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Molecular survey of tick microbiome and tick-borne pathogens in ixodid ticks and rodents collected in Sarawak, Malaysian Borneo

（マレーシアボルネオ島サラワク州におけるマダニ微生物叢ならびにマダニおよびネズミ類でのマダニ媒介性病原体の分子調査）

A Dissertation Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

By

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Summary

Malaysia is geographically located in the Southeast Asian region and is divided into Peninsular Malaysia and Malaysian Borneo. Malaysian Borneo consists of Sarawak and Sabah states and is located in Borneo Island with two other countries: Brunei and Indonesia. Sarawak is the largest state in Malaysia and has tropical geography and an equatorial climate. These relatively high daily average temperature and all-year-round humidity climatic conditions are ideal for tick survival. Furthermore, Sarawak has undergone massive forest degradation and fragmentation, mainly because of logging activities and oil palm plantations, and land conversion has been significantly related to increasing emerging or re-emerging zoonotic diseases, including vector-borne diseases.

Ticks are obligate hematophagous arthropods, feeding on a wide range of animal hosts, including humans. It is established that ticks have high public health importance by harboring various pathogenic agents and play a crucial role as a vector in disease transmission to humans and animals. Tick-borne diseases (TBDs), which afflict humans and animals, are caused by infectious agents transmitted by tick bites. These infectious agents transmitted by ticks are tick-borne pathogens (TBPs), which include bacteria, viruses, and protozoa. For example, ticks are known to harbor medically-important bacterial species from a wide range of genera, including *Anaplasma, Ehrlichia, Bartonella*, *Borrelia, Coxiella*, *Francisella*, and *Rickettsia*. In addition, *Babesia* spp. are tick-borne protozoan parasites of the phylum Apicomplexa that infect erythrocytes of mammals and birds. Despite its significant public health and veterinary implications, the tick-borne protozoal diseases caused by *Babesia* spp. have remained understudied in Malaysia. Currently, reported studies in Malaysia have been limited to bovine and canine babesiosis, with the detection of *B. bigemina* and *B. bovis* in cattle, and *B. gibsoni* and *B. vogeli* in dogs, as well as from the ticks infesting these animals.

In Malaysia, TBDs are regarded to be a potential emerging threat to public health. Thus far, tick bite cases and TBDs were rarely being reported in Malaysia, which could be expected due to a general lack of awareness of TBDs. Based on a survey conducted in 2013 on some farms in Peninsular Malaysia, it was revealed that a large number of farmworkers, including administrative workers, had experienced tick bites. With that remark, the risk of exposure to tick bites may generally be underestimated in Malaysia.

Likewise, studies eliciting tick microbiome are limited in Malaysia and not reported from Sarawak, Malaysia Borneo. The tick microbiome consists of largely non-pathogenic microorganisms essential for tick survival, development, and reproduction. Ticks acquire symbiotic microorganisms via the environment, blood meal hosts, and parents by transtandial and transovarial routes. The presence of a

high abundance of non-pathogenic organisms in ticks suggests a constant interaction between the tick, pathogen, and endosymbionts. Therefore, understanding the microbial structure in ticks may be crucial to unravel the dynamics of pathogen colonization and transmission.

Taken together, the status of TBPs in Malaysia warrants further investigation, especially in Sarawak state that has undergone massive landscape alteration in past decades due to the logging and oil palm plantations. In addition, precise identification of tick species is crucial for epidemiological investigation, prevention, and control of TBDs in general. Obtaining baseline information and insights on tick microbiome and TBPs from Sarawak, Malaysian Borneo, is pivotal for the direction and design of upcoming research from this region, for example, by pinpointing the TBPs that required immediate attention. Therefore, this study aimed to conduct a molecular survey on different tick and rodent species collected from primary forests and an oil palm plantation in Sarawak, Malaysian Borneo (Figure 1).

The present thesis consists of five chapters. In chapter I, tick species collected from flagging and rodent hosts were identified with morphological and molecular methods. In chapter II, rodents and *Ixodes* ticks were screened for *Borrelia* spp., followed by species characterization using multilocus sequence analysis. In chapter III, high-throughput screening using Next-generation sequencing (NGS) for tick microbiome and tick-borne bacterial pathogens was conducted, followed by the multi-species comparative analysis of tick microbiome. In chapter IV, a molecular survey of tick-borne bacterial pathogens was conducted in rodents. Finally, in chapter V, the tick-borne protozoal screening on *Babesia* was carried out on rodent and tick samples.

