**Ehrlichia and Anaplasma Infections: Serological Evidence and Tick Surveillance in Peninsular Malaysia**

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**Abstract**

Little information is available on human anaplasmosis and ehrlichiosis in Southeast Asia despite increasing reports of the detection of *Anaplasma* spp. and *Ehrlichia* spp. in the ticks. We report herein the serological findings against the tick-borne pathogens in a group of animal farm workers (*n* = 87) and indigenous people (*n* = 102) in Peninsular Malaysia. IgG antibodies against *Ehrlichia chaffeensis* were detected from 29.9% and 34.3% of farm workers and indigenous people, respectively, using commercial indirect immunofluorescence assays. Comparatively, only 6.9% of the indigenous people but none of the animal farm workers were seropositive to *Anaplasma phagocytophilum*. A polymerase chain reaction (PCR) assay targeting the 16S rRNA gene of Anaplasmataceae was used to identify Anaplasmataceae in ticks collected from various locations adjacent to the areas where the serological survey was conducted. In this study, a total of 61.5% of ticks infesting farm animals, 37.5% of ticks infesting peri-domestic animals in rural villages, 27.3% of ticks collected from wildlife animals, and 29.1% of questing ticks collected from forest vegetation were positive for Anaplasmataceae DNA. Sequence analyses of 16S rRNA gene region (238 bp) provide the identification for *Anaplasma marginale*, *Anaplasma bovis*, *Anaplasma plathy*, *A. phagocytophilum*, and *Anaplasma* spp. closely related to *Candidatus Cryptoplasma californiense* in ticks. *E. chaffeensis* DNA was not detected from any ticks, instead, *Ehrlichia* sp. strain EBm52, *Ehrlichia mineiresiensis* and *Candidatus* Ehrlichia shimanensis are the only *Ehrlichia* sp. identified from cattle ticks in this study. Further investigation is required to ascertain the occurrence of zoonotic transmission of *Ehrlichia* and *Anaplasma* infections in Peninsular Malaysia.

**Key words:** *Anaplasma, Ehrlichia*, Peninsular Malaysia, ticks surveillance, indigenous people, farm workers

*Anaplasma* spp. and *Ehrlichia* spp. are obligate intracellular Gram-negative, tick-borne bacteria in the family Anaplasmataceae that are potential causes of zoonotic diseases. Of the six species in the genus *Anaplasma*, *Anaplasma phagocytophilum* is the etiological agent of human and animal granulocytic anaplasmosis. *Anaplasma platys* causes infectious canine cyclic thrombocytopenia. *Anaplasma marginale*, *Anaplasma centrale*, and *Anaplasma ovis* are the causative agents for bovine anaplasmosis, whereas *Anaplasma bovis* infects a wide range of mammal species (Dumler et al. 2001). Among *Ehrlichia* spp., *Ehrlichia chaffeensis* and *Ehrlichia ewingii* are the most significant species causing human infections. The clinical presentations of human anaplasmosis and ehrlichiosis are often nonspecific and mimic other acute febrile illnesses. Fever, headache, myalgia, malaise, leucopenia, thrombocytopenia, and elevated liver enzymes are the most common manifestations of infected patients while complications such as meningoencephalitis, acute respiratory distress syndrome, septic or toxic shock-like syndrome, acute renal failure, etc. have been occasionally reported (Dumler 2005, Bakken and Dumler 2015). New species or genetic variants of *Ehrlichia* spp. have recently been described in ticks (Kawahara et al. 2006; Cabezas-Cruz et al. 2012, 2015).

Serologic and molecular evidence of human anaplasmosis and ehrlichiosis have been reported mostly in North America. Little is known...
concerning the disease burden in Southeast Asia. Tick-borne diseases are often underdiagnosed in many developing countries, owing to the lack of awareness and the absence of appropriate diagnostic assays (Blacksell et al. 2015, Ghane Kisomi et al. 2016, Kho et al. 2017). Two seroprevalence studies in Indonesia and Thailand documented antibodies to *E. chaffeensis* in 14.6% and 44.0% of healthy individuals, respectively (Heppner et al. 1997, Richards et al. 2003). A recent study in Thailand reported high IgG seropositivity of *E. chaffeensis* (up to 60.4%) but a lower IgG seropositivity (up to 5.3%) to *A. phagocytophilum* among patients with undifferentiated febrile illness (Blacksell et al. 2015).

