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Research paper

Two different genogroups of *Ehrlichia canis* from dogs in Thailand using immunodominant protein genes

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ABSTRACT

Ehrlichia canis is the causative agent of canine monocytic ehrlichiosis (CME). While there is a high prevalence of CME in Thailand, genetic diversity of *E. canis* is still poorly defined. This study examined the molecular characteristics of *E. canis* using PCR and phylogenetic analysis of the *dsb*, *gp19* and *gp36* genes. DNA was extracted from 220 whole blood samples of naturally infected dogs, and all had clinical signs compatible with tick-borne diseases. Of these, 16.4% (36/220) provided positive *E. canis* DNA via the *dsb* and *gp19* genes. However, only 13 out of the 36 samples (36.1%) were positive for the *gp36* gene. Sequences of the *dsb* gene had very high identity (99–100%) with previously deposited *E. canis* sequences. Sequences of the *gp19* gene were similar to those from US and Taiwanese genogroups (98.8–99.5% identity). Elucidation of genetic characteristics of *E. canis* based on the *gp36* gene displayed 91.4–99.1% shared identity. There were 426–429 bp of a 5′ end pre-repeat tandem region, a 27 bp repetition with variable numbers of a tandem repeat (TR) region of 9 amino acid sequences (TEDSVSAPA), and a variable 3′ end region with sequence length depending on the isolate (72–93 bp). Phylogenetic trees of *E. canis*, particularly using the *gp36* amino acid sequences, showed that the Thai strains fell into two phylogenetic clades contained within other worldwide *E. canis* strains. Alignment and phylogenetic analysis suggested that *E. canis* strains from Thailand could be divided into two genogroups, the US and Taiwanese genogroups. This study provides the first characterization of the *dsb* and *gp19* genes of *E. canis* in Thailand, the results support the conclusion that the *gp36* is a potential target for genotyping and elucidation of phylogenetic relationships among *E. canis* strains.

1. Introduction

Canine monocytotropic ehrlichiosis (CME) is a tick-borne disease in dogs. The causative agent is *Ehrlichia canis*, a small, gram-negative, obligatory intracellular bacterium in the family Anaplasmataceae (Dumler et al., 2001). CME is manifested by a wide variety of clinical and hematological signs into acute, subclinical or chronic phases. The acute phases are characterized by high fever, depression, lethargy, anorexia, lymphadenomegaly, and splenomegaly; clinical signs include dermal petechiae, ecchymosis, and epistaxis (Harrus and Waner, 2011). Thrombocytopenia is the most prominent hematological change in the acute phase (Harrus et al., 1996; Waner et al., 2000). During the subclinical phase, no clinical or overt hematological signs are explicit (Waner et al., 1997). The chronic phase is characterized by pancytopenia due to suppression or destruction of the bone marrow (Mylonakis et al., 2004). Pale mucous membranes, bleeding, significant weight loss and weakness are common clinical signs during this phase. The clinical signs in the chronic phase are similar to those of the acute phase but with greater severity (Harrus and Waner, 2011).

There are a variety of diagnostic techniques for *E. canis* infection including clinical signs with hematological findings, blood smear evaluation, serology, isolation, and molecular detection. Polymerase chain reaction (PCR) is an important technique used for the molecular detection and characterization of *E. canis* strains from different areas of the world (Harrus and Waner, 2011).

The presence of *E. canis* infection in dogs has been reported in North

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and South America, Europe, Africa and Asia (Aguirre et al., 2004; Hsieh et al., 2010; Beall et al., 2012; Aguiar et al., 2013; Kamani et al., 2013; Nazari et al., 2013). In Thailand, the prevalence rates of *E. canis* infection in stray dogs by molecular survey using PCR technique were 3.9–21.5%, depending on the geographical area (Piratae et al., 2015; Liu et al., 2016). Despite the worldwide distribution and high prevalence, there is little information concerning the molecular characterization of *E. canis* in Thailand. The 16S rRNA gene provided a highly conserved sequence, 99.76–100% identical to previous DNA samples in GenBank (Pinyoowong et al., 2008). Because of this high degree of conservation, 16S rRNA provides little information regarding the genetic diversity of *E. canis* in Thailand.

Genetic characterization and diversity of *E. canis* used to focus on a small group of genes. Currently, besides 16S rRNA, several genes have been employed for detection and characterization of *E. canis*: *dsb, gp19, gp36, gp200*, and *p28* (Doyle et al., 2005b; Hsieh et al., 2010; Huang et al., 2010; Cicuttin et al., 2016). Except for the first, the rest are immunoreactive protein of *E. canis* which have been reported to react with antibodies from *E. canis*-infected dogs and divided to major and minor immunoreactive proteins (McBride et al., 2003). Major immunoreactive proteins, gp36 and gp19, were detected antibody responses earliest in dog sera during the acute phase of canine monocytic ehrlichiosis (McBride et al., 2003; Doyle et al., 2006). These are considered as a candidate proteins for future diagnostic and vaccine development. Among the genes for immunodominant proteins of *E. canis*, *gp36* was found to be one of the most variable, and is thus likely to be used as a gene for clustering and evaluating genetic diversity. In this study, *dsb* and immunoreactive protein genes (*gp19* and *gp36*) of *E. canis* in blood samples from naturally infected dogs in Chiang Mai, Thailand were amplified using PCR. Sequencing and phylogenetic analyses were conducted to examine their phylogenetic relationships with previously deposited sequences.

