RESEARCH NOTE

IDENTIFICATION OF *EHRLICHIA* SPP IN CANINES IN THAILAND

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Abstract. Canine ehrlichiosis is an endemic parasitic disease widely found in Thailand. The causative microorganism is tick-borne *Ehrlichia* spp, an obligate intracellular rickettsia residing in leukocytes. *Ehrlichia* spp in morulae-positive canine blood samples were identified using polymerase chain reaction amplification and direct sequencing of *Ehrlichia* spp. 16S rDNA 396 bp fragment and 36 of 59 were positive for *E. canis*. *E. chaffeensis* and *E. ewingii* were not detected. Sequencing alignment and phylogenetic analysis showed that 16S rDNA sequences of *E. canis* strains are 99.1-100% identical among *E. canis* strains from different countries worldwide. Further studies are required in order to determine new target sequence for genotyping of *E. canis* strains in the dog population in Thailand.

Keywords: *Ehrlichia canis, Ehrlichia ewingii, E. chaffeensis,* 16s rDNA, phylogenetic tree, dogs, Thailand

INTRODUCTION

Ehrlichia spp are obligate intracellular gram-negative bacteria belonging to the order Rickettsiales. Monocyte, granulocyte and platelet are the target sites of these bacteria. Transmission in both animals and humans are associated with hard ticks. In Thailand, *Ehrlichia* spp that is commonly reported includes *E. canis, E. platys, E. equi* and *E. chaffeensis* (Suksawat *et al*, 2001; Parola *et al*, 2003; Pinyoowong *et al*, 2008). *E. canis* and *E. chafeensis* are causative agents of both human and canine monocytic ehrlichiosis. In Thailand, *E. canis* is considered an endemic parasitic arganism, which can be widely found in dogs and infection of this rickettsia causes various clinical symptoms varying from mild to fatal (Walker *et al*, 1970). *E. chaffeensis* is mainly the causative agent in humans, and dogs were suspected to be

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Table 1
Sequence of primers employed for PCR amplification.

Target	Primer	Sequence (5′-3′)
E. canis 16S rDNA	HE3	TATAGGTACCGTCATTATCTTCCCTAT
	ECAN5	CAATTATTTATAGCCTCTGGCTATAGGA
E. ewingii 16S rDNA	HE3	TATAGGTACCGTCATTATCTTCCCTAT
	EE52	CGAACAATTCCTAAATAGTCTCTGAC
E. ewingii 16S rDNA	HE3	TATAGGTACCGTCATTATCTTCCCTAT
	HE1	CAATTGCTTATAACCTTTTGGTTATAAAT

the reservoir host of this agent. *E.ewingii* causes granulocytic ehrlichiosis in dogs and humans (Buller *et al*, 1999; Paddock *et al*, 2001).

Diagnosis of this pathogen can be conducted by detecting morulae in leukocytes but this method is difficult to perform and time consuming, so alternative diagnosis methods have been developed, such as serology and molecular detection assays (Harrus and Waner, 2011). Positive antibody of *E. chaffeensis* and *E.canis* occur in 74% and 71% of Thai dogs, respectively (Suksawat *et al*, 2001). Polymerase chain reaction (PCR) and sequencing have revealed that *E. canis* and *A. platys* are present among stray dogs in Bangkok, Thailand but *E. chaffeensis* and *E. ewingii* have not been identified.

The purposes of this study were to identify and characterize *Ehrlichia* spp in dogs using 16S rDNA sequence. Phylogenetic tree was also constructed in order to compare Thai strains with those from other countries.

MATERIALS AND METHODS

Specimens

Blood samples were taken from dogs at the Laboratory Unit, Veterinary Teaching Hospital, Mahidol University, Thailand following routine venipuncture for hematology examination and were examined for blood parasites by thin blood smears under light microscope. Fifty-nine positive blood samples were selected based on the presence of morulae.

