ETIOLOGICAL AGENTS OF TORTOISE TICK IN THAILAND

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Abstract. Several species of bacteria and protozoa can cause infection or infestation of ticks collected from *Indotestudo* tortoises. This study surveyed etiological agents found in tortoise ticks in Thailand, which possibly could cause harm to humans, and to study the evolutionary relationships of microorganisms detected. Twenty-six *Amblyomma geoemydae* ticks were collected from three *Indotestudo elongata*: one from Satun and two from Uthai Thani provinces. Ticks were morphologically and molecularly identified. Based on PCR techniques, *Ehrlichia* and *Francisella* bacteria and *Hemolivia* protozoa were identified in *A. geoemydae*. Phylogenetic analysis indicated *Hemolivia* 18S rDNA sequences belonged to a distinct clade from genus *Hepatozoon* but related to *H. mauritanica* and *H. mariae*, and analysis of *Ehrlichia* 16S rDNA sequences revealed they formed a unique clade of *E. ruminantium* while *Francisella* 16S rDNA sequences belonged to the same group. These data will be of use in further investigations of the roles of these etiological agents in human and animal hosts.

Keywords: *Amblyomma geoemydae*, *Ehrlichia*, *Francisella*, *Hemolivia*, *Indotestudo elongata*, tortoise tick, Thailand

INTRODUCTION

Tick-borne microorganisms can cause diseases in human and animals. Several species of protozoa and bacteria have been identified in ticks collected from *Indotestudo* tortoises. Takano *et al* (2010) isolated *Borrelia* spp from *Hyalomma* ticks found on *Testudo horsfieldii* and *T. graeca* tortoises (imported into Japan). Kalmár *et al* (2015) reported *Hyalomma aegyptium* ticks collected from *T. graeca* tortoises are able to transmit *Borrelia turcica* trans-stadially, suggesting a vectorial capacity. Crimean-Congo haemorrhagic fever virus (CCHFV) was isolated from *H. aegyptium* found on *T. graeca* in Turkey (Široký *et al*, 2014) and an AP92-like CCHFV from *H. aegyptium* in Algeria (Kautman *et al*, 2016). *Anaplasma phagocytophilum* and *Ehrlichia*

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canis bacteria were isolated from *H. aegyptium* ticks on *T. graeca* in Romania (Paștiu *et al*, 2012).

Ehrlichia infection in humans in Thailand was first reported by Heppner *et al* (1997) and *Ehrlichia*-like inclusions have also been detected in peripheral blood of infected cats in Thailand (Jittapalapong and Jansawan, 1993). Other microorganisms have also been isolated from ticks in the country. *Francisella*-like bacterial endosymbionts (FLEs) were isolated from snake ticks (Sumrandee *et al,* 2014a).

Microorganisms found on tortoise ticks can possibly be etiological agents in humans. Hence, this study surveyed in *Indotestudo* tortoises in Thailand the presence of bacteria (*Ehrlichia*, *Francisella*, *Anaplasma*, and *Rickettsia*) and protozoa (*Hemolivia* and *Babesia*) and investigated their evolutionary relationships.

MATERIALS AND METHODS

Tick collection and identification

Three *Indotestudo elongata* tortoises from two provinces of Thailand were examined for tick infestation. Ticks were directly removed from the tortoises and kept in 70% ethanol at 4°C before being sent to the Faculty of Science, Mahidol University, Bangkok. The ticks were identified morphologically using taxonomic key of Kohls (1957) and molecularly as described below.

PCR-based identification of tortoise ticks and their bacterial and protozoal infections

Tick samples were washed three times in a sequence of 70% ethanol, 2% sodium hypochlorite and sterile distilled water. DNA was extracted using QIAamp DNA Extraction Kit for Tissue (Qiagen, Hilden, Germany). Tick identities were confirmed by amplification and direct sequencing of 16S mitochondrial (mt)DNA. *Hemolivia* and *Babesia* protozoa and *Ehrlichia, Francisella, Anaplasma* and *Rickettsia* bacteria were similarly identified by PCR amplification and direct sequencing of respective 16S or 18S rDNA amplicons. Primers and reaction conditions are described in Table 1. Amplicons were sequenced by the Ramathibodi Research Department, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok. Sequences were deposited at GenBank and accession numbers are indicated in the text and in Fig 1.

