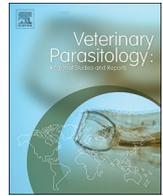




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Short Communication

Francisella spp. detected in *Dermacentor* ticks in Malaysian forest reserve areas

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ABSTRACT

Limited information is available on tropical ticks and tick-borne bacteria affecting the health of humans and animals in the Southeast Asia region. *Francisella tularensis* is a tick-borne bacterium which causes a potentially life-threatening disease known as tularemia. This study was conducted to determine the occurrence of *Francisella* spp. in questing ticks collected from Malaysian forest reserve areas. A total of 106 ticks (mainly *Dermacentor* and *Haemaphysalis* spp.) were examined for *Francisella* DNA using a Polymerase chain reaction (PCR) assay targeting the bacterial 16S rDNA. *Francisella* DNA was detected from 12 *Dermacentor* ticks. Sequence analysis of the amplified 16S rDNA sequences (1035 bp) show > 99% identity with that of *Francisella* endosymbiont reported in a tick from Thailand. A dendrogram constructed based on the bacterial 16S rDNA shows that the *Francisella* spp. were distantly related to the pathogenic strains of *F. tularensis*. Three *Francisella*-positive ticks were identified as *Dermacentor atrosignatus*, based on sequence analysis of the tick mitochondrial 16S rRNA gene. Further screening of cattle and sheep ticks (*Haemaphysalis bispinosa* and *Rhipicephalus microplus*) and animal samples (cattle, sheep, and goats) did not yield any positive findings. Our findings provide the first molecular data on the occurrence of a *Francisella* strain with unknown pathogenicity in *Dermacentor* questing ticks in Malaysia.

1. Introduction

Ticks carry a number of bacteria which may cause human and animal diseases. *Francisella* spp. are nonmotile, facultative intracellular Gram-negative coccobacilli which infect a wide variety of ticks and animals (Akimana and Kwaik, 2011). The genus consists of several pathogenic species, including *Francisella tularensis* which is highly infectious and causes a potentially life-threatening disease known as tularemia. *Francisella novicida*, *Francisella philomiragia* and *Francisella hispaniensis* are rare pathogens in patients who are severely immunocompromised (Huber et al., 2010). Tularemia is transmitted through tick bite and contact with infected animals, as well as via contaminated aerosols and freshwater (Akimana and Kwaik, 2011). *F. tularensis* infection was first reported in ticks of the species *Dermacentor andersoni* (Parker et al., 1924). *Francisella*-like endosymbionts (FLEs) closely related to *F. tularensis* have also been identified in ticks belonging to *Ornithodoros*, *Dermacentor*, *Amblyomma*, *Hyalomma*, *Rhipicephalus*, *Ixodes* and *Haemaphysalis* genera (Brevik et al., 2011; Ivanov et al., 2011; Kugeler et al., 2005; Liu et al., 2016; Noda et al., 1997; Rakthong et al., 2016; Scoles, 2004; Sumrandee et al., 2014, 2016).

Tick-borne diseases are a major public health concern and may

cause mortality and morbidity in livestock animals, leading to great economic losses. Among tick-borne pathogens that have been investigated in Malaysia (Kho et al., 2019; Koh et al., 2018), data is scarce about the prevalence of *Francisella* spp. Despite a report of *F. tularensis* subsp. *novicida* infection in a Thai patient receiving chemotherapy treatment (Leelaporn et al., 2008), cases of tularemia have not been reported in other regions of Southeast Asia. The identification of host and vector is essential for epidemiological investigation, prevention and control of tick-borne diseases in general. As part of a surveillance program for tickborne pathogens, this study was conducted to determine the occurrence of *Francisella* spp. in questing ticks collected from Malaysian forest reserve areas.

2. Materials and methods

2.1. Tick collection and identification

Ticks were collected from vegetation (herein referred as questing ticks) in two forest areas, i.e.: Krau Wildlife Reserve, Pahang (N 03°50' E 102°06') and Sungai Deka Elephant Sanctuary, Terengganu (N 05°28' E 102°44') from March to May 2013, with the permission from the

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Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN, Reference no: JPHL&TN(IP):90-1/2(23)]. Both collection sites are dipterocarp forests which support a diversity of fauna such as birds, rodents, amphibians, invertebrates, and a wide variety of tropical vegetations. The ticks were collected using tweezers, stored in cryovials and transported to laboratory in a cryoshipper. Each tick was examined under a stereomicroscope (Olympus SZX16, Japan) by referring to morphological taxonomic keys (Brahma et al., 2014; Geevarghese and Mishra, 2011; Walker et al., 2003).

