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Original article

Phylogenetic studies of bacteria (*Rickettsia*, *Coxiella*, and *Anaplasma*) in *Amblyomma* and *Dermacentor* ticks in Thailand and their co-infection

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ABSTRACT

In this study, we attempted to detect *Rickettsia, Coxiella* and *Anaplasma* bacteria in one hundred and fourteen-*Dermacentor* and thirty three-*Amblyomma* unfed adult ticks that were collected from under leaves along animal trails at different places across Thailand. PCR amplification was used to identify bacterial infection with general conserved sequences of bacteria. The results revealed single infection in *Amblyomma testudinarium* ticks with *Rickettsia* (24%) and *Coxiella* (6%). *Anaplasma* bacteria were often detected in *Dermacentor auratus* ticks (32%). *Coxiella* spp. were detected in *Dermacentor atrosignatus* (6%) and *D. auratus* ticks (3%) in this study. Moreover, we found co-infection by *Coxiella* and *Rickettsia* bacteria (39%) in *Am. testudinarium*. In contrast, *D. atrosignatus* ticks were co-infected with *Coxiella* and *Anaplasma* bacteria (3%) and *Dermacentor compactus* ticks were co-infected with *Rickettsia* and *Anaplasma* spp. (25%). Interestingly, *Am. testudinarium* ticks (12%) were found for the first time to exhibit triple infection by these three bacteria. Phylogenetic studies showed the rickettsiae from ticks causing both single and multiple infections had sequence similarity with spotted fever group rickettsial strains, including *Rickettsia massilliae*, *R. raoultii* and *R. tamurae*. In addition, the phylogenetic analysis of the *16S* rRNA gene of *Coxiella* bacteria showed that they were closely grouped with *Coxiella* endosymbionts in both *Dermacentor* and *Amblyomma*. Moreover, the *Anaplasma* identified in a *D. auratus* tick was grouped in the same clade with the pathogenic bacterium *Anaplasma phagocytophilum*. Bacterial co-infections in *Dermacentor* and *Amblyomma* ticks may cause co-transmission of some tick-borne microorganisms (pathogen and endosymbiont, whether enhance or reduce) in humans and animals and they could affect medical and veterinary health.

1. Introduction

Ticks are haematophagous ectoparasites that infest a wide variety of vertebrate hosts, including amphibians, reptiles, birds, mammals and humans. Ticks can transmit a large number of microorganisms, such as viruses, bacteria and protozoa, during blood feeding. Spotted fever group (SFG) rickettsioses are major emerging human diseases and often isolated from ticks, animals and patients. Over 25 species of *Rickettsia* are reported to cause travel-associated rickettsial infections (Eremeeva and Dasch, 2015). For example, *R. rickettsii* is the agent of Rocky Mountain spotted fever, *R. africae* is the agent of African tick-bite fever and *R. conorii* is the agent of Mediterranean spotted fever (Parola and Raoult, 2001). Furthermore, SFG rickettsiae have been determined to be human pathogens in Thailand, such as *R. felis, R. honei, R. heilongjiangensis, R. japonica, R. conorii, R. helvetica* and *R. tamurae* (Gaywee et al., 2007; Kollars et al., 2001; Lewin and Musher, 2004; Phongmany et al., 2006; Sophie et al., 2014). Additionally, rickettsiae with unknown pathogenicity have been identified from ticks in Thailand, e.g., detections of DNA of *Rickettsia* spp. RDa420 and RDa440 in *Dermacentor* ticks collected from animals near Thai-Myanmar border and *Rickettsia* sp. HOT2 in *Haemaphysalis* ticks collected from vegetation (Parola et al., 2003).