Overall, this thesis contributed to the imperative information and insights on tick species, tick microbiome, and tick-borne pathogens in Sarawak, Malaysian Borneo. This data is fundamental and valuable for the upcoming research planning from this region.

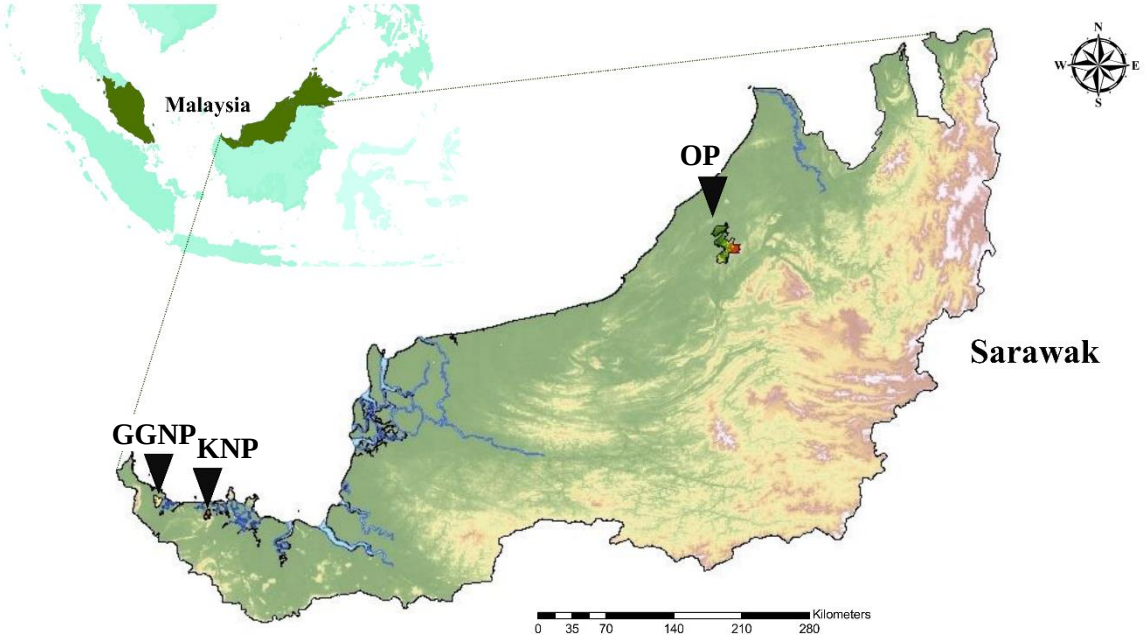

Figure 1. Map of the sampling sites. GGNP: Gunung Gading National Park; KNP: Kubah National Park; and OP: Oil palm plantation.

Chapter I

Tick species identified from primary forests and an oil palm plantation in Sarawak, Borneo

Precise identification of tick species is essential for TBP investigation and effective TBD control and prevention. However, tick species distributed in Sarawak, Borneo remains understudied. Thus, this study aimed to identify the tick species collected from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak with morphological and molecular methods. A total of 238 ticks collected from flagging and rodent hosts were first morphologically identified up to their genus or species level based on the available taxonomic keys. Molecular identification of ticks was conducted based on three genes, 16S rDNA, *CO1* gene, and 12S rDNA. Overall, nine species of four genera were collected from GGNP, KNP, and OP plantation, in which seven species, *Ixodes granulatus*, *Haemaphysalis hystricis*, *H. shimoga*, *Dermacentor compactus*, *D. steini*, *D. atrosignatus*, and, *Amblyomma testudinarium*, were successfully identified up to species level. However, one *Haemaphysalis* sp. was not be resolved to its species level. Furthermore, one *Amblyomma* sp., which revealed as closely related to *A. geoemydae* by molecular phylogeny, did not fully match the morphological description for the species. Nevertheless, this study provides the molecular evidence of multiple tick species in Sarawak, with the first report of *H. shimoga* in Malaysia. Overall, tick morphological and molecular data is crucial, especially for Southeast Asian countries like Malaysia, which will be beneficial for accurate identification of tick species in the future.

