First report of *Rickettsia asembonensis* in small ruminants

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Abstract

Rickettsioses is a group of emerging infectious diseases in Southeast Asia caused by Gram-negative obligate intracellular bacteria in the Rickettsiae tribe. However, there is limited information regarding the vertebrate hosts of *Rickettsia* spp. in this region. This study aims to detect and identify *Rickettsia* agents present in wildlife and domesticated animals in Malaysia using Polymerase Chain Reaction (PCR) and sequencing of citrate synthase gene (*gltA*), followed by genotyping and phylogenetic analysis. *Rickettsia* sp. was detected in 2 (0.67%) of 300 wildlife and domesticated animal blood samples. The positive samples were derived from a goat (5.56% of 18) and a sheep (2.22% of 45). 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 to a wider range of animals, specifically farm animals. Therefore, this bacterium may pose a zoonotic threat to the local community particularly to the farmworkers or animal handlers. The low infection rate of this pathogen across different animals highlighted the need of continuous surveillance of emerging and reemerging pathogens among animal populations.

Keywords Infectious diseases · Rickettsial · Zoonotic pathogens · Malaysia

Introduction

Rickettsioses is a neglected infectious disease in Southeast Asia despite being one of the leading causes of treatable unrecognized febrile illnesses (Aung et al. 2014; Low et al. 2020). Notably, *Rickettsia typhi* and some members of spotted fever group *Rickettsia*, exemplify important species that cause 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 (reviewed in Low et al. 2020). Essentially, human infections reported in Southeast Asia were frequently associated with wildlife, livestock, and companion animals (Edouard et al. 2014; Kho et al. 2017; Lynn et al. 2018; Tay et al. 2019). 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, farmworkers and agricultural workers who are at higher risk of developing these tick- and flea-borne diseases. Accordingly, this study aimed to detect and genotype...
**Materials and methods**

 Archived blood samples of wildlife and domesticated animals received by the Veterinary Research Institute (VRI), Department of Veterinary Services, Malaysia in 2018 were stored at −20 °C prior to extraction. Of these samples, 15 animals comprising tiger, cattle, goat, and horse presented clinical symptoms and signs such as lack of appetite, paleness, diarrhea, nasal discharge and weight loss. Other animals were physically healthy, and they were sent for routine blood protozoa screening. 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 stored at −20 °C.

*Rickettsia* DNA detection was performed using conventional polymerase chain reaction (PCR) with primers CS-239 (5′-GCT CTT CTC ATC TTA TGG CTA TTA T−3′) and CS-1069 (5′- CAG GGT CTT CGT GCA TTT CTT 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, positive control (*R. asembonensis* DNA from cat flea) and negative control (distilled water) were 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 (Apical Scientific Sdn Bhd, Kuala Lumpur, Malaysia) 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 phylogenetic tree was constructed to determine the phylogenetic position of *Rickettsia* identified in this study using the maximum likelihood (ML) method with the MEGA X software (Kumar et al. 2018). The ML bootstrap values were evaluated using 1000 replicates with Tamura-Nei model. *R. typhi* (U59714) was used as an outgroup.

**Results and discussion**

Of 300 animal blood samples provided by the diagnostic laboratory, 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 samples originated from a private and government farm, respectively, were sent for microscopic screening for detection of blood protozoa. Our records state that the goat sample presented symptoms such as lack of appetite, paleness, and weight loss while the sheep sample did not show any clinical symptoms.

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 dog flea *Ctenocephalides canis* in Peru, distinct from the original Kenya strain (Maina et al. 2016). The ML tree constructed based on the gltA gene in Fig. 1 revealed that both sequences G325 and S102, clustered with the respective reference *R. asembonensis* sequences including *R*. sp. RF2125 retrieved from the NCBI GenBank and showed a close relationship with *Candidatus* Rickettsia 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. Interestingly, this genotype differed from those detected from *Rhipicephalus linnaei* (formerly known as *Rhipicephalus sanguineus* s.l.) (Fig. 1) and *Ctenocephalides orientis* in Malaysia (Low et al. 2017).

*Rickettsia asembonensis* is a newly identified species (Maina et al. 2016), closely related to *Rickettsia 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; Foongladda et al. 2011; Tay et al. 2014). 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 (Reeves et al. 2005; Oteo et al. 2014; Tay et al. 2014; Maina...
et al. 2016). In Malaysia, *Rickettsia* sp. RF31, a strain of *Ca. R. senegalensis* and *Rickettsia* sp. RF2125, a strain of *R. asembonensis* have been reported in fleas (*C. felis* and *Cycas orientis*) and tick (*Rhipicephalus linnaei*) parasitizing dogs and cats in several studies (Low et al. 2017; Mokhtar and 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. Thus far, 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 with these programs 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 laboratory 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).

**Fig. 1** Dendogram 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.
Nevertheless, Labruna et al. (2004) reported that amplification of an 800-bp gltA fragment by conventional PCR is not as effective as amplifying a 147-bp gltA fragment by Real-time PCR, thus a false negative result from the screened samples cannot be ruled out. Further, 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 \textit{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 farmworkers or handlers with the animals. However, human pathogenesis of \textit{R. asembonensis} remains unclear. There is an urgency to identify the hosts responsible for transmission since human infections caused by this species of \textit{Rickettsia} have been reported. For instance, \textit{R. asembonensis} was identified in four patients displaying common febrile symptoms in Peru (Palacios-Salvaterra 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 favorable environment for the proliferation of \textit{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 farmworkers. 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.

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

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

**Code availability** Not applicable.

**Declarations**

**Consent to participate** Not applicable.

**Consent for publication** Permission to publish this paper was obtained from the Department of Veterinary Services, Malaysia.

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

**Conflict of interest** The authors declare that they have no conflict of interest.

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