Several human populations including farm workers, forest workers, pet owners, campers, hikers, and people with exposure to ticks or tick bites have been documented to have a higher risk of infection by tick-borne pathogens (Dumler 2005, Dinc et al. 2017). As there is no data on human infections of *Anaplasma* spp. and *Ehrlichia* spp. here in Malaysia, this study was aimed at determining the extent of human exposure to both tick-borne pathogens in the indigenous community and animal farm workers who are at high risk of tick-borne diseases. The indigenous community who lives in the rural settlements at the fringe of the forest has close contact with peri-domestic animals and activities such as hunting and collection of forest products may increase the risk to potential tick-borne diseases (Kho et al. 2017). Most of the animal farms investigated in this study are located in rural areas and farm workers may have exposure to ticks from the animals or vegetation (Ghane Kisomi et al. 2016). A tick surveillance study would be essential to determine the potential source of exposure to *Anaplasmataceae* in this region.

### Materials and Methods

#### Serum Samples

This study was conducted as a subset of a project to determine the occurrence of emerging tick-borne diseases in populations with high risk of acquiring tick-borne diseases (indigenous community and animal farm workers) in Malaysia (Lani et al. 2015, Kho et al. 2017). The villages and farms were selected based on recommendation of the Department of Orang Asli Development (JAKOA) and Department of Veterinary Services (DVS), Ministry of Agriculture, and Agro-based Industry, Malaysia. In total, 102 serum samples collected between 2012 and 2013 from indigenous people aged 8–78 years were analyzed in this study. The sampled indigenous community was from three rural villages located in the states of Johore, Pahang, and Kelantan. In total, 87 animal farm workers from six states (Johore, Negri Sembilan, Pahang, Kedah, Kelantan, and Terengganu) who were healthy and aged >18 years were enrolled in this study (Kho et al. 2017). Ethical approval was obtained from University Malaya Medical Centre (UMMC) Medical Ethics Committee (MEC Ref. No.: 944.20). For comparison, serum samples from 61 healthy blood donors residing in an urban area (Kuala Lumpur or Selangor) were kindly provided by the blood bank of the University Malaya Medical Centre for serological analysis.

#### Serological Analysis

Human sera were analyzed for IgG antibodies to *Anaplasma* spp. (*A. phagocytophilum* whole cell antigen) and *Ehrlichia* spp. (*E. chaffeensis* whole cell antigen) by using indirect immunofluorescence assay (IFA) (Fuller Laboratories, Fullerton, CA) in accordance with the manufacturer’s instructions. According to Blacksell et al. (2015) who used the similar assay to assess the serological status of Thai febrile patients, false-positive serology could occur due to cross-reactive antibodies generated by common antigens shared by *Anaplasma* and *Ehrlichia* spp. Briefly, serum samples were first diluted to 1:80 in phosphate-buffered saline (PBS) and 10 µl of each diluted sample was added to separate antigen wells. After incubation at 37°C for 30 min in a humid chamber, the wells were rinsed three times with PBS. A drop of IgG conjugate was added to each well, and the slide was incubated at 37°C for 30 min in a humid chamber. The slides were read under 400× magnification. Positive and negative sera provided in the kits were used as controls.

#### Statistical Analysis

The seropositivity to *A. phagocytophilum* and *E. chaffeensis* was determined and compared among animal farm workers, indigenous people, and blood donors. The statistical significance between age, gender, and study groups (i.e., animal farm workers, indigenous people, and blood donors) were determined using Chi-square and Kruskal–Wallis tests using the Statistical Package for the Social Science (SPSS) software program (Version 22) (SPSS Inc., Chicago, IL). The level of significance was determined based on 95% CI with a *P* value of ≤0.05. The pairwise comparisons within the study and age groups were carried out using Games–Howell post hoc test, whereby a *P* value of ≤0.05 was considered statistically significant.