Chapter II

Detection of *Borrelia burgdorferi* sensu lato and Relapsing fever *borrelia* in feeding *Ixodes* ticks and rodents in Sarawak, Malaysia: New geographical records of *Borrelia yangtzensis* and *Borrelia miyamotoi*

Members of the *Borrelia* burgdorferi sensu lato (Bbsl) complex are etiological agents of Lyme disease (LD), and *Borrelia miyamotoi* is one of the relapsing fever *Borrelia* (RFB). Despite the serological evidence of LD in Malaysia, there has been no report from Sarawak, Malaysian Borneo. Thus, this study aimed to detect and characterize *Borrelia* species in rodents and *Ixodes* ticks from primary forests and an oil palm (OP) plantation in Sarawak. *Borrelia yangtzensis* (a member of the Bbsl complex) was detected in 43.8% (14/32) of *Ixodes granulatus*, and most of the positive ticks were from the OP plantation (13/14). Out of 56 rodents, *B. yangtzensis* was detected in four *Rattus* spp. from the OP plantation, and *B. miyamotoi* was detected in one rodent, *Sundamys muelleri*, from the primary forest. Furthermore, the positive samples of *B. yangtzensis* were randomly selected for multilocus sequence analysis (MLSA). The MLSA results of successfully amplified tick samples revealed a clustering with the sequences isolated from Japan and China. This study is the first evidence of *B. miyamotoi*, a known human pathogen in Malaysia, and *B. yangtzensis*, which is circulating in ticks and rodents in Sarawak, Malaysian Borneo, and presenting a new geographical record of the *Borrelia* spp.

Chapter III

Insights on the microbiome and tick-borne pathogens of ixodid ticks in Sarawak, Malaysian Borneo

Ticks harbor a high abundance of symbiotic and commensal microorganisms that can be obligate or facultative presence. The obligate endosymbionts are maternally inherited microorganisms essential for tick survival and development. Facultative endosymbionts, on the contrary, may be involved in the manipulation of the tick immune system and reproduction. Furthermore, understanding the interaction between non-pathogenic microorganisms and pathogens in ticks may be fundamental for the control measures. There has been few studies on ticks and tick-borne pathogens, and no tick microbiome study published up-to-date from Sarawak, Borneo. Thus, this study aimed to perform the microbiome and pathogen screening of each tick species collected from two primary forests and an oil palm plantation by employing Next-generation sequencing (NGS). A total of 210 feeding and questing ticks consisting of different life stage and status of *Ixodes granulatus* (n = 32), *Haemaphysalis hystricis* (n = 36*), H. shimoga* (n = 110), *Dermacentor compactus* (n = 4), *D. steini* (n = 24), and *D. atrosignatus* (n = 4) were included for NGS. The 16S rRNA gene V3-V4 hypervariable regions were targeted, and sequencing was conducted on an Illumina MiSeq platform. The following bacteria: *Anaplasma, Ehrlichia, Bartonella, Coxiella, Francisella,* and *Rickettsia,* were verified with conventional PCR and sequencing. Data analyses were performed in QIIME 2, Decontam package and vegan package in R software, and Huttenhower lab Galaxy pipeline. Tick samples were categorized into three groups for microbial analysis. The first group included all ticks from their available life stages. The second group consisted of only the adult stage ticks because not all tick species collected had all life stages represented (*H. hystricis* excluded). Finally, since *H. shimoga* had the most comprehensive samples, the effect of different developmental stages and feeding statuses for this species were examined. The study findings revealed that microbial variations were significant between tick species. From the multispecies comparison, genus *Dermacentor* had the highest microbial diversity, while *H. shimoga* had greater microbial composition differences than other tick species. Factors contributing to the variations included the development stage, and potentially blood meal feeding may have played a role in shaping the microbiome profile. Further investigation with *H. shimoga* revealed the ontogenic and sex variations affecting microbial composition, with some bacterial taxa found more represented in one developmental stage than another. Most tick species in this study harbored one or multiple symbionts, except for *D. compactus* and *D. atrosignatus*. Finally, the microbial structure could be affected by the presence of specific bacteria taxa in high abundance, evidenced by *Borrelia*-positive

