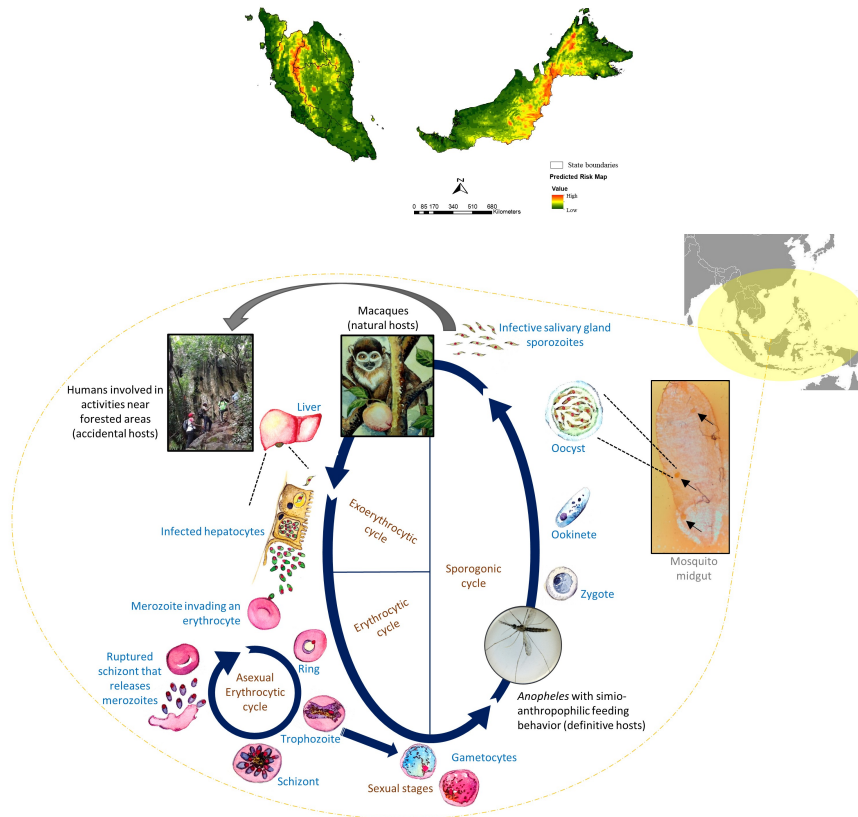


RESEARCH REPORT



Geographical distribution and bionomics of the vectors of *Plasmodium knowlesi* malaria with reference to malaria elimination in P. Malaysia

Prof. Datin Dr. Indra vythilingam, Assoc. Prof. Dr. Low Van Lun,
Dr. Wan Yusoff bin Wan Sulaiman, Assoc. Prof. Dr. Romano Ngui and Dr. Jenarun Jelip
Dept. Of Parasitology, Faculty of Medicine, Universiti Malaya
indrav@um.edu.my

ABSTRACT (120 words)

Plasmodium knowlesi, a simian malaria parasite is the predominant species being reported in Malaysia. Vector surveillance is essential in determining the geographical distribution of the vectors and to understand the dynamics of malaria transmission. Our study revealed that the *Anopheles* Leucosphyrus Group mosquitoes are highly competent vectors, and they represent a risk of human infection with zoonotic simian malaria. *Plasmodium cynomolgi* and *P. inui* were highly prevalent in mosquitoes collected from the present study and demonstrated close relatedness with those from the vertebrate hosts, suggesting ongoing transmission between vectors, macaques, and humans. This study provides a quick, fast, specific, and effective multiplex PCR test for detecting the four known Leucosphyrus Group species vectors. This assay has been confirmed to be specific for four species. Additionally, the predictive vector map correlates well with knowlesi malaria cases. This research is crucial in informing and supporting future efforts by healthcare professionals to develop effective malaria control interventions.