#### Collection and DNA Extraction of Ticks

The tick DNA extracts obtained from a previous study (Kho et al. 2017) were used for amplification of *Anaplasmataceae* DNA. These included the DNA extracts from 200 *Haemaphysalis bispinosa* and 70 *Rhipicephalus microplus* collected from cattle and sheep farms, and 65 pools of ticks collected from peri-domestic animals (dogs, cats, chickens, goats, and cattle) from six rural villages where the indigenous people were residing (Table 2).

In addition, to determine the occurrence of *Anaplasmataceae* in ticks from Malaysian forest, ticks were collected from vegetation and small mammals (cats, rats, squirrel, and skink) at two forest reserves (Kuala Lompat, Krau Wildlife Reserve, Pahang, 03°50′N, 102°06′E and Sungai Deka Elephant Sanctuary, Terengganu, 05°28′N, 102°44′E). All the ticks were handpicked using forceps from animals and vegetation. The ticks were identified morphologically to genus level according to Walker et al. (2003) and Geervarghese and Mishra (2011). For ticks that were difficult to identify, PCR followed by sequence analysis of the tick mitochondrial 16S rRNA gene was performed as described by Black and Piesman (1994).

#### Molecular Detection of *Anaplasma* spp. and *Ehrlichia* spp. in Tick Samples

Briefly, ticks were first washed in 5% bleach and 70% ethanol for 3 min, rinsed in sterile distilled water, and cut into small pieces using surgical blades (Duh et al. 2010). Each tick sample was then subjected to DNA extraction using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s protocol. All extracted DNA samples were stored at −20°C prior to molecular analysis.

Preliminary screening of *Anaplasma* and *Ehrlichia* DNA in the ticks was performed using PCR assays targeting a short fragment of the 16S rRNA gene of *Anaplasmataceae*. All the PCR annealing temperatures, primer sequences, and concentrations used in this study were listed in Supp Table 1 (online only). Amplification was performed in a reaction 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 primers. Samples that were positive in the preliminary screening were randomly selected for amplification of the full-length 16S
rRNA gene, partial fragments of heat shock protein gene (groEL), and citrate synthase gene (gltA).

For amplification of the full-length 16S rRNA gene, either ATT or fD1/EHR16SR and Rp2/EHR16SD or fD1/Rp2 primers were used. For nested PCR assays, 2 µl of the products from the first amplification were used as template for the nested amplification. Positive (A. phagocytophilum and E. chaffeensis genomic DNA extracted from commercial antigen slides (Fuller Laboratory, CA) and negative (miliQ water) controls were included in each PCR assay. Amplified PCR products were electrophoresed on a 1.5% agarose gel at 100V for 50 min, stained with GelRed (Biotium, Fremont, CA) and visualized under ultraviolet light (G-Box, Syngene).

Cloning, DNA Sequencing, and Data Analysis

The amplified fragments were either subjected to direct sequencing or cloning into pCR4-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The obtained sequences were aligned with BioEdit software (Version 7.0.5.3) and matched with those sequences deposited in GeneBank database using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 116 sequenced were constructed using the neighbor-joining method of MEGA 6.0 software (Tamura et al. 2013) for determination of the phylogenetic status of the Anaplasma and Ehrlichia spp. identified in this study.

