First Report of *Rickettsia Asembonensis* in Small Ruminants

Van Lun Low

Universiti Malaya

Siti Sarah Azhar

Universiti Malaya

Tiong Kai Tan (✉ tantk@um.edu.my)

Universiti Malaya <https://orcid.org/0000-0003-0297-1727>

Premaalatha Bathmanaban

Kementerian Pertanian dan Industri Asas Tani Malaysia

Sazaly AbuBakar

Universiti Malaya

Panchadcharam Chandrawathani

Ministry of Agriculture and Land: Kementerian Pertanian dan Industri Asas Tani Malaysia

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Ministry of Agriculture and Land: Kementerian Pertanian dan Industri Asas Tani Malaysia

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Kementerian Pertanian dan Industri Asas Tani Malaysia

Ngah Hamid Akma

Kementerian Pertanian dan Industri Asas Tani Malaysia

Mohd Noor Norlizan

Kementerian Pertanian dan Industri Asas Tani Malaysia

Yvonne Ai Lian Lim

University of Malaya: Universiti Malaya

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Abstract

Rickettsioses is an emerging infectious disease in Southeast Asia caused by different organisms in the Rickettsiae bacterial tribe, a gram-negative obligate intracellular bacterium. There is limited information regarding the vertebrate hosts of *Rickettsia* spp. This study aims to detect and identify *Rickettsia* agents present in wildlife and domesticated animals in Malaysia using Polymerase Chain Reaction (PCR) and sequencing approach with phylogenetic analysis. *Rickettsia* sp. was detected in 2 (0.67%) of 300 animal blood samples. The positive samples were derived from a goat (5.56% of 18) and a sheep (2.22% of 45). Based on the analysis of the *Rickettsia* citrate synthase gene (*gltA*), both sequences demonstrated 99.64% sequence similarity to *Rickettsia asembonensis*, a species that is known to infect humans and macaques. This study reported for the first time the detection of *R. asembonensis* in sheep and goats in Malaysian farms, suggesting this species may be adapting in a wider range of animals, specifically farm animals, therefore, poses a zoonotic threat to the local populace particularly to the farm workers or animal handlers. The low infection rate of this pathogen yet is of public health importance highlighted the needs of continuous surveillance of emerging and reemerging pathogens among animal reservoirs.

Introduction

Rickettsioses is a neglected infectious disease in Southeast Asia despite being one of the leading causes of treatable unrecognized febrile illnesses. Notably, *Rickettsia typhi* and some members of spotted fever group *Rickettsia*, exemplify important species that causes human disease among the local population (Aung et al., 2014). One of the hallmarks of *Rickettsia* is their ability to adapt to a wide variety of hosts (Weinert et al., 2009). *Rickettsia* is maintained in nature in a continuous cycle between infected arthropods, and one or several of host animals. Hence, the possibility of other animals as reservoirs of these emerging pathogens has gained the attention of researchers worldwide. Several studies across Southeast Asia have reported rickettsial infections in animals such as wild rodents, dogs, monkeys and a range of hematophagous arthropods (Low et al., 2020).

Essentially, human infections reported in Southeast Asia were frequently associated with animal interactions (Edouard et al., 2014; Kho et al., 2017). It is important to establish reservoir hosts that may participate in the enzootic maintenance of *Rickettsia* as it allows researchers and local authorities to identify risk and target control measures especially to animal handlers, farm workers and agricultural workers who are at higher risk of developing these tick and flea-borne diseases. Accordingly, this study aimed to detect and genotype *Rickettsia* DNA from various blood samples of wildlife and domesticated animals in Malaysia.

Materials And Methods

Archived blood samples of wildlife and domesticated animals which were provided by the Veterinary Research Institute (VRI), Department of Veterinary Services, Malaysia were stored at -20° C prior to extraction. DNA extraction was performed on a total of 300 animal whole blood samples (55 deer, 73

cattle, 18 goat, 45 sheep, 69 equine, 5 orangutan, 1 simian, 2 tapir, 1 otter, 6 tiger, 1 fox, 12 rabbit, 1 binturong, 6 sun bears, and 5 elephant). Genomic DNA was extracted from 200 µl of each blood sample according to the protocol of the QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA). DNA was eluted in 50 µl of elution buffer and samples were stored at -20°C.

