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Investigation of possible rickettsial infection in patients with malaria

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Abstract. Rickettsioses are a common health problem in many geographical areas, including rural areas in Southeast Asia. Co-infection of rickettsioses and malaria has been reported in Africa, where common reservoir and vectors are available. In this study, blood samples of Malaysian patients microscopically positive (n=148) and negative (n=88) for malaria parasites (*Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium falciparum*, and *Plasmodium vivax*) were screened for the presence of rickettsial DNA, using PCR assays targeting specific genes. A partial fragment of rickettsial o*mpB* gene was successfully amplified and sequenced from a patient microscopically positive for *Plasmodium* spp. and PCR-positive for *P. vivax*. BLAST analysis of the o*mpB* sequence demonstrated the highest sequence similarity (99.7% similarity, 408/409nt) with *Rickettsia* sp. RF2125 (Genbank accession no. JX183538) and 91.4% (374/409 nt) similarity with *Rickettsia felis* URRWXCal2 (Genbank accession no. CP000053). This study reports rickettsial infection in a malaria patient for the first time in the Southeast Asia region.

INTRODUCTION

Rickettsia felis (type strain URRWXCal2) is an emergent human pathogen which causes febrile illnesses in various parts of the world (Parola *et al.*, 2016). The infection has been detected more often in countries with high malaria rates whereby common reservoirs (mammals) and arthropod vectors are available, as compared with countries with low malaria rates (Mediannikov *et al.*, 2013). Co-infection of *R. felis* and *Plasmodium* spp. has been reported in malaria patients in Africa (Maina *et al.*, 2012; Mediannikov *et al.*, 2013; Mourembou *et al.*, 2015). It has been recently reported that *Anopheles gambiae* mosquitoes, the primary malarial vector in sub-Saharan Africa, may be able to transmit *R. felis* (Dieme *et al.*, 2015). Rickettsial DNA has been detected in *An. gambiae* and *Aedes albopictus* mosquitoes in Africa (Socolovschi *et al.*, 2012a, b), as well as *Culex pipiens pallens* and *Anopheles sinensis* mosquitoes in China (Zhang *et al.*, 2014).

Besides *R. felis*, genetically closely related organisms such as *Rickettsia* sp. RF2125, Rf31, *Rickettsia asemboensis*, and Candidatus Rickettsia senegalensis (collectively known as *R. felis*-like organisms) have also been reported from different arthropod vectors from various geographical regions. The uncultured *Rickettsia* sp. RF2125 was first detected in *Ctenocephalides canis* and *Ctenocephalides felis* near the Myanmar border, Thailand (Parola *et al.*, 2003a), and has since then, detected in various arthropods species (Tay *et al.*, 2014; Hii *et al.*, 2015; Kho *et al.*, 2017).

*Rickettsi*a sp. RF2125 DNA has been detected more often from arthropods such as fleas and ticks parasitizing domestic and pet animals in Malaysia, as compared

to *R. felis* (Kho *et al.*, 2017). *Rickettsi*a sp. RF2125 has also been detected from the blood samples of monkeys and a febrile patient in our previous investigations (Tay *et al.*, 2015; Kho *et al.*, 2016). As malaria and rickettsioses have been known as major causes of fever in rural Malaysia (Tay *et al.*, 2000; Kaur, 2009), this has led us to investigate the occurrence of rickettsial infection in patients clinically diagnosed as having malaria. Additionally, the presence of rickettsiae in *Ae. albopictus* and *Culex quinquefasciatus* mosquitoes was also investigated in this study.

MATERIALS AND METHODS

Blood samples

A total of 236 blood samples obtained from patients attending to several health centres in East and West Malaysia from 2015-2017 were investigated in this study. These include patients who were microscopically positive (n=148) and negative (n= 88) for *Plasmodium* spp. Ethical approval was obtained from the University of Malaya Medical Centre Ethic Committee (MEC No. 817.18). The laboratory diagnosis of malaria was based on either examination of Wright-Giemsa-stained peripheral blood smears and/or a nested PCR assay targeting 18S rRNA gene (Snounou *et al.*, 1993). DNA was extracted from the blood samples using a commercial kit (Qiagen DNeasy Blood & Tissue Kit, Germany). Additionally, DNA extracts obtained from 165 *Ae. albopictus* collected around Selangor and Kuala Lumpur and *Culex quinquefasciatus* collected from several locations (Kuala Terengganu, Malacca, Johore, Selangor, Pahang, Penang, Kelantan, Kuala Lumpur, Negeri Sembilan, Kedah, Perlis, Perak, Sarawak, and Sabah) were subjected for screening of rickettsial DNA.

