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Original article

Detection of *Coxiella*-like endosymbiont in *Haemaphysalis* tick in Thailand

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ABSTRACT

In this study, we focused on the molecular detection of *Coxiella*-like bacteria using a PCR technique to identify *Coxiella* 16S rRNA sequences in *Haemaphysalis* tick samples (105 adults, 8 nymph pools and 19 larval pools). Seven *Haemaphysalis* species obtained from 5 locations in Thailand were evaluated in this work. *Coxiella* endosymbionts could be detected in samples representing all 3 growth stages examined. The results also revealed that only 4 of 7 tick species were positive for *Coxiella*-like endosymbiont: *Haemaphysalis hystricis*, *Haemaphysalis lagrangei*, *Haemaphysalis obesa*, and *Haemaphysalis shimoga*. *Haemaphysalis shimoga* demonstrated the highest percentage of *Coxiella*-like positive samples (58.33% with $n = 24$), while *Haemaphysalis hystricis* had the lowest percentage; only 1 female tick was positive for *Coxiella*-like bacteria ($n = 6$). Interestingly, the results indicated that female *Haemaphysalis* ticks tended to harbour *Coxiella* symbionts more frequently than male ticks (59.32% of females and 21.27% of males of all species studied). Phylogenetic analyses based on 16S rRNA sequences illustrated that *Coxiella*-like spp. from the same tick species always grouped in same clade, regardless of the location from which they were isolated. Moreover, a phylogenetic tree also showed that *Coxiella*-like endosymbionts from other genera (for example, the tick genus *Rhipicephalus*) formed a separate group compared to *Coxiella*-like symbionts in the genus *Haemaphysalis*. This suggests that a high amount of DNA sequence variation is present in *Coxiella*-like bacteria harboured by ticks.

Introduction

There have been many reports that both soft and hard ticks harbour *Coxiella* bacteria. For example, the soft ticks *Ornithodoros* (Almeida et al., 2012) and *Carios* (Reeves et al., 2006) were found to harbour this bacterium. Hard ticks, such as *Amblyomma* (Jasinskas et al., 2007), *Rhipicephalus* (Bernasconi et al., 2002), *Dermacentor* (Psaroulaki et al., 2006), *Ixodes* (Spitalska and Kocianova, 2003), *Hyalomma* (Spyridaki et al., 2002) and *Haemaphysalis*, have also been found to be infected by *Coxiella* endosymbionts (Machado-Ferreira et al., 2011). This indicates that *Coxiella*-like bacteria colonise a great variety of hosts for survival and reproduction. *Haemaphysalis* ticks are part of a large group of Ixodid, or hard ticks, comprised of many species that can parasitise mammals and humans (Tanskul and Inlao, 1989). Estrada-Pena and Jongejan (1999) found that these ticks are also carriers that can transmit many types of infectious bacteria and viruses. However, most of them have rarely been described in the context of disease transmission to humans (Estrada-Pena and Jongejan, 1999). Previously, many studies reported the presence of *Coxiella*-like bacteria in *Haemaphysalis* ticks. For example, for *Haemaphysalis longicornis* from Korea, 2 out of 100 ticks were found to be positive for *Coxiella* bacteria, and these bacteria demonstrated high nucleotide sequence similarity (99.5% and 100%) when compared to the *com-1* gene of *Coxiella burnetii* Derrick reference strains (Lee et al., 2004). *Haemaphysalis coccinna* from far-east Russia was found to be positive for *Coxiella*-like bacteria based on a technique using specific primers for 16S rRNA gene sequences. In a study by Mediannikov et al. (2003), *Coxiella* bacteria belonging to novel *Coxiella*-like microorganisms with close homology to *C. burnetii* based on 16S rRNA gene sequences were discovered (Mediannikov et al., 2003). Previously, *Coxiella* are gram-negative obligate intracellular

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bacteria that are classified into the gamma subgroup of proteobacteria (Roux et al., 1997). Later on, the study by Omsland et al. (2009) reported that *C. burnetti* can be cultured on axenic (host cell-free) media. The sequence identity of the 16S rRNA gene among the *Coxiella*-like bacteria varies from species to species. When the 16S rRNA nucleotide sequences are compared with each other using *C. burnetii* as a reference genome, a maximal and a minimal nucleotide identity of 98% and 91%, respectively, is observed (Zhong, 2012). There are many animals that can be infected with this type of bacteria, such as fish, mammals, reptiles, birds, and particularly arthropods such as ticks. The only well-known species of *Coxiella* is *C. burnetii*, the Q-fever agent that causes disease in humans (Skerman et al., 1980). Generally, Q-fever patients received the bacterium following exposure to contaminated air or by consuming contaminated products (Madariaga et al., 2003). Direct transmission of this disease-causing agent from ticks is rare.

However, ticks can be important carriers of *Coxiella spp.* for the infection of livestock, which can ultimately lead to infections in humans (de Bruin et al., 2012). As mentioned, *Coxiella* bacteria can infect many types of animals. Ticks are among the most studied organisms in the context of these bacteria because *Coxiella* appear to live with ticks mutually as bacterial symbionts (Zhong et al., 2007). Ticks treated with antibiotics that affect *Coxiella* bacteria were shown to lose their fitness based on observations of delayed oviposition, decreased numbers of hatched ticks, decreased numbers of larvae per tick, and the decreased size of ticks treated with antibiotics (Zhong et al., 2007). So far, a number of studies have detected *Coxiella spp.* in many genera of ticks, demonstrating varying prevalences in different regions of the world, and have found that these bacteria can be *C. burnetii* or novel *Coxiella spp.* based on the analysis and comparison of DNA sequences with the NCBI database to evaluate sequence identity.

