**Coxiella-like bacteria in fowl ticks from Thailand**

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**Abstract**

**Background:** Coxiella bacteria were identified from various tick species across the world. Q fever is a zoonotic disease caused by the bacteria *Coxiella burnetii* that most commonly infects a variety of mammals. Non-mammalian hosts, such as birds, have also been reported to be infected with the pathogenic form of "Candidatus Coxiella avium". This research increases the list of tick species that have been found with Coxiella-like bacteria in Thailand.

**Methods:** A total of 69 ticks were collected from 27 domestic fowl (*Gallus gallus domesticus*), 2 jungle fowl (*Gallus gallus*) and 3 Siamese firebacks (*Lophura diardi*) at 10 locations (provinces) in Thailand. Ticks were identified and PCR was used to amplify *Coxiella* bacteria 16S rRNA, groEL and rpoB genes from the extracted tick DNA. MEGA6 was used to construct phylogenetic trees via a Maximum Likelihood method.

**Results:** The phylogenetic analysis based on the 16S rRNA gene showed that the *Coxiella* sequences detected in this study grouped in the same clade with *Coxiella* sequences from the same tick genus (or species) reported previously. In contrast, rpoB gene of the *Coxiella* bacteria detected in this study did not cluster together with the same tick genus reported previously. Instead, they clustered by geographical distribution (Thai cluster and Malaysian cluster). In addition, phylogenetic analysis of the groEL gene (the chaperonin family) showed that all *Coxiella* bacteria found in this study were grouped in the same clade (three sister groups).

**Conclusions:** To our knowledge, we found for the first time rpoB genes of *Coxiella*-like bacteria in *Haemaphysalis wellingtoni* ticks forming two distinct clades by phylogenetic analysis. This may be indicative of a horizontal gene transfer event.

**Keywords:** Coxiella-like bacteria, Fowl ticks, Thailand

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**Background**

Ticks are ectoparasites of vertebrates transmitting pathogens like protozoa, viruses and bacteria which cause zoonotic diseases in domestic animals and humans. Both hard and soft tick species have been documented to harbour *Coxiella* bacteria. For example, the soft ticks *Ornithodoros capensis* (s.l.), *O. rostratus* and the hard ticks *Dermacentor atrosignatus* and *Amblyomma testudinarium* have been found to be infected with *Coxiella* bacteria [1–4].

Q fever (Query fever) is a zoonotic disease caused by *Coxiella burnetii* that most commonly infects a variety of mammals throughout the world. *Coxiella burnetii* affects the reproductive system in animals causing stillbirths and miscarriages. Infection of *C. burnetii* in humans results from inhalation of contaminated aerosols in nature or from direct contact with infected domestic animals products or formites [5, 6]. Individuals infected with *C. burnetii* were detected among rice farmers who raised cattle and chickens in northeastern Thailand. A seroepidemiological survey of *C. burnetii* in cattle and chickens in Thailand was carried out using an indirect fluorescent antibody test. Only one out of 113 serum samples from fowl was seropositive [7]. Moreover, non-mammalian hosts, such as birds, have also been reported to be infected with “Candidatus Coxiella avium” [8].

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Coxiella bacteria were identified from various species of Haemaphysalis and Rhipicephalus sanguineus (s.l.) ticks in Thailand [3, 9]. Nevertheless, in Thailand, infection of Coxiella bacteria in fowl ticks has rarely been investigated. Therefore, the aims of this work were to determine the presence of Coxiella bacteria in fowl ticks and to study their evolutionary relationships in phylogenetic analyses based on partial 16S, rpoB (RNA polymerase beta-subunit), and groEL (the chaperonin family) gene sequences.

Methods
Tick samples and identification
A total of 69 ticks were collected from 27 domestic fowl (Gallus gallus domesticus), 2 jungle fowl (Gallus gallus) and 3 Siamese firebacks (Lophura diardi) at 10 locations (provinces) in Thailand during 2014–2016: (i) Chaiyaphum; (ii) Chumphon; (iii) Krabi; (iv) Pattani; (v) Rayong; (vi) Satun; (vii) Songkhla; (viii) Surat Thani; (ix) Trang; and (x) Yala (Table 1). The ticks were removed by using forceps, stored in 70% alcohol and preserved at -20 °C awaiting further identification and molecular assays. Ticks were classified by developmental stage and sex and identified based on morphology using standard identification keys [10, 11]. Ticks positive for Coxiella bacteria were also molecularly identified using a primer set consisting of 16S + 1 and 16S - 1 to detect tick 16S mitochondrial DNA (16S mDNA) [12].