DNA of 100 ticks obtained from cattle and sheep from several farms in Malaysia in a previous investigation (Kho et al., 2015) were also included for detection of *Francisella* DNA. These ticks were identified as *Rhipicephalus microplus* and *Haemaphysalis bispinosa* using the approach as described above.

2.2. DNA extraction and molecular identification of ticks

Briefly, each tick was first washed in 5% sodium hypochlorite and rinsed in 70% ethanol (v/v) prior to trituration using a sterile surgical blade. DNA extraction of the ticks was carried out using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's instruction. Molecular identification of ticks was carried out for randomly selected *Francisella*-positive ticks based on amplification and sequence analysis of the tick mitochondrial 16S rRNA gene (Black and Piesman, 1994). Primers 16S + 2 (5'- TTG GGC AAG AAG ACC CTA TGA A -3') and 16S-1 (5'- CCG GTC TGA ACT CAG ATC AAG T -3') (Black and Piesman, 1994) which amplified a portion of the gene (approximately 300 bp) were used. The thermal cycling profile for amplification of tick mitochondrial 16S rRNA gene consisted of an initial denaturation step at 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. Amplification was performed in a Veriti thermal cycler (Applied Biosystems, Foster City, CA) and the products were purified using GeneAll Expin™ Combo GP kit (GeneAll Biotechnology, South Korea) prior to sequencing on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, Foster City, CA), using both forward and reverse primers. The sequences obtained were subjected to BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search for homologous sequences in the GenBank database.

2.3. Molecular detection of *Francisella* spp.

For the detection of *Francisella* DNA, a primer set (F5: 5'- CCT TTT TGA GTT TCG CTC C -3' and F11: 5'- TAC CAG TTG GAA ACG ACT GT -3') targeting the 16S rRNA gene was used (Forsman et al., 1994). The expected size of the amplified DNA is 1104 bp. The PCR amplification conditions included initial denaturation at 95 °C for 10 min, followed by 30 cycles, each consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. All PCR assays were performed in a final volume of 25 µL containing 2 µL of DNA template, 1 U GoTaq Flexi DNA Polymerase (Promega, Madison, WI), 1 × Green GoTaq Flexi Buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 0.2 µM of forward and reverse primers, in a Veriti thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were purified for sequence determination using both forward and reverse primers. To determine the phylogenetic placement of *Francisella* spp. identified in this study, a dendrogram was constructed based on the bacterial 16S rDNA sequences using the neighbor-joining method of MEGA software (Tamura et al., 2013).

2.4. PCR screening of *Francisella* DNA from blood samples of livestock animals

Additionally, blood DNA samples of 184 cattle, 40 sheep and 40 goats previously collected from livestock farms across several states in Malaysia (Kho et al., 2015) were also subjected to PCR screening for

Francisella DNA.

3. Results

A total of 106 questing ticks consisting of 68 (40 *Dermacentor* spp., 26 *Haemaphysalis* spp. and two *Amblyomma* spp.) from Krau Wildlife Reserve, Pahang and 38 (26 *Dermacentor* spp., 9 *Haemaphysalis* spp. and three *Amblyomma* spp.) from Sungai Deka Elephant Sanctuary, Terengganu were examined in this study. The ticks were mainly adults with an equal proportion of male and female. *Francisella* DNA was amplified from 12 (11.3%) questing ticks, i.e., 11 (3 male and 8 female) ticks from Krau Wildlife Reserve and one female tick from Sungai Deka Elephant Sanctuary, Terengganu. All *Francisella*-positive ticks were identified morphologically as *Dermacentor* spp. Three (25%) *Francisella*-positive ticks randomly selected for sequence analysis of the tick mitochondrial 16S rRNA gene (approximately 230 bp) demonstrated the highest similarity (99–100%) to that of *Dermacentor atrosignatus* from wild boar in Thailand (KC170745). All tick and blood samples from livestock farms were tested negative for *Francisella* DNA.

The sequences for nine amplified *Francisella* DNA fragments were successfully determined (GenBank accession nos.: MH270616-24) in this study. Two sequence variants (with one nucleotide difference) exhibiting 97.2–97.3% similarity to that of *F. tularensis* type strain (Z21931) were identified. All sequences show the closest match (99.8–99.9%, 1035 bp or 1036 bp over 1037 bp) with a *Francisella* endosymbiont (isolate DASSD4) previously reported in *D. atrosignatus* questing tick collected from Thailand [KC170748; Sumrandee et al., 2016].