1. INTRODUCTION

Despite increasing cases of knowlesi malaria and the possibility for the emergence of other zoonotic simian malaria in Malaysia, there is limited vector studies to understand the transmission dynamic of this disease. Previous vector studies in Malaysia had focused only on few localities in Peninsular Malaysia, while concentrated primarily on Malaysian Borneo due to the relatively high number of knowlesi malaria cases. Besides, due to the widespread use of PCR to detect the presence of *Plasmodium* parasites, many vector studies presently do not include mosquito dissection, leaving out crucial information such as mosquito parity rate, which is the key index to estimate mosquito longevity and vector competency. Thus, there is a major knowledge gap which is still unanswered especially on the entomological characteristics of the vectors in Peninsular Malaysia. For an extensive entomological study, an effective mosquito collection tool is necessary. Unfortunately, the best collection tool for *Anopheles* mosquito, vectors of simian malaria is still undetermined. Thus, trap comparison study is necessary to determine the trap efficiency in collecting the local *Anopheles* mosquitoes. Besides, there is a paucity of information regarding the blood meal preference of the local vectors. Lack of information regarding the entomological characteristics and the hematophagic preferences of the vector especially in Peninsular Malaysia are some of the issues which still need a clear answer. To the best of my knowledge, there was no previous study on the blood meal preference of field caught *Anopheles* mosquitoes in Peninsular Malaysia, mainly due to the difficulties in finding resting *Anopheles* mosquitoes in forested areas. All these entomological parameters are very important, especially considering that numerous development activities are taking place along the forest fringes in Peninsular Malaysia, increasing human contact with macaques, the host of simian malaria. Furthermore, there is also very limited information available regarding the prevalence and genetic diversity of the simian *Plasmodium* from the mosquitoes.

Correct vector identification is crucial to incriminate the vector. Thus, expertise is needed for proper identification, especially in the Leucosphyrus Group, whose morphological characters are very similar. DNA-based techniques play an ever-increasing role in the study of malaria transmission and are also used to identify vectors. Many of the main malaria vectors belong to complexes or groups of cryptic species that cannot be identified easily using morphological markers. For many of these species complexes, the problem of vector identification has been resolved by developing species-specific genomic DNA probes and polymerase chain reaction (PCR) technologies. With PCR identification, the region of the genome that has received considerable attention is the ribosomal DNA (rDNA) [1]. The rDNA internal transcribed spacer (ITS) regions have been informative when examining different taxa in Diptera [2-4] and subsequently used for phylogenetic analysis [4].

Besides correct identification, the distribution of the vectors throughout the country needs to be addressed. Overall, the spatial distribution of vectors is influenced by climatic and land cover factors [5]. Understanding the spatial distribution of vectors is vital not only for detecting cases but also for effectively incorporating control strategies. Due to environmental factors, the distribution of various species may differ from one another. Different behaviours such as biting times or breeding sites may influence vector capacity and adaptation to changing environments [6]. Remote sensing techniques provide valuable information on such environmental conditions. Several studies have used remote sensing imagery to map vector species distribution at different spatial scales. It helps in analysing the relationship between the environment and potential vector distribution, thereby providing vital information on the risk of simian malaria emergence and a better understanding of the ecology of the invasive vector species. This information will assist in predicting future potential zoonotic malaria cases. This study aims to determine and quantify the spatial distribution of the Leucosphyrus Group of *Anopheles* population in Malaysia from different habitats.

2. RESEARCH METHODOLOGY

The methodology of the current project comprised seven main studies based on the seven specific research objectives. Study 1 involved evaluation of Mosquito Magnet against different trapping methods in collecting *Anopheles* mosquitoes. On the other hand, Study 2 aimed to carry out entomological investigation on the simian malaria vectors at the longitudinal study locations in Peninsular Malaysia and to identify malaria parasites in all the positive *Anopheles* mosquitoes. Besides entomological parameters, the hematophagous behaviour of the *Anopheles* mosquitoes was also investigated using blood meal analysis under Study 3. In addition, molecular study was conducted under Study 4 to characterize the *18S SSU rRNA* gene of simian malaria parasites found in the *Anopheles* mosquitoes collected throughout the study in Peninsular Malaysia. In study 5, multiplex PCR was carried out to identify the vector species, while in study 6, *COI* and *ITS2* genes were sequenced for *An. balabacensis*, *An. cracens*, *An. introlatus*, and *An. latens* specimens to study the genetic diversity of the vectors. In study 7 Geospatial analysis of *Anopheles* mosquitoes using the Geographic Information System (GIS) and Remote Sensing (RS) Derived Environmental Data.