DNA extraction from tick samples and PCR
Ticks were washed with 70% ethanol and 10% sodium hypochlorite and rinsed three times with sterile distilled water. Then, ticks were immediately homogenized using the TissueLyser system (QiagenGmbH, Hilden, Germany). One 3-mm tungsten carbide bead (Qiagen GmbH, Germany) was added to each tube (collection microtubes; Qiagen GmbH, Germany) and ticks (individual for adult, individual for Amblyomma nymph and a pool of 5 for nymph of Haemaphysalis) were homogenized for 4 min at 30 Hz. After a short centrifugation step (5 s at 3220×g), the supernatants were collected in separate collection microtubes and DNA extracted using Qiagen’s DNeasy Blood and Tissues Kit (Qiagen GmbH, Germany) following the manufacturer’s instructions. Genes and primers used to amplify Coxiella DNA were used as in a previously reported protocol [13].

| Location no. / Province | No. of hosts | No. of ticks tested | Tick species | No. of ticks positive for Coxiella |
|-------------------------|--------------|---------------------|-------------|-----------------------------------|
|                         |              | M       | F     | N     |                                   |                          |
| 1. Chaiyaphum           | Siamese fireback (n = 3) | 0 | 0 | 3 | A. testudinarium | 1N |
|                         |               | 0 | 1 | 0 | H. wellingtoni | – |
|                         |               | 0 | 0 | 5 | H. obesa | 5N |
| 2. Chumphon             | Domestic fowl (n = 2) | 1 | 2 | 0 | H. wellingtoni | 1F |
| 3. Krabi                | Domestic fowl (n = 3) | 3 | 2 | 0 | H. wellingtoni | 1M, 1F |
|                         |               | 0 | 0 | 3 | H. bispinosa | 1N |
| 4. Rayong              | Domestic fowl (n = 1) | 0 | 1 | 0 | H. wellingtoni | 1F |
| 5. Satun               | Domestic fowl (n = 5) | 5 | 4 | 0 | H. wellingtoni | 1F |
|                         |               | 0 | 0 | 3 | H. wellingtoni | – |
| 6. Trang               | Domestic fowl (n = 2) | 0 | 2 | 0 | H. wellingtoni | 2F |
| 7. Pattani             | Domestic fowl (n = 2); | 0 | 2 | 0 | H. wellingtoni | – |
|                         | jungle fowl (n = 1) | 5 | 6 | 0 | R. microplus | – |
|                         |               | 0 | 0 | 3 | H. wellingtoni | – |
| 8. Songkhla            | Domestic fowl (n = 9) | 7 | 4 | 0 | H. wellingtoni | – |
| 9. Surat Thani         | Domestic fowl (n = 2) | 2 | 0 | 0 | H. wellingtoni | – |
|                         |               | 0 | 0 | 1 | H. wellingtoni | – |
| 10. Yala               | Domestic fowl (n = 1); | 0 | 1 | 0 | H. wellingtoni | – |
|                         | jungle fowl (n = 1) | 1 | 2 | 0 | R. microplus | – |
| Total                   | 32            | 24 | 27 | 18 | 14 |

Ticks were obtained from 10 locations in Thailand. The number of ticks tested and positive results for Coxielia bacteria are shown. Abbreviations: M, male; F, female; N, nymph; A., Amblyomma; H., Haemaphysalis; R., Rhipicephalus.
Purification and sequencing of PCR products

After PCR amplification and gel electrophoresis, DNA bands corresponding to positive amplification results were excised. Purified DNA samples (using Purification kit from Roche, Basel, Switzerland) were sent to the Ramathibodi Research Department (Ramathibodi Hospital, Bangkok, Thailand) for DNA sequencing. The results were analysed and compared with other DNA sequences from GenBank in the National Center for Biotechnology Information database (NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Phylogenetic analyses

DNA sequences were edited and aligned with MEGA6 using ClustalW multiple sequence alignment algorithm. DNA sequences from this study, along with selected reference strains from GenBank, were used to construct a phylogenetic tree via Maximum Likelihood (Kimura 2-parameter model) and determining the confidence value for each branch of the phylogenetic tree with bootstrap analysis by using 1000 pseudoreplicates of the original alignment.