Nucleotide Sequence Accession Numbers


Results

Serological Analysis of Human Samples

Table 1 shows the demographic data of the indigenous community and animal farm workers investigated in this study. There were 143 males (57.2%) and 107 females (42.8%) with ages ranging from 8 to 78 years. The subjects were divided into five age groups: those ≤20 years (n = 37), 21–30 years (n = 75), 31–40 years (n = 60), 41–50 years (n = 39), and ≥51 years (n = 39). IgG antibodies against E. chaffeensis were detected from 29.9% and 34.3% of animal farm workers and indigenous people, respectively. The seropositivity to E. chaffeensis was significantly higher in the indigenous people, as compared to the blood donors (P = 0.002). Comparatively, only 6.9% of the indigenous people and none (0%) of the animal farm workers were IgG positive to A. phagocytophilum (Table 1). Lower seropositivity to A. phagocytophilum (1.6%) and E. chaffeensis (9.8%) were detected in the urban blood donors in this study (Table 1). The seropositivity to E. chaffeensis ranged from 20.0 to 43.2% for different age groups, with a high percentage of seropositivity noted among those aged below 20 years. Low seropositivity to A. phagocytophilum (ranging from 0.0 to 5.4%) was noted across all age groups. There was no significant difference in the seropositivity to E. chaffeensis and A. phagocytophilum by gender and age groups (Table 1).

Analysis of Tick DNA Samples

Haemaphysalis spp. were the main tick species identified in this study (Table 2). Screening of tick samples using EHR primers showed the detection of Anaplasmataceae 16S rDNA in 61.5% of ticks infesting farm animals, 37.5% of ticks infesting peri-domestic animals in rural villages, 27.3% of ticks collected from wildlife animals, and 29.1% of questing ticks collected from forest vegetation (Table 2).

Table 3 shows the results of BLAST analyses of Anaplasmataceae 16S rDNA sequences obtained in this study. Of the 22 amplified fragments from cattle ticks (7 R. microplus and 15 Haemaphysalus spp.), the sequences of 8 (36.4%) samples were found to match that of A. marginale type strain (AF311303). Further confirmation of A. marginale was obtained based on nearly full-length sequences data of the 16S rDNA (1275 bp) and gltA (511 bp) genes exhibiting 99.8% and 100% similarity with those of A. marginale strain Dawn (CP006847), respectively. The sequences obtained from three cattle ticks exhibit the highest sequence similarities (99–100%) to that of A. bovis type strain (U03775). Two sequence types of A. bovis were identified, of which two sequences were identical to that of a monkey

Table 1. Seropositivity to A. phagocytophilum and E. chaffeensis in indigenous people, farm workers, and urban blood donors investigated in this study

<table>
<thead>
<tr>
<th>Categories</th>
<th>A. phagocytophilum</th>
<th>E. chaffeensis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>P value</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 143)</td>
<td>3 (2.1)</td>
<td>0.254</td>
</tr>
<tr>
<td>Female (n = 107)</td>
<td>5 (4.7)</td>
<td>1–5.4</td>
</tr>
<tr>
<td>Age group (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20 (n = 37)</td>
<td>2 (5.4)</td>
<td>0.726</td>
</tr>
<tr>
<td>21–30 (n = 75)</td>
<td>3 (4.0)</td>
<td>0.85</td>
</tr>
<tr>
<td>31–40 (n = 60)</td>
<td>2 (3.3)</td>
<td>0–8.0</td>
</tr>
<tr>
<td>41–50 (n = 39)</td>
<td>1 (2.6)</td>
<td>0–7.8</td>
</tr>
<tr>
<td>≥51 (n = 39)</td>
<td>0 (0.0)</td>
<td>0</td>
</tr>
<tr>
<td>Study group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indigenous people (n = 102)</td>
<td>7 (6.9)</td>
<td>0.020</td>
</tr>
<tr>
<td>Farm workers (n = 87)</td>
<td>0 (0.0)*</td>
<td>0</td>
</tr>
<tr>
<td>Blood donors (n = 61)</td>
<td>1 (1.6)</td>
<td>0–4.9</td>
</tr>
<tr>
<td>Total</td>
<td>8 (3.2)</td>
<td>1.0–5.4</td>
</tr>
</tbody>
</table>

CI, confidence level.