Q fever is a zoonotic disease caused by *Coxiella burnetii*; infection primarily occurs through inhalation of airborne particles contaminated with bacteria. The clinical features of Q fever involve flu-like symptoms to pneumonia and granulomatous hepatitis in severe cases (Duron et al., 2015). However, ticks have never been reported to transmit *C. burnetii* to humans and animals directly in the field, although their vectorial competence was demonstrated in the laboratory condition. Furthermore, ticks may be involved and play an important role in the transmission of *C. burnetii* to vertebrate hosts (Duron et al., 2015). Over 40 ticks have been reported to carry *C. burnetii* in Argentina, Egypt and Ethiopia (Loftis et al., 2006; Pacheco et al., 2014). *Coxiella burnetii* infections have been found in humans and other animals in Thailand (Porntip et al., 2014; Pachirat et al., 2012; Suputtamongkol et al., 2003; Yingst et al., 2013). For example, *Coxiella*-like endosymbionts were

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detected in *Haemaphysalis shimoga, H. lagrangei, Rhipicephalus sanguineus* s.l. and *Rh. microplus* ticks. Although humans and animals have been infected with *C. burnetii*, the existence of this causative agent in ticks has been inadequately reported in Thailand (Ahantarig et al., 2008; Arthan et al., 2015).

Anaplasma phagocytophilum has been reported to cause canine anaplasmosis disease in dogs (Ahantarig et al., 2008; Suksawat et al., 2001). Additionally, *An. platys* and *An. bovis* which are the causative agents of canine cyclic thrombocytopenia and bovine ehrlichiosis have been detected in domestic dogs and cats, and in *H. lagrangei*, *H. obesa* and *H. shimoga* ticks collected from Sambar deer and vegetation (Foongladda et al., 2011; Malaisri et al., 2015; Sumrandee et al., 2016). *Anaplasma phagocytophilum* mainly replicates in mammalian white blood cells. The clinical characteristics of this infection are similar to those of the viral haemorrhagic fever and leptospirosis as well as severe fever with thrombocytopenia syndrome virus in China, easily leading to misdiagnosis by physicians (Li et al., 2011). Therefore, they need to be diagnosed by molecular techniques (Rikihisa, 2010; Wormser, 2016).

In addition, individual ticks can also be infected by multiple microorganisms, causing co-transmission to their host during blood feeding (Randolph et al., 1996; Sonenshine, 2004). The risks caused by tick-borne pathogens can strongly harm both animals and humans. In this study, we attempted to identify bacterial infection and co-infections in field-collected questing *Dermacentor* and *Amblyomma* ticks in Thailand.

2. Materials and methods

2.1. Tick collection and identification

During summer and rainy seasons (June-October) of the years 2008–2013, *Dermacentor* and *Amblyomma* ticks were randomly picked from under leaves along the tourist and animal trails at 9 different locations in Thailand (Fig. 1): 1) Nakhon Nayok, 2) Chanthaburi, 3) Phetchaburi, 4) Tak, 5) Kanchanaburi, 6) Sa Kaeo, 7) Chiang Mai, 8) Prachuap Khiri Khan and 9) Phang Nga. All tick samples were morphologically identified (Arthur, 1960; Wassef and Hoogstraal, 1983, 1984) before being preserved at −20 °C for further studies.

2.2. DNA extraction and PCR amplification

Ticks were surfaces sterilized with 70% ethanol and 10% sodium hypochlorite for 3–5 min followed by three times with sterile distilled water for 5 min each. Whole individual adult ticks were ground with pestles in individual Eppendorf tubes before DNA extraction using the QIAamp DNA tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplifications were performed using specific primer sequences for bacterial detection, including *Rickettsia* spp., *Anaplasma* spp. and *Coxiella* spp. (Table 1). The PCR mixtures were composed of 18 µl of reaction mixture containing 11.3 µl of PCR-grade sterile distilled water, 2 µl of 25 mM $MgCl_2$, 2 µl of 10X *Taq* buffer, 0.5 µl of dNTP mix, 1 µl of forward primer, 1 µl of reverse primer, 0.2 µl of *Taq* DNA polymerase and 2 µl of DNA template (tick genomic DNA). PCR amplifications were cycled by an MJ Mini™ Personal Thermocycler (BioRad, California, USA) for each bacterial primers with PCR cycling conditions were as follows: 95 °C for 3 min; 35 cycles of 95 °C for 20 s, 50 °C for 30 s, 60 °C for 2 min; and final extension at 72 °C for 7 min. The DNA fragments of the PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide before being visualized with a UV transilluminator. Positive control was the DNA samples tested positive for each bacterium and negative control was distilled water.