Phylogenetic analysis

Nucleotide sequences were aligned using MEGA6 ClustalW multiple sequence alignment algorithm (Tamura *et al*, 2011) and neighbor-joining (NJ) method was employed to generate phylogenetic relationships using PAUP4.0 program (Swofford, 2002).

RESULTS

Bacteria and protozoa in tortoise ticks

During June 2016, 26 ticks (25 males and 1 female) collected from three *Indotestudo elongata* tortoises (one infected with 6 ticks) from Satun Province, southern Thailand and two (one with 6 and the other with 14 ticks) from the edge of the forest in Uthai Thani Province, western Thailand were identified as *Amblyomma geoemydae* from morphology and 16S mt DNA gene sequences (GenBank accession no. MG971297). Tick-infecting protozoa and bacteria identified from 18S and 16S rDNA sequences, respectively were *Hemolivia* sp (in 13 ticks, 4 from Satun tortoise), *Ehrlichia* sp (3 ticks; all from Uthai Thani tortoises) and *Francisella* sp (5 ticks; 2 from from Uthai Thani tortoises). Results were negative for *Babesia* protozoa and *Anaplasma* and *Rickettsia* bacteria. Double

Table 1
Primers used for PCR reactions in the study.

Primer	Target gene	Sequence (5′–3′)	Amplicon size (bp)	Reference
16s+1 16s-1	Tick 16S mitochondrial DNA	CTGCTCAATGATTTTTTAAATTG CTGTGG CCGGTCTGAACTCAGA TCAAGT	460	Black and Piesman (1994)
ge9f ge2r	*Anaplasma* spp 16S rDNA	AACGGATTATTCTTTATAGCTTGCT GGCAGTATTAAAAGCAGCTCCAGG	546	Sun *et al* (2008)
F11 F5	*Francisella* spp 16S rDNA	TACCAGTTGGAAACGACTGT CCTTTTTGAGTTTCGCTCC	1,142	Forsman *et al* (1994)
HepF300 HepR900	*Hepatozoon* 18S rDNA	GCTAATACATGAGCAAAATCTCAA CGGAATTAACCAGACAAAT	600	Vilcins *et al* (2009)
EHR16SD EHR16SR	*Ehrlichia* spp 16S rDNA	TAGCACTCATCGTTTACAGC GGTACCYACAGAAGAAGTCC	345	Parola *et al* (2000)
BAB GF2 Ba721R	*Babesia* spp 18S rDNA	GTCTTGTAATTGGAATGATGG CCCCAGAACCCAAAGACTT TGATTTCTCTCAAG	559	Anderson *et al* (1992)
RR17R RR17F	*Rickettsia* spp 17 kDa antigen	CATTGTTCGTCAGGTTGGCG GCTCTTGCAACTTCTATGTT	434	Williams *et al* (1992)

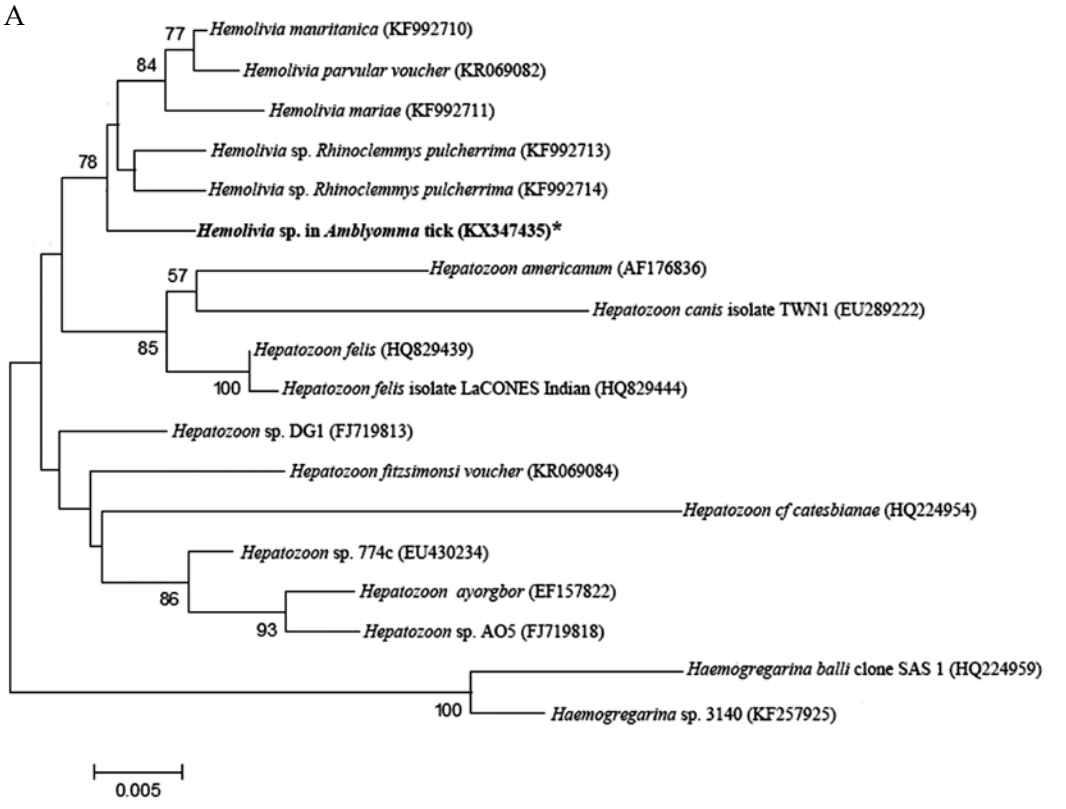
infections of *Hemolivia* sp and *Ehrlichia* sp were detected in three ticks, all from Uthai Thani tortoises; and double infections of *Hemolivia* sp and *Francisella* sp in three ticks, one from Satun tortoise and two from Uthai Thani tortoises. However, *Ehrlichia* and *Francisella* bacteria were not found to co-exist in any of the ticks examined.