*Significant difference in the seropositivity rate when compared with the indigenous people (Games-Howell post hoc test).
strain (KM114612, tentatively designated as *A. bovis* sequence type 1) and one to that of a wild boar strain (KU189194, tentatively designated as *A. bovis* sequence type 2). The sequences of three amplified fragments from *H. bispinosa* ticks were identical to that of *A. platys* type strain (AF303467), whereas the 16S rDNA sequences obtained from two sheep ticks were identical (238/238 bp) to the wild boar strain of *A. bovis* in Malaysia (tentatively designated as *A. bovis* sequence type 2) (KU189194).

Of the ticks examined, the 16S rDNA sequences obtained from three cattle ticks were found to match those of *Ehrlichia* sp. strain EBm52 (100%; AF497581) (Parola et al. 2003). Two ticks showed the highest sequence similarity with that of *Ehrlichia mineirensis* (99.5%; JX629805) (Cabezas-Cruz et al. 2012), while three ticks matched that of *Candidatus Ehrlichia shimanensis* (100%; AB074439) (Kawahara et al. 2006). The identification of these *Ehrlichia* spp. was further supported by sequence analysis of the gltA and groEL genes (data not shown).

Of the 64 pools of ticks collected from peri-domestic animals in rural villages, 24 pools (37.5%) were positive for Anaplasmataceae DNA (Table 2). Two sequences obtained from *R. sanguineus* and *Haemaphysalis* spp. ticks were found to match that of *A. bovis* (KM114612) (Table 3). The 16S rRNA partial gene fragment obtained from a *Haemaphysalis* cat tick (designated as *Anaplasmataceae* sp. strain SP002; KY046288) exhibits a low sequence similarity (94.1%; 224/238 nt) to that of *A. phagocytophilum* strain Webster (U02521), suggesting that it may be a potentially novel strain of Anaplasmataceae. The msp4 sequence obtained from the same tick shows only 85.7% similarity (MF314191; 215/251 nt) to *A. phagocytophilum* strain Webster (U02521).

Questing ticks comprised 77 *Dermacentor* spp., 35 *Haemaphysalis* spp., and 5 *Amblyomma* spp. that are collected from forest vegetation from the forest areas. The DNA of Anaplasmataceae was detected from 34 (29.1%) (20 *Dermacentor* spp., 12 *Haemaphysalis* spp., and 2 *Amblyomma* spp.) of the 117 questing ticks collected from the forest vegetation (Table 2). Six (27.3%) of 22 ticks collected from small mammals were positive for Anaplasmataceae-PCR (Table 2). Eleven of the 15 16S rDNA sequences obtained from questing ticks collected from forest vegetation were found to match (251/251 bp) that of *A. phagocytophilum* strain Webster (U02521), while the remaining 4 sequences exhibited the highest sequence similarity (99–100%) to that of *A. platys* (AF303467) (Table 3). The 16S rDNA sequence obtained from a tick infesting a wild rat matched that of *A. platys* type strain (AF303467) (Table 3).

Dendrograms were constructed based on short 16S rDNA sequences (238 bp) of *Anaplasma* spp. (Fig. 1) and *Ehrlichia* spp. (Fig. 2) obtained in this study (Table 3). In general, *Anaplasma* spp. and *Ehrlichia* spp. were clustered according to their respective type strains. *A. bovis* was differentiated according to sequence types 1 and 2, respectively, as shown in Fig. 1. *A. bovis* sequence type 1 included strains detected from ticks infesting cattle, monkey, goat, and forest vegetation, whereas sequence type 2 included strains previously reported from ticks infesting wild boar, cattle, and sheep. Interestingly, *Anaplasmataceae* sp. strain SP002 formed a cluster with *Candidatus Cryptoplasma californiense* (isolates MR-9, CC-14 and CP-1; KP276585-KP276587) (Eshoo et al. 2015) that was detected from *Ixodes pacificus* ticks in California, together with the China and South Korea strains of *Anaplasmataceae* sp. (clone BJ01; JN715833; clones HLA133 and HLA134; GU075703-GU075704). The *Ehrlichia* sp. identified from cattle ticks were separated into three clusters as represented by *Ehrlichia* sp. strain EBm52, *E. mineirensis* and *Candidatus* E. *shimanensis* (Fig. 2).