Rickettsia DNA detection was performed using conventional polymerase chain reaction (PCR) with primers CS-239 (5'-GCT CTT CTC ATC CTA TGG CTA TTA T -3') and CS-1069 (5'- CAG GGT CTT CGT GCA TTT CTT -3'), targeting an 800-bp fragment of the citrate synthase gene (*gltA*) (Labruna et al., 2004). All reactions were performed in a final volume of 25 µl involving 25-50 ng of genomic DNA, 12.5 µl of MyTaq Red Mix (Bioline Reagents Ltd., London, UK) and 10 pmol of each forward and reverse primer using the Applied Biosystems Veriti 96-Well Thermal Cycler (Applied Biosystems, Inc., Foster City, CA). For each set of reactions, a positive control and a negative control was included. The final thermal-cycling conditions were set to an initial denaturation at 95 °C for 3 mins followed by 40 cycles of denaturing at 95 °C for 20s, annealing at 52 °C for 20s, extension at 72 °C for 40s, and a final extension at 72 °C for 5 mins. The PCR products were visualized in a 2% agarose gel. Positive amplicons were then sent to a local company for bi-directional sequencing using both forward and reverse primers.

Sequences generated from the present study were deposited into the GenBank database under accession numbers OL502704-OL502705. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify homologous sequences. The sequences were trimmed and aligned using the BioEdit Sequence Alignment Editor Software (Hall, 1999). A dendrogram was constructed to determine the phylogenetic position of *Rickettsia* identified in this study using the neighbour-joining method with the MEGA X software (Kumar et al., 2018). The NJ bootstrap values were evaluated using 1000 replicates with Kimura's two-parameter model of substitution (K2P distance). *Rickettsia typhi* (U59714) was used as an outgroup.

Results And Discussion

Of 300 animal blood samples provided by the diagnostic lab, two samples were successfully amplified based on the *gltA* gene of *Rickettsia* with an overall infection rate of 0.67% (2/300). The positive samples were derived from a goat with an infection rate of 5.56% (1/18) and a sheep with an infection rate of 2.22% (1/45). None of the blood samples of wildlife animals reported rickettsial infections. The goat and sheep sample originated from a private and government farm, respectively. Our records state that the goat sample was sent for a diagnostic test as it presented symptoms such as lack of appetite, paleness, and weight loss while the sheep sample was sent for routine screening.

Through BLAST analysis, the sequence obtained from the goat (G325) and sheep (S102) both demonstrated 99.64% sequence similarity to *Rickettsia asembonensis* (MK923743), a recently discovered isolate from the ectoparasites of domestic animals in Peru, distinct from the original Kenya strain (Maina et al., 2016). The neighbour joining dendrogram constructed based on the *gltA* gene sequence in Fig. 1 revealed that both sequences G325 and S102, clustered with the respective reference *R. asembonensis*

sequences (KY445724, KY445725, KX196267, AF516333) retrieved from the NCBI GenBank and showed a close relationship with *R. senegalensis* thus, confirming the findings obtained from the BLAST analysis. We reported the first detection of a genotype of *Rickettsia asembonensis* in sheep and goat in Malaysian farms.

Rickettsia asembonensis is a newly identified species (Maina et al., 2016), closely related to *R. felis*, and together with *Ca. R. senegalensis* and other genetically related species grouped as *R. felis*-like organisms (RFLO). There have been several reports of partially characterized agents that were closely related to *R. asembonensis* prior to its first complete characterization including the *Rickettsia* sp. RF2125, which was first identified in the flea specimens along the Thai-Myanmar border (Parola et al., 2003). Reports of *Rickettsia asembonensis* and those genetically related, have been identified in various flea species (predominantly *Ctenocephalides felis*) collected from domestic animals across the world (Maina, et al., 2016). In Malaysia, *Rickettsia* sp. Rf31, a strain of *R. senegalensis* and *Rickettsia* sp. RF2125, a strain of *R. asembonensis* have been reported in fleas (*Ctenocephalides felis* and *Ctenocephalides orientis*) and tick (*Rhipicephalus sanguineus*) parasitizing dogs and cats in several studies (Low et al., 2017; Mokhtar & Tay, 2011; Tay et al., 2014). In a vector surveillance study among farm animals and peri-domesticated animals in rural villages of Peninsular Malaysia, *Rickettsia* sp. RF2125 was detected in ticks (predominantly *Haemaphysalis* spp.) parasitizing cattle and the peri-domesticated animals (Kho et al., 2017). As a result, the potential of ectoparasites of domestic animals as vectors of this emerging pathogen should not be disregarded.

