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

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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

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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). 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., 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* molecularily 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-white 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°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 MgCl2. Touchdown PCR amplification was performed using MyCycler (BioRAD, Applied Biosystems, USA). An initial at 94°C for 3 min was followed by 10 cycles of denaturation at 94°C for 15 sec, annealing at 65°C for 30 sec with a decrease in temperature at 0.5°C/cycle and extension at 72°C for 30 sec, and then followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Final extension was done at 72°C for 7 min, followed by a hold step at 4°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 with 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).

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Babesia canis</em>-specific primer (BcSP)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bab-f</td>
<td>AAG-TAC-AAG-CTT -TTT-ACG-GTG</td>
<td></td>
</tr>
<tr>
<td>Bab-c</td>
<td>CCT-GTA-TTG-TTA-TTT-CTT-GTC-ACT-ACC-TC</td>
<td>394</td>
</tr>
<tr>
<td>Piroplasm-specific primer (PSP) (Földvári et al., 2005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piro-A1</td>
<td>AGG-GAG-CCT-GAG-AGA-CGG-CTA-CC</td>
<td>450</td>
</tr>
<tr>
<td>Piro-B</td>
<td>TTA-AAT-ACG-AAT-GCC-CCC-AAC</td>
<td></td>
</tr>
<tr>
<td>Babesia species-specific primer (BsSP) (Santos et al., 2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babgen-81</td>
<td>GAA-GCT-GCG-AAT-GGC-TCA-CCA</td>
<td>642</td>
</tr>
<tr>
<td>Babgen-722</td>
<td>CCA-TGC-TGA-AGT-ATT-CAA-GAC</td>
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</tbody>
</table>
Specificity of individual sets of primers used to determine the presence of \textit{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 \textit{Babesia canis} specific primer amplifying a 394-bp product. Lane 2, 5, 8 and 11 were detected by using a \textit{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 \textit{B. canis vogeli} in dog blood samples: Blood samples of 102 stray dogs were examined for the presence of \textit{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 \textit{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 \textit{B. canis vogeli}: The 18S rDNA gene sequence of Thai \textit{B. canis vogeli}, sequenced from three primers (703 bp), was compared to other \textit{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 \textit{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 \textit{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).

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

<table>
<thead>
<tr>
<th>\textit{B. canis} strains</th>
<th>GenBank accession numbers</th>
<th>Identity (%)*</th>
<th>125</th>
<th>458</th>
<th>461</th>
<th>488</th>
<th>599</th>
<th>632</th>
<th>669</th>
<th>760</th>
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<tr>
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<td>JF825145</td>
<td>100</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>-</td>
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<tr>
<td>China</td>
<td>HM590440</td>
<td>99.86</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
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<td>T</td>
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<tr>
<td>Japan</td>
<td>AY077719</td>
<td>99.86</td>
<td>●</td>
<td>●</td>
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<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>T</td>
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<tr>
<td>Venezuela</td>
<td>DQ297390</td>
<td>99.86</td>
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<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>T</td>
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<tr>
<td>Brazil</td>
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<td>99.86</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
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<td>T</td>
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<tr>
<td>USA</td>
<td>AY371198</td>
<td>99.72</td>
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<td>●</td>
<td>C</td>
<td>●</td>
<td>●</td>
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<td>AY072925</td>
<td>99.72</td>
<td>●</td>
<td>●</td>
<td>C</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>T</td>
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<tr>
<td>Spain</td>
<td>DQ439545</td>
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<td>●</td>
<td>●</td>
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<td>Egypt</td>
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<td>C</td>
<td>G</td>
<td>C</td>
<td>●</td>
<td>●</td>
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</table>

*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 BsSP 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 DQ97390). Carret et al. (1999) and Flóldavári 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; Foldvári 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; Gülüanber 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 (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; Gülüanber et al., 2006; Beck et al., 2009).

In conclusion, BsSP 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 BsSP) 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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