### Table 2. Proportion of ticks positive for Anaplasmataceae DNA (amplification of 16S rDNA) in this study

<table>
<thead>
<tr>
<th>Sources</th>
<th>Tick species (no. PCR-positive ticks/ticks tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farms</td>
<td><em>Haemaphysalis Amblyomma Dermacentor Rhipicephalus</em></td>
</tr>
<tr>
<td>Cattle</td>
<td>EBm52 (100%); AF497581 (Parola et al. 2003)</td>
</tr>
<tr>
<td>Sheep</td>
<td>103/156</td>
</tr>
<tr>
<td>Rural villages</td>
<td></td>
</tr>
<tr>
<td>Goats</td>
<td>3/3</td>
</tr>
<tr>
<td>Dogs</td>
<td>2/15</td>
</tr>
<tr>
<td>Cats</td>
<td>8/18</td>
</tr>
<tr>
<td>Chickens</td>
<td>8/19</td>
</tr>
<tr>
<td>Cow</td>
<td></td>
</tr>
<tr>
<td>Forest reserve</td>
<td></td>
</tr>
<tr>
<td>Small mammals</td>
<td>3/15</td>
</tr>
<tr>
<td>Vegetation</td>
<td>12/35</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The results of BLAST analyses of Anaplasmataceae 16S rDNA sequences obtained from 43 tick samples

<table>
<thead>
<tr>
<th>Source of ticks</th>
<th><em>A. bovis</em></th>
<th><em>A. platys</em></th>
<th><em>A. marginale</em></th>
<th><em>A. phagocytophilum</em></th>
<th>Uncharacterized Anaplasmata sp.*</th>
<th><em>Ehrlichia</em> sp. strain EBm52</th>
<th><em>E. mineirensis</em></th>
<th><em>Candidatus E. shimanensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle in animal farms (n = 22)</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Sheep in animal farms (n = 2)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peri-domestic animals in rural villages (n = 3)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vegetation in a forest reserve (n = 15)</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Small mammals from a forest reserve (n = 1)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Demonstrating close genetic relationship with *Candidatus Cryptoplasma californiense* isolates from *I. pacificus* ticks in California, USA (Eshoo et al. 2015) (Fig. 1).
Discussions

This study shows detectable IgG antibodies to *E. chaffeensis* and its closely related species in a substantial proportion of Malaysian indigenous community (34.3%) and animal farm workers (29.9%) (Table 1). Although no significant difference was noted between *Ehrlichia* seropositivity with age, a high seropositivity (~40%) to *Ehrlichia* spp. among indigenous people below 20 years of age was noted (Table 1), suggesting early exposure of the indigenous people to *Ehrlichia* spp. This finding is in contrast to a Thailand study, whereby *Ehrlichia* infection was found to be more prevalent among older patients (>50 years old) (Blacksell et al. 2015). The varying degrees of immune responses toward *Ehrlichia* spp. might be due to different extents of exposure to ticks and unknown host factors.

The low seropositivity (6.9%) to *A. phagocytophilum* and its closely related species in the indigenous people was comparable to the serological data obtained from Thai febrile patients (ranging from 3.7 to 5.3%) (Blacksell et al. 2015) and farm workers in Tianjin, China (8.8%) (Zhang et al. 2008). The overall findings in this study show higher seropositivity to *Ehrlichia* spp. in both indigenous people and animal farm workers as compared to the seropositivity to *Anaplasma* spp. Interestingly, a similar seropositivity pattern was also reported in Thai febrile patients (Blacksell et al. 2015), suggesting that ehrlichiosis is serologically more prevalent as compared to anaplasmosis in this region. Higher seropositivity to *Ehrlichia* spp. has also been reported in stray dogs in our previous study, whereby antibodies against *E. canis* and *A. phagocytophilum* were detected in 39.5% and 9.3% of stray dogs, respectively, as determined using SNAP 4Dx kits (IDEXX Laboratories, Westbrook, ME) (Koh et al. 2016a).