Results and Discussion

A total of 69 ticks collected from domestic and jungle fowl and Siamese firebacks belong to 3 genera: *Haemaphysalis; Amblyomma;* and *Rhipicephalus* (Table 1). The 51 adult ticks included 14 *R. microplus*, 37 *H. wellingtoni* and the remaining 18 ticks were nymphs of *H. wellingtoni* (*n* = 7), *H. obesa* (*n* = 5), *H. bispinosa* (*n* = 3) and *A. testudinarius* (*n* = 3). The 16S mDNA sequences of ticks were submitted to the GenBank database under the accession numbers MG865746 (*H. wellingtoni*), MG865747 (*A. testudinarius*), MG865748 (*H. bispinosa*), MG865749 (*H. obesa*), MG865750 (*R. microplus*), MG865751 (*H. wellingtoni*), MG865752 (*A. testudinarius*), and MG865753 (*R. microplus*).

Fig. 1 Phylogenetic tree for Coxieilla-like bacteria 16S rRNA gene sequences constructed with the Maximum Likelihood method using MEGA6 software. Bootstrap analysis was performed with 1000 pseudoreplicates. *Rickettsia rickettsii* was used as the outgroup. Coxieilla-like bacteria isolates from this study are indicated in bold and with asterisks.
MG874025, MG910463 and MG874022 (H. obesa, H. bispinosa, and A. testudinarium, respectively). A total of 14 out of 69 ticks tested were positive for Coxiella bacteria, as defined by the amplification of 16S rRNA sequences. Positive results were found in H. wellingtoni from Chumphon, Krabi, Rayong, Satun and Trang, H. obesa from Chaiyaphum, H. bispinosa from Krabi and A. testudinarium from Chaiyaphum (Table 1).

Coxiella bacteria-positive samples were sequenced, and a phylogenetic tree was constructed based on their analysis (Fig. 1). Coxiella DNA sequences were submitted to the GenBank database, including H. wellingtoni from Trang (TRG32 and TRG33), H. obesa and A. testudinarium from Chaiyaphum (PK179-183 and PK190), H. wellingtoni from Rayong (RYG1), H. wellingtoni from Satun (STN77) and H. bispinosa from Krabi (KBI32) (see Fig. 1 for accession numbers).

The phylogenetic analysis, based on the 16S rRNA gene, showed that Coxiella bacteria from Haemaphysalis ticks of domestic fowl and Siamese firebacks were grouped with Coxiella bacteria of the same corresponding tick species previously reported (Fig. 1). In addition, the 16S rRNA sequence of A. testudinarium of our study was in the same group with those reported by Nooroong et al. [3] and Khoo et al. [14] (Fig. 1).

Phylogenetic analyses of rpoB and groEL genes of Coxiella-like bacteria were also performed. The results are shown in Figs. 2 and 3. Most of Coxiella rpoB sequences from this study were in the same group and exhibited 88–89% identity with Coxiella-like endosymbiont of Argas reflexus (isolate Areflex2, GenBank: KY677983) and Coxiella endosymbiont of ticks of the genus Ixodes (GenBank: KP985313, KP985318 and KP985320) (Fig. 2). However, rpoB gene sequences of Coxiella-like bacteria in H. wellingtoni from Trang (TRG33) clustered in a different clade and was closely related to Coxiella endosymbiont of Rhipicephalus sp. isolate (Tchien14; GenBank: KP985345; 96% identity) (Fig. 2). The rpoB gene sequences of Coxiella bacteria detected in this study did not cluster together with those previously reported in the same tick species by Khoo et al. [14] (H. bispinosa and Amblyomma spp.). Instead they seemed to be clustering by their geographical distribution forming a Thai cluster (the present study) and a Malaysian cluster (data by Khoo et al. [14]). Coxiella groEL gene sequences detected in ticks from domestic fowl and Siamese firebacks from this study were clustered in the same clade (three sister groups) and exhibited about 89% DNA sequence identity with “Candidatus Coxiella avium” from seabird ticks (GenBank: KJ459059) (Fig. 3).