and negative *I. granulatus*. This study also managed to characterize human and animal pathogens from genera *Coxiella*, *Francisella*, *Rickettsia*, *Anaplasma*, *Ehrlichia*, and *Bartonella*. Amongst, *Coxiella*-like endosymbiont from *H. shimoga* and *Francisella*-like endosymbionts from *D. steini* and *I. granulatus* were characterized. Furthermore, human pathogens such as *Rickettsia heilongjiangensis* and *Ehrlichia* sp. closely related to *E. ewingii* were also identified. This study is the first initiative to outline different tick microbiome profiles in addition to the pathogens screening from this region and included tick species that have never been studied previously. More works are required to unravel the factors associated with the variations we observed in this study.

Chapter IV

Molecular survey of tick-borne bacterial pathogens in rodents collected in Sarawak

Worldwide, tick-borne diseases are a persistent growing problem at the One Health interface. The role of rodents has been highlighted as important reservoir hosts in maintaining and circulating tick-borne pathogens. In this study, a molecular survey was conducted on the rodents collected from two primary forests (GGNP and KNP) and an oil palm (OP) plantation in Sarawak, Borneo, for the tick-borne bacteria from the following genera, *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Francisella*, and *Rickettsia*. Overall, 55 rodent spleens were screened with PCRs. As a result, *Anaplasma bovis* and *Ehrlichia chaffeensis* were detected based on 16S ribosomal DNA, three *Bartonella* spp., namely *B. rattimassiliensis*, *B. phoceensis*, and *B. coopersplainsensis*, were confirmed with transfer messenger RNA, *ssrA* gene, and *Coxiella sp.* closely related to *C. burnetii* was detected by using the chaperone protein, *groEL* gene. None of the rodent samples were positive for *Francisella* and *Rickettsia* spp. Nevertheless, this study provides the first evidence of *E. chaffeensis*, a causative agent of human monocytic ehrlichiosis in Malaysia. In addition, potentially *C. burnetii*, a causative agent for Q fever in humans, was also identified in two rodent samples. Furthermore, all positive samples belonged to rodent species collected from the oil palm plantation, *Rattus* spp., except for one *Bartonella*-positive sample identified in *Sundamys muelleri* from a primary forest. Despite the small sample size limitation, this is significant evidence that the rodent species in Sarawak harbored multiple causative agents, in which *E. chaffeensis* and potentially *C. burnetii* pose zoonotic threats to public health. These findings warrant more investigation to determine the prevalence and infection risk in the next step.

Chapter V

Detection of a *Babesia* sp. genotype closely related to marsupial-associated *Babesia* spp. in *Haemaphysalis shimoga* from Sarawak, Borneo

Babesia spp. are tick-borne Apicomplexan parasites that infect erythrocytes of mammals and birds. Since their discovery, over 100 *Babesia* species have been reported worldwide from a vast range of vertebrate hosts. However, research into *Babesia* spp. in Malaysia has been limited to dogs and livestock and the ticks collected from these animals. Thus, we investigated *Babesia* spp. in different rodent and tick species collected from primary forests and an oil palm plantation in Sarawak, Borneo. A total of 55 rodents and 160 questing and engorged ticks were included for *Babesia* spp. detection by PCR targeting the 18S ribosomal DNA. The presence of *Babesia* spp. DNA were detected in two questing *Haemaphysalis shimoga* collected from the oil palm plantation. Sequence analysis revealed that both sequences obtained were identical and showed 98.6% identity with *Babesia macropus* detected from eastern grey kangaroos (*Macropus giganteus*) in Australia. Phylogenetic tree showed that the *Babesia* sp. in this study and marsupial-associated *Babesia* spp. clustered together and were positioned in the *Babesia* sensu stricto clade. Adult *H. shimoga* ticks are known to feed primarily on sambar deer (*Cervus unicolor*), but other hosts such as cattle, sheep, goats, and humans have also been documented. Furthermore, *Babesia* spp. of the *Babesia* sensu stricto clade are capable of transovarial and transtandial transmission, which increase the capacity of *Babesia* transmission in ticks. Whether or not *H. shimoga* is the competent vector and the importance of the *Babesia* sp. detected in this study warrants more investigation.