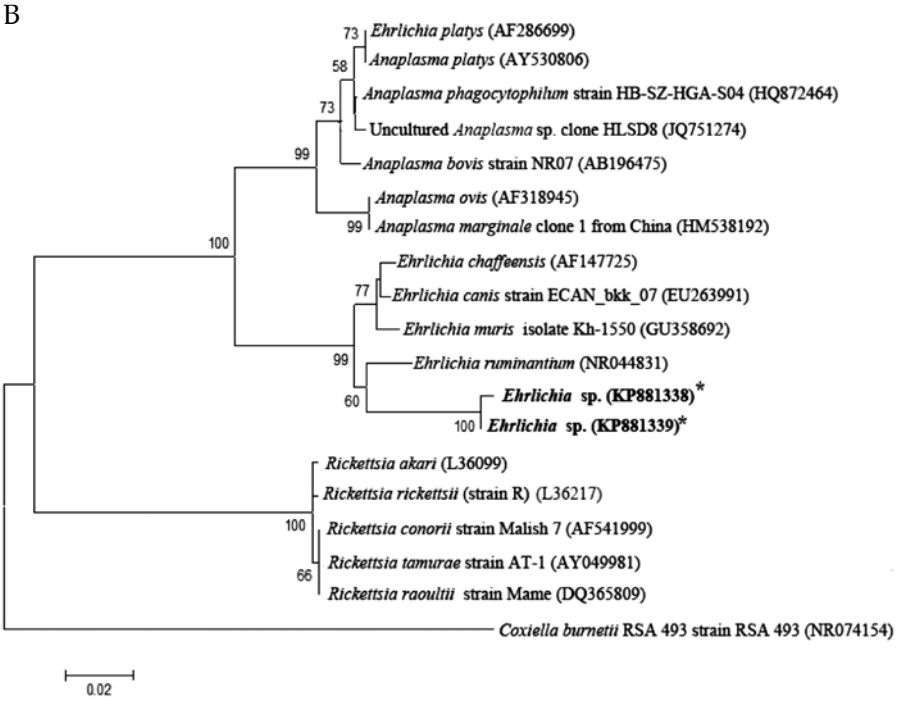
Phylogenetic analysis

Hemolivia sp 18S rDNA consensus fragment (631 bp, GenBank accession no. KX347435) showed highest sequence identity (99%) with *Hemolivia* sp ex *Rhinoclemmys pulcherrima* isolate 5054 and formed a distinct clade of *Hepatozoon* genus and related to other *Hemolivia* species previously reported (*H. mariae* and *H. mauritanica*) (Fig 1A). There were two *Ehrlichia* sp 16S rDNA fragments sequences (254 bp, GenBank accession nos. KP881338 and KP881339) and these showed highest sequence identity (97%) with *Ehrlichia* sp TC251-2 representing a unique clade of *E. ruminantium* (Fig 1B). *Francisella* sp 16S rDNA consensus fragment (993 bp, GenBank accession no. KP881337) showed highest sequence identity (100%) to *Francisella* endosymbiont of *A. geoemydae* from Thailand and was grouped with FLEs from various tick genera with a close relationship to FLE of *A. geoemydae* infesting compressed tortoises in Thailand but in a different group from (pathogenic) *F. tularensis* (Fig 1C).