PCR amplification

Three rickettsial-specific genes i.e., citrate synthase gene (*gltA*) (Labruna *et al.*, 2004), 190-kDa outer membrane protein gene (*ompA*) (Regnery *et al.*, 1991) and 135-kDa outer membrane protein gene (*ompB*) (Roux & Raoult, 2000) were targeted for amplification from the blood and mosquito DNA samples. All PCR assays were performed in a final volume of 20 µL containing 2 µL of DNA template, 1X ExPrime *Taq* DNA polymerase (GENET BIO, South Korea) and 0.2 µM of each primer, in a Veriti thermal cycler (Applied Biosystems, USA). Cloned PCR4-TOPO TA plasmids (Invitrogen, Carlsbad, CA, USA) with amplified *gltA* fragment from *R. honei* (strain TT118) and *ompB* fragment from a rickettsial endosymbiont (98% similarity to *R. raoultii*) were used as positive controls. Sterile distilled water was used as the negative control in each PCR reaction. PCR products (5 µl) were separated on a 1.5 % (w/v) agarose gel and purified using GeneAll Expin™ Combo GP kit (GeneAll Biotechnology, South Korea) prior to sequencing on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems, USA), using both forward and reverse PCR primers. The sequence obtained was subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for homologous sequences in the GenBank database. The genetic relatedness of the rickettsial strain with other *R. felis* reference strains was determined by constructing a dendrogram using neighbour-joining method of the MEGA software and bootstrap analysis with 1,000 re-samplings (Tamura *et al.*, 2013). *Rickettsia rickettsii ompB* gene sequence (X16353) was used as an outgroup for comparison.

RESULTS

The amplification of rickettsial *gltA* and *ompA* genes was negative for all patients' blood DNA samples. An *ompB* gene fragment was successfully amplified from the blood sample of a patient (SBH0005) microscopically positive for *Plasmodium* spp. (*P. malariae/P. knowlesii*), but was positive for *P. vivax* in the nested PCR assay (Snounou *et al.*, 1993). Comparative analysis of 409 nucleotides of the amplified

ompB gene (Table 1) demonstrates the highest similarity (99.7%, 408/409 nt) with a rickettsial strain from a Malaysian patient (*Rickettsia* sp. clone Mal, Genbank accession no. KU255717), and monkey (*Rickettsia* sp. 0095, Genbank accession no. KP126804), as well as *Rickettsia* sp. RF2125 (Genbank accession no. JX183538). The sequence demonstrates 99.5% (407/409 nt) similarity with *Rickettsia asemboensis* isolate F30, CF#22, and VGD7 (Genbank accession nos. JN315972, KY445737, KY650699), 91.4% with *Rickettsia felis* URRWXCal2 (Genbank accession no. CP000053) and 88.7% with *Rickettsia* sp. clone HL15c (Genbank accession no. KF963608) from Malaysia and Candidatus Rickettsia senegalensis strain PU01-02 (Genbank accession no. KF666470) from Africa. Figure 1 is a dendogram showing the closely relatedness of the rickettsial strain (SBH0005) detected in this study with *Rickettsia* sp. RF2125 and other rickettsial species, based on *ompB* partial gene sequences. None of the mosquito DNA (*Ae. albopictus* and *Culex quinquefasciatus*) screened in this study was positive for rickettsial DNA.

Table 1. Percentage sequence similarity of rickettsial *ompB* partial gene sequence (SBH0005) with *R. felis* and its genetically closely related species

Rickettsia species (sequence length)	SBH0005 (409 nt)
Rickettsia sp. RF2125 (JX183538) (409 nt)	99.7
Rickettsia sp. 0095 (KP126804) (409 nt)	99.7
Uncultured *Rickettsia* sp. clone Mal (KU255717) (409 nt)	99.7
Rickettsia asemboensis isolate F30 (JN315972) (409 nt)	99.5
Rickettsia asembonensis isolate CF#22 (KY445737) (409 nt)	99.5
Rickettsia asembonensis isolate VGD7 (KY650699) (409 nt)	99.5
Rickettsia felis URRWXCal2 (CP000053) (409 nt)	91.4
Candidatus Rickettsia senegalensis strain PU01-02 (KF666470) (418 nt)	88.7
Uncultured Rickettsia sp. clone HL15c (KF963608) (418 nt)	88.7
R. rickettsii (X16353) (418 nt)	85.8