Coxiella bacteria have been reported in several tick species, such as Rhipicephalus sanguineus (s.l.), Amblyomma americanum, Ixodes uriae and the soft tick O. rostratus [1, 2, 15, 16]. In addition, Coxiella-like bacteria were also detected in Haemaphysalis ticks, such as H. lagrangei, H. obesa, H. shimoga and H. hystricis [3]. In the present study, the rate of

![Fig. 2 Phylogenetic tree for Coxiella-like bacteria rpoB gene sequences constructed with the Maximum Likelihood method using MEGA6 software. Bootstrap analysis was performed with 1000 pseudoreplicates. Rickettsia raoultii was used as the outgroup. Coxiella-like bacteria isolates from this study are indicated in bold and with asterisks](image-url)
Coxiella-like bacteria in ticks collected from fowl was rather high because about 20% of ticks of the 4 species (H. wellingtoni, H. bispinosa, H. obesa and A. testudinarium) were positive for Coxiella-like bacteria. Thus, our results seem to agree with those of Arthan et al. [4] who demonstrated that the prevalence was not dependent on tick species.

Analyses of rpoB sequences revealed that most of Coxiella-like bacteria exhibited 88–89% identity with Coxiella-like endosymbiont from Argas reflexus (isolate Areflex2). However, rpoB gene of Coxiella bacteria in H. wellingtoni from Trang (TRG33) was clustered in the different group in the phylogenetic analysis and related to Coxiella-like bacteria of Rhipicephalus spp. isolate Tchien14 rpoB gene, partial cds (96% identity). This result may simply be indicative of a horizontal gene transfer event. Since only a small number of sequences is reported here, and only one with these characteristics is shown, it remains to be determined what is the real impact of this observation. The interesting point is that Coxiella rpoB sequences from different H. wellingtoni ticks belong to a different clade (even from the same tick species). The roles of these Coxiella-like bacteria in ticks and their fowl hosts are still unclear and needs further investigation.

Conclusions
To our knowledge, we found for the first time that Coxiella rpoB gene sequences from different H. wellingtoni ticks belong to two different clades and that rpoB sequence of the Coxiella bacteria detected in this study did not cluster together with those previously reported in the same tick species.

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Availability of data and materials
Data supporting the conclusions of this article are included within the article and its additional files. The DNA sequences of ticks (16S mDNA) were submitted to the GenBank database under the accession numbers MG865746 for H. wellingtoni adult, MG874025 for H. obesa nymph, MG910463 for H. bispinosa nymph and MG874022 for A. testudinarium nymph. Coxiella-like bacteria genes were submitted to GenBank as follows: Cox-16S rRNA (in H. bispinosa (KBI32: MG871184); in H. obesa (PK179-183: MG871183); in A. testudinarium (PK190: MG871182); in H. wellingtoni (RYG1: MG871190; TRG33: MG871405; TRG32: MG871191; STN77: MG871192). Coxiiella in H. bispinosa (KBI32: MG871184); in H. obesa (PK179-183: MG871183); in A. testudinarium (PK190: MG871182); in H. wellingtoni (RYG1: MG871190; TRG33: MG871405; TRG32: MG871191; STN77: MG871192). Coxiiella in H. bispinosa (KBI32: MG871405; TRG32: MG871191; STN77: MG871192). Cox-groEL: Coxiiella in H. bispinosa (KBI32: MG871405; TRG33: MG871405; TRG32: MG871191; STN77: MG871192). Cox-rpoB: Coxiiella in H. bispinosa (KBI32: MG893014; in H. obesa (PK179-183: MG893014); in A. testudinarium (PK190: MG893013); in H. wellingtoni (RYG1: MG921604); in H. bispinosa (TRG33: MG921603; TRG32: MG893017; STN77: MG893016).

Authors’ contributions
WT and AA planned and designed the study. WT, AA and SM carried out the majority of the laboratory and tick identification work. WK and PU performed phylogenetic analyses. WT and AA wrote the manuscript with advice from VB. All authors read and approved the final manuscript.
Ethics approval and consent to participate
The study was carried out according to the license number U1-05257-2559 from NRCT, Thailand.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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