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Molecular investigation of *Anaplasma* spp. in domestic and wildlife animals in Peninsular Malaysia

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ABSTRACT

Anaplasma spp. are Gram-negative obligate intracellular, tick-borne bacteria which are of medical and veterinary importance. Little information is available on *Anaplasma* infection affecting domestic and wildlife animals in Malaysia. This study investigated the presence of *Anaplasma* spp. in the blood samples of domestic and wildlife animals in Peninsular Malaysia, using polymerase chain reaction (EHR-PCR) assays targeting the 16S rRNA gene of *Anaplasmataceae*. High detection rates (60.7% and 59.0%, respectively) of *Anaplasma* DNA were noted in 224 cattle (*Bos taurus*) and 78 deer (77 *Rusa timorensis* and one *Rusa unicolor*) investigated in this study. Of the 60 amplified fragments obtained for sequence analysis, *Anaplasma marginale* was exclusively detected in cattle while *Anaplasma platys/Anaplasma phagocytophilum* was predominantly detected in the deer. Based on sequence analyses of the longer fragment of the 16S rRNA gene (approximately 1000 bp), the occurrence of *A. marginale*, *Anaplasma capra* and *Candidatus* Anaplasma camelii in cattle, *Candidatus* A. camelii in deer and *Anaplasma bovis* in a goat was identified in this study. To assess whether animals were infected with more than one species of *Anaplasma*, nested amplification of *A. phagocytophilum*, *A. bovis* and *Ehrlichia chaffeensis* DNA was performed for 33 animal samples initially screened positive for *Anaplasmataceae*. No amplification of *E. chaffeensis* DNA was obtained from animals investigated. BLAST analyses of the 16S rDNA sequences from three deer (*R. timorensis*), a buffalo (*Bubalus bubalis*) and a cow (*B. taurus*) reveal similarity with that of *Candidatus* Anaplasma boleense strain (GenBank accession no.: KX987335). Sequence analyses of the partial gene fragments of major surface protein (*msp4*) gene from two deer (*R. timorensis*) and a monitor lizard (*Varanus salvator*) show the detection of a strain highly similar (99%) to that of *A. phagocytophilum* strain ZJ-China (EU008082). The findings in this study show the occurrence of various *Anaplasma* species including those newly reported species in Malaysian domestic and wildlife animals. The role of these animals as reservoirs/maintenance hosts for *Anaplasma* infection are yet to be determined.

1. Introduction

Anaplasma spp. and *Ehrlichia* spp. are Gram-negative obligate intracellular, tick-borne bacteria in the family of *Anaplasmataceae* (Dumler et al., 2001). Of the six species in the genus *Anaplasma, Anaplasma phagocytophilum* is the etiological agent of human and animal granulocytic anaplasmosis and has been reported in a wide range of animals including horses, cats, canids, ruminants, rodents, birds and reptiles (Stuen et al., 2013). *Anaplasma marginale, Anaplasma centrale* and *Anaplasma ovis* are the causative agents for bovine anaplasmosis. *Anaplasma platys* has been reported to cause infectious canine cyclic thrombocytopenia, as well as infections in deer, cats, camels and red foxes (Atif, 2016; Cardoso et al., 2015; Li et al., 2015a; Li et al., 2016; Salakij et al., 2012). The presence of *Anaplasma bovis* in diverse animal hosts including ruminants, cottontail rabbits, dogs, raccoons and cats has also been documented (Atif, 2016). In recent years, new species of *Anaplasma* such as *Anaplasma capra* and *Candidatus* Anaplasma camelii have also been associated with human and animal infections (Bastos et al., 2015; Li et al., 2015b). *Anaplasma capra* was first isolated from humans and goats in a forest area in the northeast China (Li et al., 2015a). The ticks carrying the novel *Anaplasma* species, *Haemaphysalis longicornis* ticks, have been widely distributed in the Asian region (Sun et al., 2015). *Candidatus* Anaplasma camelii was first reported by Bastos et al. (2015) as a closely related species to *A. platys* and *Ehrlichia canis* in

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the dromedary camel (*Camelus dromedaries*). *Candidatus* Anaplasma boleense was first identified in *Hyalomma asiaticum* ticks sampled from Bole in the Xinjiang Uygur Autonomous Region, China (Kang et al., 2014).