Amplification and sequence analysis of *E. canis*, *E. chaffeensis* and *E. ewingii* specific gene fragment

DNA was extracted from 200 l of blood and eluted in 100 l of distilled water using QIAamp® DNA blood Mini Kit (QIAGEN, Hilden, Germany) and the samples were stored at -20ºC until further processing. DNA from each blood sample was amplified in three individual PCR with primers specific for a portion of the 16S rDNA gene as previously described (Murphy *et al*, 1998). The primer sequences specific for *E. canis*, *E. chaffeensis* and *E. ewingii* 16S rDNA are listed in Table 1. Amplification was performed in a total volume of 25 l containing 2 l of template DNA, 2 l of 2.5 mM dNTP, 2 l of 25 mM $MgCl_2$, 5 pmol of each primer (Bio basic, Tai Chung City, Taiwan), 2.5 l of 10x PCR buffer, 15.125 l of water and 2.5 U *Taq* polymerase (i-Tag® DNA polymerase, Intron Biotechnology, Seoul, Korea). Thermocycling consisted of 30 cycles of 94ºC for 45 seconds, 59ºC for 30 seconds for *E. canis* and *E. ewingii* or 55ºC

for 30 seconds for *E. chaffeensis* and at 72ºC for 45 seconds. PCR amplicons (396 bp) were eletrophoresed in 2.0% agarose gel, stained with GelRed™ (Biotium, Hayward, CA) and visualized under UV light (Gene genius, Cambridge, UK).

PCR amplicons were purified using a PCR clean-up gel extraction kit Nucleospin® Extract II (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's instruction. DNA was sequenced in both directions using primers employed in PCR (Bio Basic, Tai Chung City, Taiwan. Sequence data were analyzed using BioEdit software version 7.0.8. The sequences were matched with public *Ehrlichia* spp sequences from a BLAST search of the National Center for Biotechnology Information (NCBI) database and multiple sequence alignments were performed using ClustalW2 from EMBL-EBI. Phylogenetic trees were generated using UPGMA method analysis with MEGA software Version 5.0 (Tamura *et al*, 2011).