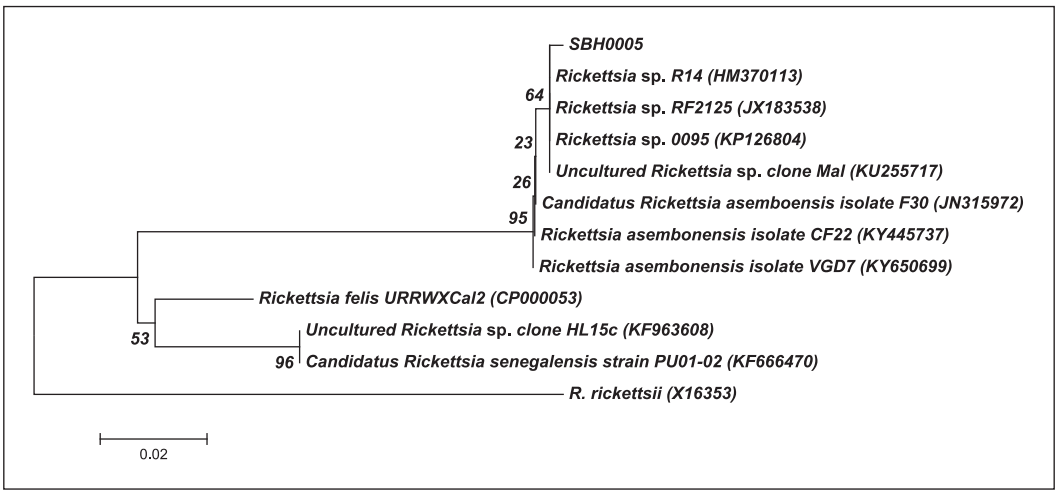

Figure 1. Dendrogram showing the closely relatedness of rickettsial species based on *ompB* partial gene sequences. The sequence (SBH0005) was clustered in the same group with *Rickettsia* sp. RF2125.

DISCUSSION

The clinical manifestations of *R. felis* infection, i.e., fever, headaches, chills, muscle aches and joint pains (Maina *et al.*, 2012) can often be misdiagnosed for other tropical diseases including malaria, dengue and other vector-borne diseases. The first report of *R. felis* infection has been reported amongst rural residents of the central Thai-Myanmar border (Parola *et al.*, 2003b), since then, the infection has been diagnosed in febrile patients from several Asian countries, including Korea (Choi *et al.*, 2005), Thailand (Sophie *et al.*, 2014), Laos (Dittrich *et al.*, 2014) and China (Zhang *et al.*, 2014). Although direct evidence of *R. felis* infection has not been obtained in Malaysia, significantly higher seroprevalence to *R. felis* has been reported among rural populations (indigenous population, 22.5% and animal farm workers, 16.5%) as compared to urban blood donors (0%) (Kho *et al.*, 2017).

This study reports the detection of a rickettsial strain genetically closely related to *R. felis* in a malaria patient from East Malaysia (Sabah) presenting with fever and chills. Although the patient was microscopically positive for *P. malariae/P. knowlesi* infection, *P. vivax* was detected from the patient' blood sample. The species misidentification of malaria parasites by microscopy is not uncommon and has been reported in malaria patients especially those infected with *P. vivax*, *P. falciparum* and *P. knowlesi* (Barber *et al.*, 2013). The *Rickettsia* sp. detected in this study had the highest sequence similarity with the *ompB* gene of *Rickettsi*a sp. RF2125, which has been previously detected in monkeys, fleas and ticks (Tay *et al.*, 2014; Kho *et al.*, 2017), as well as in a Malaysian patient with persistent fever, conjunctival suffusion, petechiae, low platelet count, elevated hepatic transaminases, and hypoalbuminemia (Kho *et al.*, 2016). As different antimicrobials are used for treatment of malaria and rickettsioses, co-infection of *Plasmodium* and *Rickettsia* spp. may contribute to "clinical resistance" if malaria drug is used alone. In fact, clinical resistance to chloroquine in *P. vivax* infection has been reported in Indonesia (Maguire *et al.*, 2002; Asih *et al.*, 2011). This study highlights the importance of awareness and the availability for appropriate laboratory diagnostic tests for rickettsial infections and other tropical diseases in malaria endemic regions.

R. felis has been postulated as a pathogen with a global impact based on the fact that mosquitoes can act as a vector for rickettsioses (Parola *et al.*, 2016; Dieme *et al.*, 2015; Socolovschi *et al.*, 2012a, b). *A. albopictus* mosquitoes (also native in Southeast Asia) from Gabon, Africa and *Culex pipiens* mosquitoes from China were tested positive for *R. felis* in previous investigations (Angelakis *et al.*, 2016; Zhang *et al.*, 2014). However, none of the *A. albopictus* and *Culex* mosquitoes screened in this study were positive for rickettsial DNA. This may be owing to the small number and limited species of mosquitoes investigated in this study or seasonal variation, as seen in mosquitoes infected with *Rickettsia* (Mediannikov *et al.*, 2013) and dengue virus (Angel & Joshi, 2008). The finding on co-infection of *R. felis* and *Plasmodium* spp. in malaria patients in Africa (Maina *et al.*, 2012; Mediannikov *et al.*, 2013; Mourembou *et al.*, 2015) suggests that *R. felis* infection should be included in the differential diagnosis in malaria endemic areas, especially when there are shared reservoirs (mammals) and arthropod vectors. The overall incidence of rickettsial infection in malaria patients has been low in this study. It is also possible for patients to acquire multiple infections through simultaneous exposure to various infected arthropod vectors including mosquitoes, fleas and ticks in the tropical region. Further studies are required to identify common reservoirs and arthropod vectors for both *Rickettsia* and *Plasmodium* spp. and to investigate the clinical impact and management of co-infection.

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