2. Materials and methods

2.1. Blood sample collection and DNA extraction

A total of 220 dog whole blood samples were collected from naturally infected dogs, both male and female, brought to seven animal hospitals or clinics in Chiang Mai, Thailand. All dogs displayed clinical signs compatible with tick-borne diseases, including pale mucous membrane, dermal petechiae, ecchymosis and epistaxis; non-specific signs include fever, depression, anorexia and lymphadenomegaly. In addition, there were hematological abnormalities suspected blood parasite infection; anemia, thrombocytopenia, or pancytopenia. Samples were collected by veterinarians who examined the suspected dogs from April 2013 to July 2017, inclusive. The blood collection tubes containing EDTA anticoagulant were stored at 4 °C until the extraction of genomic DNA on the same day using a PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The eluted genomic DNA was then stored at − 20 °C until further process.

2.2. Primer selection and PCR amplification of dsb, gp19 and gp36 genes

The specific oligonucleotide primers used for amplification and sequencing of *dsb, gp19,* and *gp36* of *E. canis* (Hsieh et al., 2010; Kamani et al., 2013) were designed using primer design software (PrimerSelect; DNASTAR, Madison, WI, USA) and related reference sequence from the *E. canis* genome strain Jake (accession number CP000107) (Mavromatis et al., 2006). The primers used in this study are shown in Table 1. For each PCR amplification, 5 µl of extracted DNA was used as a template in 25 µl of reaction mixture containing 1× PCR buffer, 1 µmol of each primer, 0.2 µM deoxynucleotide triphosphates (dNTPs), 2.5 µM magnesium chloride ($MgCl_2$) and 1.25 U *Taq* DNA polymerase (Invitrogen). The PCR reactions were carried out in a T100™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) according to conventional PCR cycling conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, ending with a final extension at 72 °C for 5 min for *dsb* and *gp19* gene. For *gp36* gene, the temperature used for annealing in each cycles were 56 °C for 60 s. The amplified PCR products were electrophoresed on 2% agarose gels and stained with Novel Juice in 6× loading buffer (GeneDireX, Vegas, NV, USA). Electrophoresis had been employed for 40 min at 100 V to check the product size by comparison to a DNA molecular size marker (100 bp DNA Ladder; Promega, Madison, WI, USA); bands were visualized under UV (UVP Benchtop UV Transilluminators; Fisher Scientific, Upland, CA, USA).

Table 1
PCR and oligonucleotide sequences of primers targeting *dsb*, *gp19* and *gp36* genes of *Ehrlichia canis* used in this study.

Nested PCR	Primer name	Oligonucleotide sequence (5′-3′)	Target gene	Product size
1st round	EC-dsb-F1	5′- CCA ATC ACA GGA TTC CAC TCA G − 3′	*dsb*	1533 bp
	EC-dsb-R1	5′- GCT GTT GTA TTA CAG CCA TGC -3′	*gp19*	414 bp
	EC-gp19-F1	5′- ATT AGT GTT GTG GTT ATG CAA −3′	*gp36*	1069 bp
	EC-gp19-R1	5′- TAC GCT TGC TGA ATA TCA TGA −3′		
	EC36-F1	5′- AGA TTC TAT GGG ACA TAA TTT GT −3′		
	EC36-R1	5′- ACA CAG TAA CAT ATT GCA ATA AG −3′		
2nd round	EC-dsb-F3	5′- CGC GGC AGT AAT AAT GCT AAG G − 3′	*dsb*	911 bp
	EC-dsb-R2	5′- CCA GAA GGA TGA TGT TGC TGA G − 3′	*gp36*	969 bp
	EC36-F2	5′- GTA TGT TTC TTT TAT ATC ATG GC -3′		
	EC36-R5	5′- GGT TAT ATT TCA GTT ATC AGA AG −3′		

2.3. DNA sequencing and analysis

A single amplified fragment was purified using a PCR purification kit (Invitrogen) following the manufacturer's protocol. Direct sequencing was performed using an ABI PRISM® 3730 capillary sequencer (Applied Biosystems, Waltham, MA, USA) and BigDye™ Terminator cycle sequencing kit (Applied Biosystems) with the same primers. All sequences were manually edited to resolve ambiguities after comparing both the sense and antisense sequences. Sequence identification and multiple alignment analysis were performed using the BLAST program (http://blast.ncbi.nlm.nih.gov) of the National Center for Biotechnology Information (NCBI) and multiple sequence comparison by log-expectation (MUSCLE), respectively.

2.4. Phylogenetic analysis

After the sequences had ambiguities resolved and were aligned with MUSCLE, phylogenetic trees were constructed for *gp19* and *gp36* sequence analysis using the maximum likelihood method based on the Kimura 2-parameter model by MEGA7 software (Kumar et al., 2016). Reliability of phylogenetic relationships was assessed by the bootstrapping method (1000 bootstrap replications).

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