DISCUSSION

This survey of etiological agents in


A
77
Hemolivia mauritanica (KF992710)
84
Hemolivia parvular voucher (KR069082)
Hemolivia mariae (KF992711)
Hemolivia sp. Rhinoclemmys pulcherrima (KF992713)
78
Hemolivia sp. Rhinoclemmys pulcherrima (KF992714)
Hemolivia sp. in Amblyomma tick (KX347435)*
57
Hepatozoon americanum (AF176836)
Hepatozoon canis isolate TWN1 (EU289222)
85
Hepatozoon felis (HQ829439)
100
Hepatozoon felis isolate LaCONES Indian (HQ829444)
Hepatozoon sp. DG1 (FJ719813)
Hepatozoon fitzsimonsi voucher (KR069084)
Hepatozoon cf catesbianae (HQ224954)
Hepatozoon sp. 774c (EU430234)
86
Hepatozoon ayorgbor (EF157822)
93
Hepatozoon sp. AO5 (FJ719818)
Haemogregarina balli clone SAS 1 (HQ224959)
100
Haemogregarina sp. 3140 (KF257925)
0.005


B
73
Ehrlichia platys (AF286699)
58
Anaplasma platys (AY530806)
Anaplasma phagocytophilum strain HB-SZ-HGA-S04 (HQ872464)
73
Uncultured Anaplasma sp. clone HLSD8 (JQ751274)
99
Anaplasma bovis strain NR07 (AB196475)
Anaplasma ovis (AF318945)
99
Anaplasma marginale clone 1 from China (HM538192)
100
Ehrlichia chaffeensis (AF147725)
77
Ehrlichia canis strain ECAN_bkk_07 (EU263991)
Ehrlichia muris isolate Kh-1550 (GU358692)
99
Ehrlichia ruminantium (NR044831)
Ehrlichia sp. (KP881338)*
60
100
Ehrlichia sp. (KP881339)*
Rickettsia akari (L36099)
Rickettsia rickettsii (strain R) (L36217)
100
Rickettsia conorii strain Malish 7 (AF541999)
Rickettsia tamurae strain AT-1 (AY049981)
66
Rickettsia raoultii strain Mame (DQ365809)
Coxiella burnetii RSA 493 strain RSA 493 (NR074154)
0.02