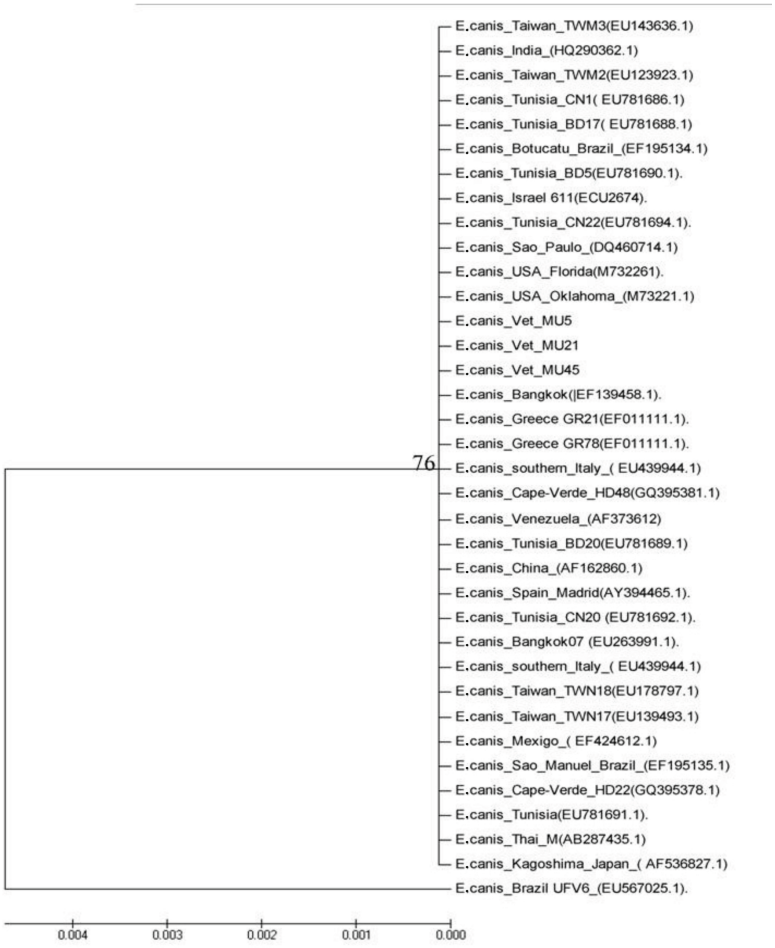

Fig 1–Phylogenetic tree of *Ehrlichia canis*. Sequences of 16S rDNA from different geographic regions were compared using UPGMA method with distance matrix calculation by Kimura-two parameters by MEGA software (version 5.0).

RESULTS

From direct sequencing of PCR amplicons of a 396 bp fragment of 16S rDNA, 36/59 (61%) of suspected morulae positive samples were shown to be *E. canis*, which were named *E. canis*_Vet_Mu. *E. chaffeensis* and *E. ewingii* were not detected. For alignment comparisons a length of 333 bp was used. Thirty-five *E. canis*_Vet_Mu sequences were identical, except *E. canis*_Vet_Mu5 that has an insertion (A) at position 332.

A phylogenetic tree was generated using *E. canis* 16S rDNA sequences and *E. canis*_Vet_Mu5, 21 and 45 were compared with other sequences of *E.canis* strains from different countries. The resultant

phylogenetic tree revealed that almost all *E. canis* strains were grouped in a single clade, with *E. canis* strain from Brazil (EU567025) classified as an outgroup (Fig 1).

DISCUSSION

Ehrlichia spp are considered the agents of emerging tick-borne disease in humans and animals, especially dogs and livestocks. In this study, although the buffy coat smear examination indicated 59 samples were morulae-positive in monocytes. PCR amplification and direct sequencing of 396 bp fragment of 16S rDNA revealed 36 samples were *E. canis*. Buffy coat smears are difficult to distinguish morulae from platelets, lymphocytic azurophilic granules and phagocytosed nuclear materials (Woody and Hoskins, 1991; Mylonakis *et al*, 2004; Harrus and Waner, 2010).

Serological evidence of *E. chaffeensis* has been reported in humans and dogs (Suksawat *et al*, 2001) in Thailand, but in this report both *E. chaffeensis* and *E. ewingii* showed negative results by PCR assay. The vector for *E. canis* is brown dog tick, *Rhipicephalus sanguineus* (Groves *et al*, 1975), which is widespread in domestic animals in Thailand, while the tick vector, *Amblyomma* spp, of both *E. chaffeensis* and *E. ewingii* (Anziani *et al*, 1990; Ewing *et al*, 1995) is not abundantly found in domestic animals.

Alignment of 333 bp 16S rDNA sequences of *E. canis* strains revealed 99.1 - 100% identity with those from Asia (China, India, Israel, Japan, Taiwan and Thailand), Africa (Cape Verde and Tunisia), Europe (Greece, Italy and Spain), North America (Mexico, Florida and Oklahoma), South America (Brazil and Venezuela). The phylogenetic tree and sequence alignments showed low diversity in *E.canis* strains in concordance with previous reports (Parola *et al*, 2003; Unver *et al*, 2003; Aguirre *et al*, 2004; Pinyoowong *et al*, 2008; Romero *et al*, 2011). However, it would be of interest to examine longer 16S rDNA sequences of *E. canis* strains for detecting genetic diversity of this bacterium in Thai dogs.

In summary, our study showed all dogs studied were infected with *E. canis* and the genetic diversity of this bacterium is closely similar with *E. canis* from other locations worldwide. Further studies are required in order to determine new target sequence for genotyping of *E.canis* strains in Thai dogs.

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