Despite the extensive research on the ectoparasite vectors of *R. asembonensis*, there is limited study on its animal reservoirs. Thusfar, it has been detected in the blood sample of healthy cynomolgus monkeys in Malaysia (Tay et al., 2015) and in blood samples of cats in Thailand (Phoosangwalthong et al., 2018). The identification of *R. asembonensis* in a goat and a sheep in this study therefore suggests that the infection caused by this species may be present in a wider range of animals, specifically farm animals.

The absence of rickettsial infections in other domestic animals such as cattle, may be due to better tick control or compliance to the Herd Health Program in the farms (Rohaya et al., 2017; Tay et al., 2014). Their compliance to these programmes encourages the implementation of additional control measures to avoid any disease-causing factors, such as eliminating potential vectors. Admittedly, these samples were sent to the diagnostic lab on account of suspicion of a parasitical infection, implying that these samples may not be a good representation of the population for a bacterial study. The PCR assays in this study were based on the *gltA* gene fragment of *Rickettsia* spp. as it is well-conserved, and a more extensive reference sequences are available in the NCBI GenBank for phylogenetic analysis. A *gltA* gene amplicon provides conclusive evidence that the agent belongs to the genus, *Rickettsia* (Parola et al., 2005). However, previous studies have used *gltA* PCR assay for initial screening of *Rickettsia* DNA before amplifying other *Rickettsia* specific genes such as *ompA* and *ompB* gene fragment (Kho et al., 2017). Since the detection was solely by *gltA* gene amplification, it is possible that it has remained undetected in most of the blood samples.

Our findings pinpoint the need of continuous surveillance of emerging and reemerging pathogens in animal reservoirs. Although low infection rate of *R. asembonensis* was detected in sheep and goat samples in this study, its zoonotic potential should not be disregarded in view of the close contact of animal farm workers or handlers with the animals. However, human pathogenesis of *R. asembonensis* remains unclear. There is an urgency to identify the true hosts responsible for transmission since human infections caused by this species of *Rickettsia* have been reported. For instance, *R. asembonensis* was identified in four patients displaying common febrile symptoms in Peru (Palacios-Salvatierra et al., 2018) and two human blood samples in Zambia (Moonga et al., 2021). Similarly, in Malaysia, the first report of this infection was in a 15-year-old boy presenting typical febrile symptoms however, with no signs of a tick bite (Kho et al., 2016). Interestingly, another case presented a co-infection with malaria in Sabah (Tay et al., 2019).

Our study has revealed the possibility of domestic animals (sheep and goat) as a potential host of rickettsial infections in Malaysia. The tropical climate of Malaysia creates a favourable environment for the proliferation of *Rickettsia*-infected ticks and fleas. An increased exposure to these vectors and its reservoirs, therefore, pose a zoonotic threat to the local population, especially animal handlers, and farm workers. This preliminary study has yet to establish the competency of the goat and sheep as reservoirs of this emerging pathogen however, the molecular evidence of rickettsioses in these samples introduces concern over the complexity of the host-reservoir system of this bacterium.

Declarations

Availability of data and material

The corresponding author declares that all information as regards this study is available online for public view.

Code availability

Not applicable.

Author' contribution

Conceived or designed study: VLL, TKT, SAB, PC, HNQN, MSFH, NHA, MNN, YAIL. Performed research: VLL, SSA, TKT, PB. Analyzed data: VLL, SSA, TKT, PB. Contributed new methods or models: SAB, PC, HNQN, MSFH, NHA, MNN, YAIL. Wrote the paper: VLL, SSA, TKT

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Statement of animal right

This study was approved by the Department of Veterinary Services, Malaysia [Reference Number: JPV:BPI/100-1/12JLDIXXXX(15)]

Consent to participate

Not applicable.

Consent for publication

Permission to publish this paper was obtained from the Department of Veterinary Services, Malaysia

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Dendrogram depicting the phylogenetic position of *Rickettsia* detected from the goat (G325) and sheep (S102) sample with other published *Rickettsia* species (based on comparison of *gltA* gene sequences).

The dendogram was constructed using the neighbor-joining method with Kimura's two parameter model of substitution (K2P distance). *Rickettsia typhi* (U59714) was used as an outgroup for comparison.