H., Roberts, T.K. and Brown, G.K. 2006. *Babesia canis vogeli*: A novel PCR for its detection in dogs in Australia. Exp Parasitol. 112(1): 63-65.

Niwetpathomwat, A., Techangamsuwan, S., Suvarnavibhaja, S. and Assarasakorn, S. 2006. A retrospective study of clinical hematology and biochemistry of canine babesiosis on hospital populations in Bangkok, Thailand. Comp Clin Pathol. 14(4): 110–112.

Passos, L.M., Geiger, S.M., Ribeiro, M.F., Pfister, K. and Zahler-Rinder, M. 2005. First molecular detection of *Babesia vogeli* in dogs from Brazil. Vet Parasitol. 127(1): 81-85.

Sambrook, J. and Russell, D.W. 2001. Commonly Used Techniques in Molecular Cloning. Molecular Cloning, Volume 3. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Santos, F., Coppede, J.S., Pereira, A.L.A., Oliveira, L.P., Roberto, R.G., Benedetti, R.B.R., Zucoloto, L.B., Lucas, F., Sobreira, L. and Marins, M. 2009. Molecular evaluation of the incidence of *Ehrlichia canis, Anaplasma platys* and *Babesia* spp. in dogs form Ribeirao Preto, Brazil. Vet J. 179(1): 145-148.

Schetters, T.P., Kleuskens, J., Scholtes, N.C., Gorenflot, A., Moubri, K. and Vermeulen, A.N. 2001. Vaccination of dogs against heterologous *Babesia canis* infection using antigens form culture supernatants. Vet Parasitol. 100(1-2): 75-86.

Schetters, T.P., Moubri, K., Précigout, E., Kleuskens, J., Scholtes, N.C. and Gorenflot, A., 1997. Different *Babesia canis* isolates, different diseases. Parasitology 115(5): 485-493.

Simking, P., Wongnakphet, S., Stich, R.W. and Jittapalapong, S. 2010. Detection of *Babesia vogeli* in stray cats of metropolitan Bangkok, Thailand. Vet Parasitol. 173(1-2): 70-75.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 24(8): 1596-1599.

Uilenberg, G., Franssenm, F.F.J., Perié, N.M. and Spanjer, A.A.M. 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet Q. 11(1): 33-40.

Walter, L., Heinz, M. and Zweygarth, E. 2002. Electron microscopic investigations on stages of dog piroplasms cultured in vitro: Asian isolates of *Babesia gibsoni* and strains of *B. canis* from France and Hungary. Parasitol Res. 88(1): 32-37.

Waters, A.P. 1994. The ribosomal RNA genes of *Plasmodium*. Adv Parasitol. 34: 33-79.