Recent reports on animal anaplasmosis are available from China, Japan and Korea (Chae et al., 2008; Kawahara et al., 2006; Liu et al., 2012; Moustafa et al., 2015; Ooshiro et al., 2008; Yang et al., 2015a; Yang et al., 2015b), however; data is scarce on the circulation of *Anaplasma* spp. in the domestic and wildlife animals in Southeast Asia including Peninsular Malaysia. The emergence of *Anaplasmataceae* as human pathogens has gained the attention of scientific community. A recent serosurvey reported high antibody prevalence to *Ehrlichia chaffeensis* in Malaysian indigenous people (34.3%) and animal farm workers (29.9%); however, only 6.9% of the indigenous people but none of the animal farm workers were seropositive to *A. phagocytophilum* (Koh et al., 2018). The scarce data on the maintenance hosts of *Anaplasma* spp. and *Ehrlichia* spp. has led us to investigate the occurrence of these bacteria in domestic and wildlife animals in Malaysia.

2. Materials and methods

2.1. Sample collection

Malaysia is a tropical country with hot and humid climate and little seasonal variation throughout the year. In this study, the occurrence of *Anaplasma/Ehrlichia* in the blood samples of domestic animals (224 cattle and 40 goats) in livestock farms (six cattle and a goat farm) at different geographical regions in Peninsular Malaysia [Kedah, Kelantan, Terengganu, Pahang, Negeri Sembilan and Johore; Kho et al., 2015] were examined using a PCR assay targeting a fragment of *Anaplasmataceae* 16S rRNA gene (Parola et al., 2000). All the cattle (majority were Nellore, Kedah-Kelantan, Yellow cattle cross with Kedah-Kelantan and Mafriwal breeds) were managed by rotational grazing system while the goats (majority were Boer breed) were managed by zero grazing practice. The age of the cattle and goats ranged from 6 to 147 months and 8 to 32 months, respectively. Although all farms had fixed schedule (every 1-, 2- and 6-monthly) for acaricide treatment, some farms provided treatment for their farm animals whenever tick infestation was high. Ticks were frequently observed under the ear, eyes, flank, abdomen, tail and perineal regions of cattle (Kho et al., 2015), however, clinical symptoms related to tick-borne infections in the animals have not been reported.

Random blood sampling was performed by veterinarians from February to September 2013, with the approval from the Director, Department of Veterinary Services, Ministry of Agriculture and Agro-Based Industry, Malaysia (Reference no.: JPV/PSTT/100-8/1). Approximately 1–3 mL whole blood sample was collected from each animal via jugular vein in EDTA-coated tube and kept in ice for transportation to the laboratory.

Additionally, 276 animal blood samples [78 deer (77 *Rusa timorensis* and one *Rusa unicolor*), 75 goats (*Capra hircus*), 55 buffaloes (*Bubalus bubalis*), 46 horses (*Equus caballus*) and 22 cattle (*Bos taurus*)] in EDTA-coated tubes were provided by the Parasitology department of the Veterinary Research Institute (VRI), Ipoh, Malaysia [Reference no.: JPV: VRI/197/PA/141Jid.11 (48)]. Twenty-six blood samples from a variety of wildlife animals [ten monitor lizards (nine *Varanus salvator* and one *Varanus bengalensis*), nine rats (eight *Rattus rattus* and one *Maxomys surifer*) and seven squirrels (*Rhinosciurus laticaudatus*)] on Whatman® cards were provided by the Department of Wildlife and National Parks (PERHILITAN), Peninsular Malaysia [Reference no.: JPHL&TN (IP): 80-4/2 Jilid 15(51)]. These samples were collected from Salang, Pulau Tioman, Pahang during a wildlife surveillance program in March 2013.

