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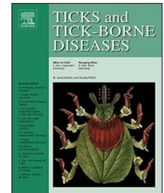

Original article

Natural infection of questing ixodid ticks with protozoa and bacteria in Chonburi Province, Thailand

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ABSTRACT

Ixodid ticks are important vectors of tick-borne disease agents affecting humans and animals, with wildlife often serving as important reservoirs. This study examined protozoal and bacterial infection in questing ticks in forest habitats in Chonburi Province, Thailand in 2015, using PCR and DNA sequencing techniques. A total of 12,184 ticks were morphologically identified to species and a subset of ticks were confirmed by PCR, targeting the tick mitochondrial 16S rRNA gene. Tick species collected included *Haemaphysalis lagrangei* (92.8%), *H. wellingtoni* (0.1%), and *Rhipicephalus microplus* (7.0%). In total, 419 tick pools [ELM(1] [ST2] were examined by PCR amplification of a fragment of the 18S rRNA gene of *Babesia* and *Theileria* species, and the 16S rRNA gene of bacteria in the family Anaplasmataceae. Results revealed a tick infection rate for the tick pools of 57.0% (239/419) including four pathogens and one bacterial symbiont. The highest infection rate in *H. lagrangei*, *H. wellingtoni*, and *R. microplus* pools was recorded for *Anaplasma* spp. at 55.6% (233/419) including three *Anaplasma* species genotype groups *Anaplasma* spp. closely related to *A. bovis*, *A. platys*, and unidentified *Anaplasma* spp. *Theileria* spp. showed a lower infection rate in *H. lagrangei* at 4.3% (18/419) with three *Theileria* species genotypes closely related to *T. cervi*, *T. capreoli*, and unidentified *Theileria* spp. Only 0.2% (1/419) of *H. lagrangei* pools contained *Babesia* spp., *Ehrlichia* spp., or *Wolbachia* spp. [ELM(3] [ST4] These findings provided information on tick species in wildlife habitats and detected protozoa and bacteria in the ticks. The results suggest that these tick species are possible vectors for transmitting tick-borne disease agents in Thailand wildlife habitats.

1. Introduction

Hard ticks (Acari: Ixodidae) are important vectors for viral, bacterial, and protozoal disease agents affecting humans and animals (Brites-Neto et al., 2015; de la Fuente et al., 2008). Tick-borne diseases are distributed worldwide, including in Thailand (Ahantarig et al., 2008). Tick-borne protozoal and bacterial hemoparasites of veterinary importance, including protozoa of the genera *Babesia* and *Theileria*, and bacteria of the genera *Ehrlichia* and *Anaplasma*, can cause severe anemia and death in infected animals (Atif, 2016; McQuiston et al., 2003). Some diseases have considerable medical importance such as zoonotic babesiosis and anaplasmosis, and wild animals are considered important reservoirs of the causative agents (Mans et al., 2015; McQuiston et al., 2003; Penzhorn, 2006; Yabsley and Shock, 2013).

Babesia can infect a wide variety of animals, and occasionally humans. Babesial infection in wildlife varies from subclinical to clinical symptoms such as fever, hemolytic anemia, and death (Schnittger et al., 2012). *Theileria* are economically important in domestic ruminants due to anemia and loss of production, whereas wild ruminants are usually asymptomatic and serve as a source of infection (Bishop et al., 2004). *Theileria cervi* and *Theileria capreoli* have been found in a wide variety of cervids and might be a reservoir for infection in domestic animals with various clinical signs from benign to moderately pathogenic (Li et al., 2014; Liu et al., 2016). Transstadial survival of *Theileria* in ticks was previously recorded (Mans et al., 2015).

The family Anaplasmataceae comprises tick-borne bacterial parasites that include *Ehrlichia*, *Anaplasma*, *Wolbachia*, *Neorickettsia,* and *Candidatus* Neoehrlichia (Kocan et al., 2010). *Anaplasma* species are

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obligate intra-erythrocytic bacterial parasites that can infect animals and humans. *Anaplasma marginale*, *A. centrale*, *A. ovis*, and *A. bovis* infect both domestic and wild ruminants, while cervids are usually reservoir hosts (Rymaszewska and Grenda, 2008). *Ehrlichia* species are intracellular bacterial parasites that predominantly infect leukocytes of animals and humans (Rar and Golovljova, 2011). A wide variety of wildlife can be infected with pathogenic or zoonotic *Ehrlichia* with no clinical signs and symptoms and served as source of zoonotic infection (Kruse et al., 2004). *Wolbachia* are intracellular bacterial endosymbionts, commonly found in arthropods and nematodes. Although *Wolbachia* are non-pathogenic species for vertebrate hosts, they live in various arthropods and ticks as reproductive parasites.

Ticks can spread disease agents between wild animal reservoirs and other hosts; therefore, detection of pathogens in tick vectors are important to demonstrate potential for disease in a given area. This study examined pathogen infection in questing ticks in wildlife habitats in Thailand using PCR and DNA sequencing techniques.