Different *Ehrlichia* spp. and *Anaplasma* spp. in ticks infesting cattle and sheep, peri-domestic animals in rural villages, and wildlife are identified in this study. In the current study, *A. marginale* was detected in 38.3% of ticks, *A. centrale* in 32.5%, and *A. phagocytophilum* in 29.2% (Table 1). The varying degrees of immune responses toward *Ehrlichia* spp. might be due to different extents of exposure to ticks and unknown host factors.

Fig. 1. Phylogenetic relationships among various *Anaplasma* spp. based on partial sequences of the 16S rRNA gene (238 bp). The dendrogram was constructed using the neighbor-joining method of the MEGA software with the maximum composite likelihood substitution model, and bootstrapping with 1,000 replicates. *R. rickettsii* (U11021) and *Ehrlichia sennetsu* (MT3225) was used as an outgroup.
was the predominant species detected from tick populations (both \textit{H. bispinosa} and \textit{R. microplus}) in the animal farms, followed by \textit{A. bovis} and \textit{A. platys}. \textit{R. microplus} ticks have been known as a vector to \textit{A. marginale}. The presence of \textit{A. ovis} and \textit{A. centrale} could not be ruled out as these species share almost similar sequences with \textit{A. marginale} in the 16S rRNA gene. In studies conducted previously, \textit{A. marginale} has been identified from cattle blood samples and \textit{R. microplus} ticks from cattle in Malaysia (Tay et al. 2014) and Philippines (Ybañez et al. 2013) and from water buffaloes in Northeast Thailand (Saetiew et al. 2015).

\textit{A. bovis} has been reported in both domestic and wild animals in various parts of the world (Liu et al. 2012), including the blood samples of monkeys and wild boars in Malaysia (Tay et al. 2015, Koh et al. 2016b). On the basis of a minor difference in the 16S rDNA sequence, \textit{A. bovis} is differentiated into two sequence types, as shown in Fig. 1. However, the significance of this strain differentiation is not clear yet.

Recently, a number of \textit{Anaplasma} strains closely related to the canine, platelet-infecting \textit{A. platys} species has been detected in ruminants (Zobba et al. 2014). In this study, \textit{A. platys} was detected from ticks collected from a variety of animals, including cattle, rat, and questing ticks in the forest vegetation, suggesting that the organism might have a wider distribution in our ecosystem than initially thought. In this study, \textit{A. phagocytophilum} was only detected in \textit{Dermacentor} and \textit{Haemaphysalis} questing ticks in forest vegetation. The organism was also detected in the blood samples of stray dogs and \textit{R. sanguineus} ticks in our previous investigation (Koh et al. 2016a). The detection of \textit{A. phagocytophilum} in a wide variety of ticks including \textit{Ixodes dentatus}, \textit{Amblyomma americanum}, \textit{Dermacentor variabilis}, and \textit{Dermacentor occidentalis} in Europe (Goethert and Telford 2003, Stuen et al. 2013) as well as \textit{H. longicornis}, \textit{Haemaphysalis megaspinosa}, \textit{Haemaphysalis douglasi}, \textit{Haemaphysalis japonica}, \textit{Ixodes persulcatus}, \textit{Ixodes ovatus}, \textit{Ixodes nipponensis}, and \textit{Dermacentor silvarum} in Asia has been documented (Stuen et al. 2013). Further investigation on the possible transmission of \textit{A. phagocytophilum} through questing ticks is important to prevent human anaplasmosis, especially for indigenous communities who live in close proximity to forests.