A dendrogram was constructed based on *Francisella* 16S rDNA sequences (Fig. 1). The phylogenetic analysis showed that the Malaysian strains were genetically closely related to a group of *Francisella* endosymbionts reported from various tick species in Thailand, which include *Dermacentor auratus* from wild boars [JQ764628-9; Sumrandee et al., 2016], *D. atrosignatus* (KC170748), *Haemaphysalis asiatica* (KC170752), *Haemaphysalis shimoga* (KC170755), *Rhipicephalus haemaphysaloides* (KC170751), *Haemaphysalis hystricis* (KC170753), *Haemaphysalis papuana* (KC170754) and *Dermacentor compactus* (KC170750) from vegetation. Together with *Francisella* endosymbiont from other geographical regions, i.e., Bulgaria (Ivanov et al., 2011), Japan (Noda et al., 1997), Namibia (Brevik et al., 2011), USA (Kugeler et al., 2005), China (Liu et al., 2016) and Thailand (Rakthong et al., 2016; Sumrandee et al., 2014, 2016), the *Francisella* strains investigated in this study are considered to be distantly related from *F. tularensis* type strain and other pathogenic strains of *Francisella* species (Fig. 1).

4. Discussion

Dermacentor and *Haemaphysalis* are ticks which are commonly encountered in Malaysia forest areas. Both tick genera have previously reported in flagging vegetation and wildlife animals such as sambar deer and wild pigs (Mariana et al., 2008). Four *Dermacentor* species, *D. auratus*, *D. compactus*, *D. atrosignatus* and *Dermacentor steini* have been previously reported in Malaysia (Nadchatram, 2008). However, there has not been any intensive study on the detection of potential human pathogens from *Dermacentor* ticks in Malaysia, except for rickettsiae and anaplasma (Kho et al., 2019; Koh et al., 2018). The negative findings of ticks and animal samples from livestock farms in this study suggest that *Francisella* spp. are so far restricted to *Dermacentor* questing ticks and have not spread to other ticks and livestock animals. However, in Thailand, a neighboring country located at northern of Malaysia, *Francisella* spp. have been reported in *Dermacentor* ticks infecting wild boars with an infection rate of 18% (2/11 ticks). The bacteria were also detected in 11 out of 24 (46%) adult ticks (*Amblyomma varanense* and *Amblyomma helvolum*) collected from four species of snake (Sumrandee et al., 2014) and a *Rhipicephalus sanguineus* tick collected from a chicken (*Gallus domesticus*) (Rakthong et al., 2016). Among the *Dermacentor*