2.2. DNA extraction

DNA was extracted from 200 µL of animal blood samples using QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance to the manufacturer's protocol. All DNA samples were stored at −20 °C prior to PCR amplification. Ten discs were excised from each FTA card and washed thrice with 200 µL FTA Purification Reagent (GE Healthcare, UK) at room temperature for 5 min to remove cell debris and PCR inhibitors. After washing twice with 200 µL 1 × TE buffer at room temperature for 5 min, the discs were dried at 50 °C for 15 min. A single disc was used as the DNA template for PCR amplification.

2.3. Molecular detection of Anaplasma spp. and Ehrlichia spp. from animal blood samples

Preliminary screening of *Anaplasmataceae* DNA in the animal blood samples was performed by polymerase chain reaction (PCR) using primers targeting 345 bp of the 16S rRNA gene of *Anaplasmataceae* (including *Anaplasma* spp. and *Ehrlichia* spp.). Samples that were positive in the preliminary screening were subjected to further characterization by amplification of the full length 16S rRNA gene, using either ATT or fD1/EHR16SR and Rp2/EHR16SD or fD1/Rp2 primers (Table 1). Amplification was performed in a reaction volume of 25 µL containing two microliters of DNA template, 1 U GoTaq® Flexi DNA Polymerase (Promega, WI, USA), 1 × Green GoTaq® Flexi Buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and primers. Cloned DNA fragments of *A. bovis* in pCR4-TOPO plasmids were used as positive controls in the PCR assays.

To assess whether animals were infected with more than one species of *Anaplasma*, nested PCR assays targeting the 16S rRNA gene of *A. phagocytophilum*, *A. bovis* and *E. chaffeensis* and major surface protein 4 (*msp4*) gene of *A. phagocytophilum* were performed for 33 EHR-positive samples. Two microliters of the products from the first amplification was used as the template for the nested amplification. Positive controls, i.e., DNA extracted from *A. phagocytophilum* and *E. chaffeensis* on immunofluorescence assay slides (Fuller Laboratories, CA, USA) and negative (miliQ water) controls were included in each PCR assays.

PCR products were electrophoresed in a 1.5% agarose gel at 100 V for 50 min, stained with GelRed™ (Biotium, CA, USA) and visualized under UV light (G-Box, Syngene, England). Amplified fragments were sequenced on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, USA), using both forward and reverse primers of each PCR assay. The sequences were matched to those deposited in the GenBank database using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To determine the phylogenetic status of the *Anaplasma* spp. identified in this study, a dendrogram was constructed based on the long fragments of the *Anaplasma* 16S rRNA gene using the neighbor-joining method of the MEGA 6.0 software (Tamura et al., 2013).

3. Results

The results of molecular detection for *Anaplasmataceae* DNA are presented in Table 2. High detection rates were obtained in cattle (136/224, 60.7%) from livestock farms in this study. None of the 40 goats investigated in this study were positive for *Anaplasmataceae* DNA. The *Anaplasmataceae* detection rates ranged from as low as 37.5% (Johore) to as high as 96.2% (Negeri Sembilan). Of the 276 animal samples provided by VRI, 26.8% (74/276) were positive for EHR-PCR assays. The positive samples were derived from the deer (46/78, 59.0%), goats (2/75, 2.7%), buffaloes (12/55, 21.8%) and cattle (14/22, 63.6%). Of the wildlife examined, all ten monitor lizards were positive in the EHR-PCR assays. *Anaplasmataceae* DNA was not amplified from any of the rats and squirrels.

The sequences for 42 partially amplified 16S rRNA gene fragments obtained from cattle were subjected to BLAST analyses (Table 2). *A. marginale* was identified in 37 (88.1%) cattle with 99–100% sequence