The DNA of human tick-borne pathogens, \textit{E. chaffeensis} and \textit{E. ewingii}, was not detected from ticks investigated in this study, instead, several \textit{Ehrlichia} sp. with unknown pathogenicity (i.e., \textit{Ehrlichia} strain EBm52, \textit{E. mineirensis}, and \textit{Candidatus E. shimanensis}) were detected from cattle ticks (\textit{R. microplus} and \textit{H. bispinosa}). \textit{Ehrlichia} sp. strain EBm52 was first described in \textit{R. microplus} ticks infesting cattle in Thailand (AF497581) (Parola et al. 2003). \textit{E. mineirensis}, isolated from the hemolymph of \textit{R. microplus} (Cabezas-Cruz et al. 2012, 2013; Zweygarth et al. 2013) in Brazil, is phylogenetically close to \textit{E. canis} (Cabezas-Cruz et al. 2012). \textit{Candidatus E. shimanensis} (AB074459) was first reported by Kawahara et al. (2006) in a \textit{H. longicornis} tick collected in the Shimane Prefecture in 1999. The zoonotic or veterinary potential of the \textit{Ehrlichia} sp. is not known. As \textit{R. microplus} rarely bite people, it is thus unlikely to transmit human pathogens (Parola et al. 2003). However, \textit{H. bispinosa}, which is prevalent in the Asian region, requires a new host during each stage.
The tick has been associated with Ehrlichia, Anaplasma, and Rickettsia spp. in Thailand (Malaisri et al. 2015; Theileria sergenti, Borrelia burgdorferi, and Babesia bigemina in China (Yu et al. 2015, 2016); and A. bovis in Bangladesh (Qu et al. 2016). Interestingly, the tick surveillance in this study has also detected an uncharacterized Anaplasma sp. (strain SP002) from a Haemaphysalis cat tick, which is genetically closely related to a novel Anaplasmataceae species, Candidatus Cryptoplasma californiensis (Eshoo et al. 2015) (Fig. 1), first reported in I. pacificus ticks in California.

The serological status of Malaysian indigenous people and farm workers against Anaplasma spp. and Ehrlichia spp. has been determined using IFA in this study. The limitation of the assay is the lack of specificity due to cross-reactivity between E. chaffeensis, E. ewingii, E. canis, and A. phagocytophilum (Blacksell et al. 2015). Development of serological tests specific for E. chaffeensis and A. phagocytophilum are thus essential to facilitate epidemiological study of anaplasmosis and ehrlichiosis. In addition, the primer sets used for PCR assays in this study were not specific enough to differentiate between different species of Anaplasma and Ehrlichia, for example, A. marginale, A. centrale, and A. ovis and E. chaffeensis and E. ewingii are closely related species that shared almost similar sequences in the 16S rDNA fragments. Due to the potential PCR bias, PCR assays tend to amplify the most abundant organism present in a mixed sample. As a result, some other organisms with lower abundance can be missed in the detection. DNA hybridization assay, for example, PCR-RLB (Bekker et al. 2002) will be useful, whereby more than one Anaplasma spp. or Ehrlichia spp. in the ticks can be detected simultaneously. Besides that, molecular identification of ticks to the species level is challenging due to the lack of sequences for comparison.

In summary, the serological data in this study suggests high exposure of our indigenous community and animal farm workers to E. chaffeensis. This finding is consistent with the published data of Blacksell et al. (2015) for Thai patients. Although the tick surveillance study provides valuable data on the type and distribution of Anaplasma spp. and Ehrlichia spp. present in this region, we do not have any direct evidence to show the association of any tick species with zoonotic transmission of Anaplasma or Ehrlichia infections. The finding of A. phagocytophilum in ticks from the forest area investigated in this study suggests a possible source of exposure for indigenous community who engage in activities such as hunting or collection of forest products. The fact that none of the animal farm workers are seropositive to A. phagocytophilum implies that Anaplasma sp. harbored by cattle ticks (including A. marginale, A. platys, and A. bovis) are unlikely to be the source of exposure. None of the ticks examined in this study had E. chaffeensis DNA detected. The possible zoonotic potential of several Ehrlichia sp. detected from cattle ticks (particularly H. bispumosa) is yet to be determined. Future research should emphasize surveillance for human cases and improving public awareness and knowledge of tick-borne diseases.

**Supplementary Material**

Supplementary material can be found at *Journal of Medical Entomology* online.

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