In Thailand, one study reported on *Coxiella*-like symbionts in *Haemaphysalis* ticks using a molecular technique, PCR which suggested that *Haemaphysalis shimoga* ticks harbour a novel *Coxiella*-like symbiont (Ahantarig et al., 2011). This was the first report of the existence of *Coxiella*-like bacteria in *Haemaphysalis* ticks in Thailand. The study described here focused on the detection of *Coxiella*-like *spp.* in various *Haemaphysalis* spp. in Thailand isolated from various locations. The infection rate of *Coxiella*-like bacteria in *Haemaphysalis* ticks and the genetic diversity of these bacteria were also evaluated using a molecular technique (PCR).

Materials and methods

Tick collection and identification

Haemaphysalis ticks were collected from five locations in Thailand in 2010 (Location 1: Khao Yai National Park, 2: Kaeng Krachan National Park, 3: Khao Soi Dao Wildlife Sanctuary, 4: Taksin Maharaja National Park, and 5: Pang Sida National Park). The samples were collected and identified at the species level using Keys to the Adult Ticks of *Haemaphysalis*, 1844, in Thailand with Notes on Changes in Taxonomy (Acari: Ixodoidae: Ixodidae) (Tanskul and Inlao, 1989). For the developmental stages, ticks were identified to the genus *Haemaphysalis* with short palpi, no eyes and morphologically similar to the adult of the same genus but smaller size. Larva has six legs while nymph has eight legs. Nymph has no genital opening compared to the adult *Haemaphysalis*.

DNA extraction and Polymerase Chain Reaction (PCR)

Ticks were cleaned by sequential surface washes with 70% ethanol for 5 min, 10% sodium hypochlorite for 5 min, and sterile distilled water three times for 5 min each. DNA extraction was performed (individually for the adults) using the QIAamp DNA Extraction Kit for Tissue (Qiagen, Hilden, Germany) following the manufacturer's protocol. Primers used in PCR reaction for COX-16S rRNA were: 5′ GGGGAAGAAAGTCTCAAGGGTAA 3′ (forward primer) and 5′ TGCATCGAATTAAACCACATGCT 3′ (reverse primer) (Almeida et al., 2012).

The PCR reaction mixtures were comprised of 2 μl extracted tick DNA and 18 μl reaction mixture containing 11.3 μl of deionised distilled water grade 3, 2 μl of $MgCl_2$, 2 μl of 10× buffer, 0.5 μl dNTP solutions, 1 μl of forward primer, 1 μl of reverse primer and 0.2 μl of *Taq* polymerase. PCR reactions were performed with a Bio-Rad thermocycler (Bio-Rad, California, USA). DNA was first denatured at 95 °C for 1 min. In next step, DNA was annealed with the primers at 58 °C for 1 min, and extension of DNA took place at 72 °C for 2 min. For the larval and 8 nymph pools, each pool contained either 20 larvae or 20 nymphs.

DNA purification and sequencing

Representative positive samples were selected based on the criteria of location, species, sex and stage for DNA purification. PCR reactions were first performed to amplify interest DNA region (16S rRNA from *Coxiella*-like *spp.*) from selected representatives. The PCR reaction mixtures were purified using a Qiagen kit. Purified DNA samples in solution were sent to the Ramathibodi Research Department (Ramathibodi Hospital, Bangkok, Thailand) for DNA sequencing. The results were then analysed and compared with other DNA sequences from GenBank in the National Center for Biotechnology Information database (NCBI: http://www.ncbi.nml.nih.gov/BLAST/).

Analysis of results and construction of phylogenetic trees

To perform phylogenetic analyses, 302-nucleotide DNA sequences (after primer trimming) were used. Representative DNA sequences were edited and aligned with MEGA 5 using ClustalW Multiple Alignment. DNA sequences from this study and selected reference strains from GenBank were used to construct a phylogenetic tree via the neighbour-joining method by applying 1000 pseudoreplicates and determining the confidence value for each branch of the phylogenetic tree with bootstrap analysis from the MEGA 5 program.

Results

Tick samples

One hundred and thirty two *Haemaphysalis* tick samples (105 adults, 8 nymph pools and 19 larval pools) from 5 locations in Thailand were collected from vegetation (under leaf blades) in parks. The tick samples were identified to the species level. There were 7 species of *Haemaphysalis* ticks identified in this study: *Haemaphysalis lagrangei* (HL), *Haemaphysalis shimoga* (HS), *Haemaphysalis obesa* (HO), *Haemaphysalis hystricis* (HH), *Haemaphysalis asiaticus* (HAs), *Haemaphysalis aborensis* (HAb), and *Haemaphysalis papuana* (HP). The sexes, life stages, numbers and species of the *Haemaphysalis* ticks are summarised in Table 1.

Molecular detection of Coxiella bacteria

A Cox-16S rRNA primer pair was used to detect *Coxiella*-like bacteria in *Haemaphysalis* ticks. Overall, 56 out of 132 tick samples (42.42%) were positive for *Coxiella*-like bacteria as shown in Table 1. For each *Haemaphysalis* species, 39.47% (15/38) of *H. lagrangei* adults, 58.33% (14/24) of *H. shimoga* adults, 46.88% (15/32) of *H.*