2. Materials and methods

2.1. Tick collection and species identification

Ixodid ticks were sampled every two months during the year of 2015 from wildlife habitats in Chonburi Province, in Eastern Thailand. The study site was dry evergreen forest with a variety of wildlife including an abundant population of deer. Questing ticks were collected from three sampling locations by vegetation dragging combined with eye searching for ticks on vegetation by randomly walking along animal trails for a minimum of one hour per location. All collected ticks were stored in 70% ethanol. This study was conducted in compliance with the National Parks, Wildlife and Plant Conservation Department and Chulalongkorn University Animal Care and Use Committee[ELM(5] [ST6], Thailand (Animal Use Protocol No. 1631030). All collected ticks were counted and identified to genus and/or species, sex, and life stage under a stereo microscope using morphological identification keys (Hoogstraal et al., 1973; Tanskul and Inlao, 1989; Tanskul et al., 1983; Yamaguti et al., 1971). Due to limitations of taxonomic data for immature ticks, eight individual representative species with similar morphologies and life stages were confirmed by polymerase chain reaction.

2.2. Tick sample size for pathogen detection

Because of high numbers, the collected larvae were split into pools of 1–50 larvae according to species, location, collection time, and collector. Quota sampling was used to gather representative data from each stage of tick, location, and sampling time to detect the pathogens. For each location and sampling time, ten each of males, females, nymphs, and larval pools were the quota selected as representative for tick sample pathogen detection. If the numbers of collected ticks did not reach this quota, then as many as possible were selected.

2.3. Polymerase chain reaction (PCR) and DNA sequencing

Each individual tick sample was washed in 70% ethanol, rinsed three times with sterile distilled water, air dried, and then stored at −20 °C. For DNA extraction, the tick sample was manually homogenized and genomic DNA was extracted from each sample using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany[ELM(7] [ST8]) according to the manufacturer's procedure.

To confirm tick species, an individual representative of each species and each life stage was tested by PCR using 16S + 1 and 16S-1 primers (Table 1). *Rhipicephalus sanguineus* sensu lato obtained from rearing in our facility and deionized distilled water were included in the PCR reaction as positive and negative control, respectively. To detect protozoa and bacteria, PCR was performed in 419 tick pools to detect *Babesia* spp. and *Theileria* spp. with KB−16 and KB−17 primers and to detect members of family Anaplasmataceae with EHR16SD and EHR16SR primers (Table 1). *Babesia bovis*, *B. bigemina*, *Theileria* spp., and *Anaplasma marginale* provided by the Parasitology Section, National Institute of Animal Health were used as positive controls with deionized distilled water included as negative control.

PCR was performed for both species confirmation and pathogen detection in a final volume of 25 μl containing 1X High Fidelity PCR buffer, 2.0 mM $MgSO_4$, 0.2 mM of each dNTP, 0.2 μM of each primer, 1U of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), and 2 μl of template DNA. Thermal cycling conditions were 94 °C for 30 s followed by 45 cycles of 94 °C for 15 s; 55 °C for 30 s; 68 °C for 30 s, and a final extension at 68 °C for 7 min. After agarose gel electrophoresis, DNA bands at the expected product size were cut out of the gel and purified using GenepHlow™ Gel/PCR Cleanup Kit (Geneaid, New Taipei City, Taiwan[ELM(9] [ST10]) according to the manufacturer's procedure. The DNA purified product was submitted to a commercial service for DNA sequencing (First Base Laboratories, Kuala Lumpur, Malaysia).

2.4. Molecular analysis

The obtained nucleotide sequencing results were aligned and trimmed using the ClustalW multiple alignment of BioEdit (Thompson et al., 1994), and compared with available DNA sequences in the GenBank database to define genus and/or species of ticks and pathogens using the Basic Local Alignment Search Tool (Altschul et al., 1990). For phylogenetic analysis, the optimal model of nucleotide substitution was determined by Find Best DNA/Protein Model implemented in Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar et al., 2016). The model with the lowest Bayesian Information Criterion (BIC) score was selected as best fitted model to construct the phylogenetic tree of each dataset. The phylogenetic trees were generated using the maximum likelihood method based on the Kimura two-parameter model for each of protozoal and bacterial DNA sequences and based on the General Time Reversible model for tick species DNA sequences by using MEGA 7. A discrete Gamma distribution was used to model evolutionary rate difference among sites (5 categories). The robustness of the phylogenetic tree was estimated by 1000 bootstrap replicates. The DNA sequences retrieved were submitted to NCBI as accession numbers KY766213−KY766244 and MF374638 for protozoal and bacterial DNA sequences and MG788690−MG788692 for tick species DNA sequences.

3. Results

3.1. Tick species

Due to the incapability to identify immature stage ticks to species by morphology alone, eight individual representative ticks were identified to species using a DNA-based method. Of eight NCBI BLAST acquired mitochondrial 16S rRNA gene sequences, four DNA sequences showed 99% identity with *Haemaphysalis lagrangei* (male, female, nymph, larva), two DNA sequences showed 99% identity with *Haemaphysalis wellingtoni* (male, female) and one DNA sequence showed 100% identity with *Rhipicephalus microplus* (2 larvae). The mitochondrial 16S rRNA gene sequences of *H. lagrangei* (MG788690), *H. wellingtoni* (MG788691) and *R. microplus* (MG788692) were used to construct a phylogenetic tree to compare with other ixodid tick species available in the GenBank database (Fig. 1).

A total of 12,184 ticks were collected belonging to two genera and three species: *Haemaphysalis lagrangei* (92.8%; 192 males, 179 females, 107 nymphs, and 10,831 larvae), *Haemaphysalis wellingtoni* (0.1%; 8 males and 8 females), and *Rhipicephalus microplus* (7.1%; 859 larvae). From all collected ticks in this study, larvae (96.0%; 11,690/12,184) were most abundant followed by males (1.6%; 200/12,184), females

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