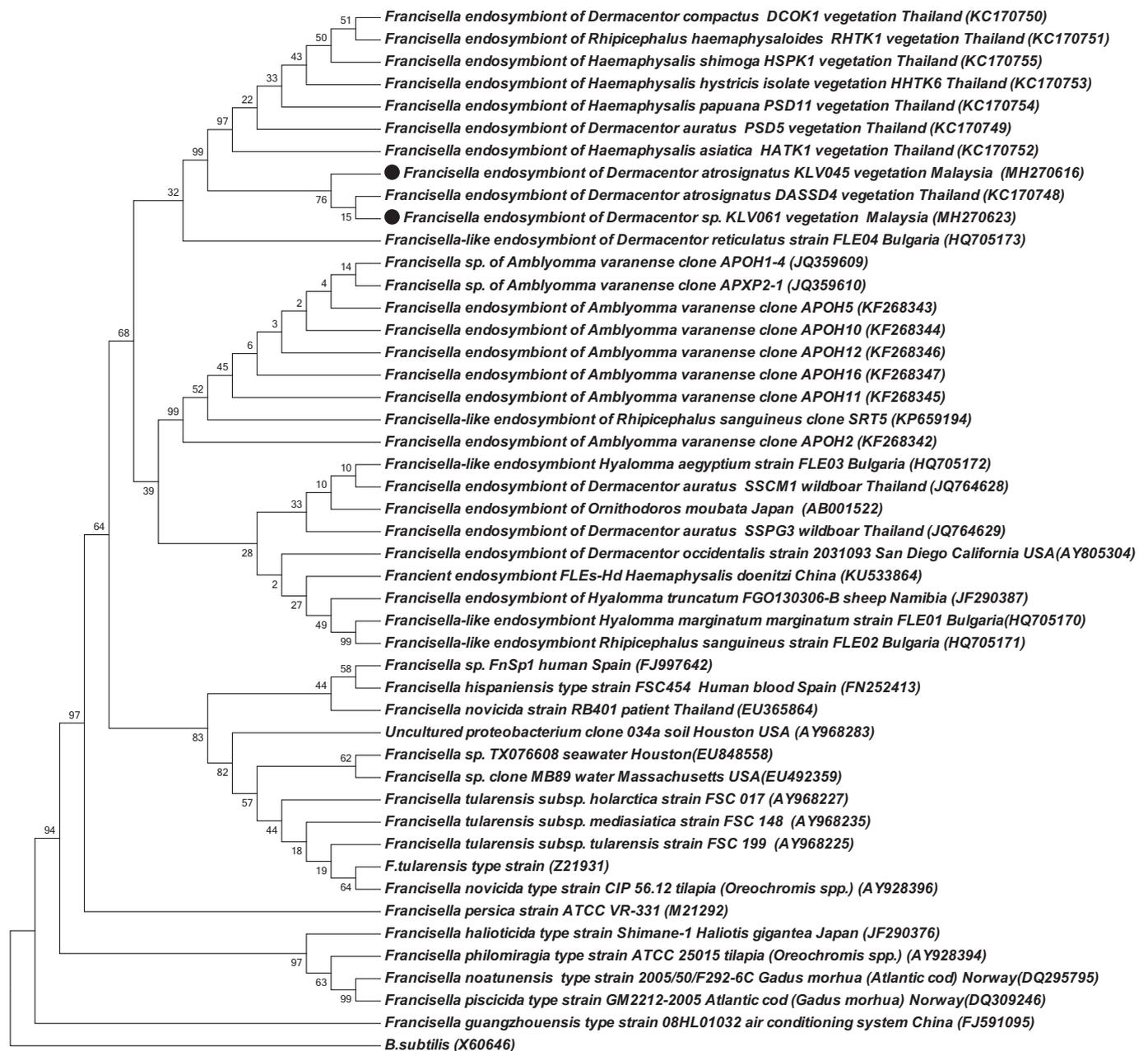


Fig. 1. Dendrogram depicting the phylogenetic relationship of Malaysian *Francisella* spp. (● KL045 and KL061) with *Francisella* endosymbionts from other geographical regions. *Bacillus subtilis* (X60646) was used as an outgroup. Numbers in brackets are GenBank accession numbers.

spp., *D. auratus* has been associated with multiple tick-borne infections including *Anaplasma*, *Rickettsia* and *Francisella* (Sumrandee et al., 2016). Comparatively, little data is available on *D. atrosignatus* tick.

The occurrence of *Francisella* spp. in questing ticks suggests that the *Dermacentor* ticks may have acquired the bacteria through animals via blood meals when the tick develop from larva and the nymph stages, or through transovarial transmission. The detection of *Francisella* spp. in different tick genera (*Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Amblyomma* spp.) indicates possible spread of the bacteria through ticks and potential animal hosts, such as wild boar, snake and chicken in Thailand (Rakthong et al., 2016; Sumrandee et al., 2014; Sumrandee et al., 2016). The phylogenetic tree analysis of the partial 16S rDNA gene sequences of Malaysian strains concurs with the finding of Sumrandee et al. (2016), whereby *Francisella*-like endosymbionts (FLEs) from different tick species (*Dermacentor*, *Amblyomma*, *Haemaphysalis*, *Rhipicephalus* and *Hyalomma*) were separated from pathogenic

Francisella species including *F. tularensis* type strain (Z21931), *F. hispaniensis* (FN252413) and *F. novicida* (EU365864).

Ticks that have been reported in the transmission of tularemia in the Northern hemisphere include *Amblyomma americanum*, *D. andersoni* and *D. variabilis* (Burgdorfer et al., 1973; Petersen et al., 2009). Besides arthropod transmission, contact with infected animals and ingestion of contaminated food or water has been associated with tularemia transmission in certain geographical regions (Hubalek et al., 1996; Petersen et al., 2009; Tarnvik et al., 2004) and occupational groups, such as laboratory workers, farmers, veterinarians, hunters, cooks and meat handlers (Nigrovic and Wingerter, 2008).

In conclusion, this study provides molecular data on the occurrence of *Francisella* strains with unknown pathogenicity in *Dermacentor* questing ticks in Malaysian forest reserve areas. As ticks are known to carry bacterial endosymbionts for survival, growth and immunity (Bonnet et al., 2017), further research on the host-parasite relationship

and pathogenic potentials of the *Francisella* sp. is essential.

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Declaration of interest

The authors declare that there is no conflict of interests.

Ethical statement

Tick sampling has been conducted with the permission from the Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN, Reference no: JPHL&TN(IP):90-1/2(23)] and the approval is acknowledged within the manuscript.

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