2.3. DNA purification and sequencing

Positive PCR of bacterial DNA detections were excised from the agarose gel before being purified using a Qiagen kit. The purified DNA samples were sent for DNA sequencing at the Ramathibodi Research Department (Ramathibodi Hospital, Bangkok, Thailand). The resulting DNA sequences were searched with the BLAST program to determine their relationships with other reported DNA sequences in GenBank.

2.4. Sequence analysis and phylogenetic tree construction

All DNA sequences were trimmed and edited before alignment with other referenced sequences from GenBank by the MEGA7 program (Kumar et al., 2016) using CLUSTALW multiple alignment. The aligned DNA sequences were used to construct phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the branches. Evolutionary analyses were conducted in MEGA7 based on 17-kDa antigen (*Rickettsia*), COX-*16S* rRNA (*Coxiella*) and EHR-16S rRNA genes (*Anaplasma*). All DNA sequences were submitted to GenBank (Table 2).

3. Results

3.1. Tick samples

One hundred and forty-seven questing ticks were collected from under leaves along animal trails at different places in Thailand. All adult ticks belonged to the genera *Amblyomma* and *Dermacentor*. There were 17 males and 16 females of *Am. testudinarium*. The remaining 114 ticks were *Dermacentor* of three species, i.e., *D. auratus* (35 males and 41 females), *D. atrosignatus* (14 males and 20 females) and *D. compactus* (2 males and 2 females) (Table 3).

3.2. Bacterial detection

The results revealed single infection in *Am. testudinarium* ticks with *Rickettsia* (24%) and *Coxiella* (6%) (Fig. 2A). *Anaplasma* bacteria was often detected in *D. auratus* tick (32%) (Fig. 2C). *Coxiella* spp. were detected in *D. atrosignatus* (6%) and *D. auratus* (3%) (Fig. 2B) ticks in this study. Moreover, we found co-infection of *Coxiella* and *Rickettsia* bacteria (39%) in *Am. testudinarium*. The percentages were calculated from single infection of each bacterium. In contrast, *D. atrosignatus* ticks were co-infected with *Coxiella* and *Anaplasma* bacteria (3%) whereas *D. compactus* ticks were co-infected with *Rickettsia* spp. and *Anaplasma* spp. (25%) (Fig. 2D). Interestingly, *Am. testudinarium* ticks were found for the first time to exhibit triple infection by these three bacteria (12%) (Fig. 2A and Table 3).

Multiple bacterial infections were detected in *Am. testudinarium*. Double infection occurred in *Am. testudinarium* at location no. 1 which were mainly co-infected with *Rickettsia* and *Coxiella* spp. (39%) (Fig. 2A). Moreover, *D. atrosignatus* ticks were co-infected with *Coxiella* spp. and *Anaplasma* spp. (3%) (Fig. 2B) and one *D. compactus* was co-infected with *Rickettsia* and *Anaplasma* spp. (25%) (Fig. 2D). Interestingly, triple infection by *Rickettsia*, *Coxiella* and *Anaplasma* spp. was found in *Am. testudinarium* (12%) (Fig. 2A). Infection rates of bacterial detection were calculated and shown in Fig. 2.

3.3. DNA sequencing and phylogenetic analysis

3.3.1. Rickettsia

Rickettsia spp. were obtained from positive samples that were amplified using the 17-kDa gene. Sequence analysis revealed that *Rickettsia* isolated from a single infected *D. atrosignatus* collected from location no. 4 (DASTK7) showed 99% identity to a partial DNA sequence from *R. honei* strain *marmionii* KB (AY737683) and also displayed 99% with partial DNA sequences of *R. heilongjiangensis* and *R. japonica*. From multiple DNA alignment results, all of 4 sequences were grouped in the same clade (Fig. 3). Another single *Rickettsia* infected *D. atrosignatus*