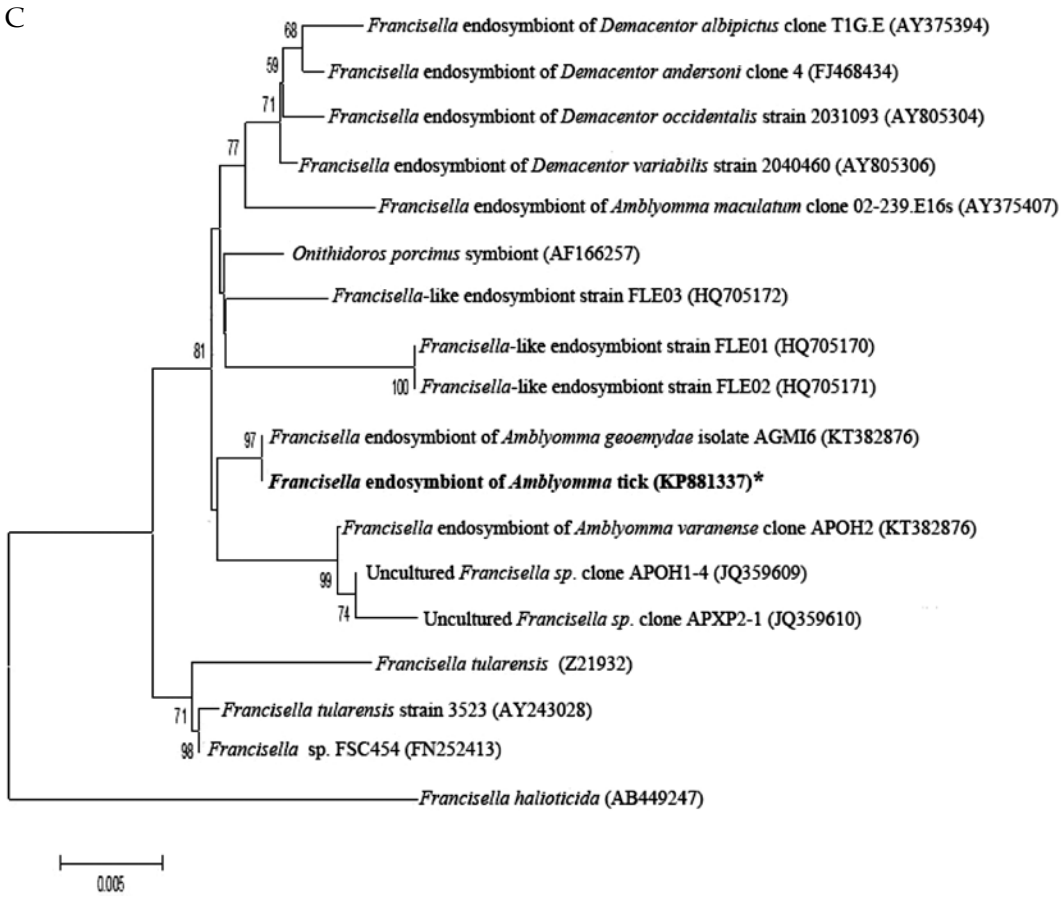


Fig 1-Phylogenetic relationships of (A) *Hemolivia* sp 18S rDNA, (B) *Ehrlichia* sp 16S rDNA and (C) *Francisella* sp 16S rDNA sequences (bold and asterisk) obtained from *Amblyomma geoemydae* tick infesting *Indotestudo elongata* tortoise in Thailand. Neighbor-joining method was used to generate the phylogenetic trees. A. *Haemogregarina* spp were chosen as outgroups. B. *Coxiella burnetii* was selected as outgroup. C. *F. halioticida* was selected as outgroup. Bootstrap tests of 1,000 pseudoreplicates are shown at branch nodes. Scale bar denotes percent nucleotide substitutions per site . GenBank accession number is shown in parenthesis.

I. elongata tortoise ticks reveals the presence of *Ehrlichia* and *Francisella* bacteria and *Hemolivia* (protozoa). For the first double infection by either *Ehrlichia* or *Francisella* bacteria and *Hemolivia* protozoa were observed in such *A. geoemydae* ticks collected in Thailand; however, double infection by both bacteria species was not found. Closely related microorganisms are more likely to interfere with each other in arthropod vectors than are distant relatives (Azad and Beard, 1998; de la Fuente *et al*, 2003). Thus, endosymbionts could possibly be exploited to render ticks incapable of transmitting closely related pathogens (Macaluso *et al*, 2002). Pastiu *et al* (2012) reported *Anaplasma phagocytophilum* and *Ehrlichia canis* in *H. aegyptium* ticks from

T. graeca in Romania, but have no evidence of the presence of *Francisella* spp.

Phylogenetic analysis of 16S rDNA sequences from *Ehrlichia* sp in *A. geoemydae* infesting *I. elongata* show they form a unique clade with *E. ruminantium* and distinct from *E. canis*, as has been previously reported in Thailand (Pinyoowong *et al*, 2008). In addition, FLE in *A. geoemydae* from our work was in a different group from pathogenic *Francisella* sp but closely related to FLEs of *A. geoemydae* and *A. varanense* obtained from snakes (Sumrandee *et al*, 2014b).

Positive results for *Hemolivia* detection have never been reported before in Thailand. From this study, *A. geoemydae* could potentially play an important role in *Ehrlichia*, *Francisella* and *Hemolivia* spp epidemiology. As mentioned above, we did not detect any co-infection of *Ehrlichia* and *Francisella* spp in the same *A. geoemydae* tick. Whether these two bacteria are capable of co-infection requires further investigations. The roles of co-infections of these bacteria and protozoa in tortoise ticks may play a significant role in the epidemiology of diseases brought about by these tick-borne microorganisms in the Southeast Asian region.

In conclusion, this survey extends current knowledge in the field of ticks and tick-borne bacteria and protozoa with their tortoise host. The roles of these bacteria and protozoa in ticks and humans warrant further investigations.

ACKNOWLEDGEMENTS

This research project was supported by Mahidol University, Thailand Higher Education Research Promotion Program and BDC PERDO (grant BDC-PG2-160008 and BDC-PG3-161006), Thailand.

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