The overall studies were carried out in seven different states in Peninsular Malaysia, namely Johor, Kedah, Kelantan, Negeri Sembilan, Pahang, Perak and Selangor between June 2019 and March 2022. A total of 81 sampling locations were selected to collect *Anopheles* mosquitoes from various districts in the seven states of Peninsular Malaysia as shown in Figure 1. The sampling locations were selected based on the past human cases of knowlesi malaria and through discussion with the district health officers. The areas were scouted to check if they were suitable for mosquito collections as well as for the presence of macaques.

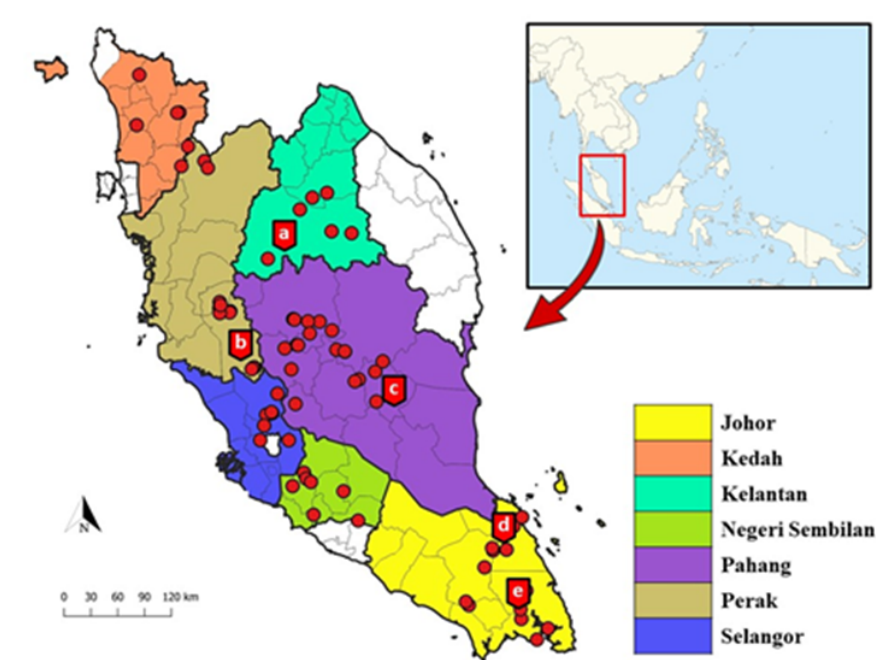


Figure 1. Map of Peninsular Malaysia showing the mosquitoes sampling locations (marked in red dots) with the longitudinal sites for entomological investigations (a) Kampung Lalang, Gua Musang Kelantan, (b) Sungai Dara, Muallim, Perak, (c) Kem Sri Gading, Jengka Pahang, (d) Bukit Tinggi, Mersing Johor and (e) Gunung Panti, Kota Tinggi Johor.

Study 1

A randomized 4 x 4 Latin square designed experiment was conducted to compare the efficiency of the Mosquito Magnet against three other common trapping methods; human landing catch (HLC), CDC light trap and human baited trap (HBT). The experiment was conducted over six replicates where sampling within each replicate was carried out for four consecutive nights. Additional four nights of sampling were used to further evaluate Mosquito Magnet against the “gold standard” HLC. The abundance of *Anopheles* sampled by different methods was compared and evaluated with focus on the *Anopheles* from the Leucosphyrus group; the vectors of knowlesi malaria. For detailed methodology please refer to [7].

Study 2

We conducted longitudinal studies to investigate the entomological parameters of the simian malaria vectors and to examine the genetic diversity and evolutionary pattern of their simian *Plasmodium*. All the captured *Anopheles* mosquitoes were dissected to examine for the presence of oocysts, sporozoites and to determine the parous rate. The mosquitoes were examined for the presence of sporozoites in the salivary glands and for oocysts in the midgut. Genomic DNA was extracted from the parasite-positive guts and glands using the DNeasy tissue kit (Qiagen, Germany) according to the manufacturer’s protocol. Nested PCR assay was performed targeting the *Plasmodium* small subunit ribosomal RNA (18S rRNA) gene to identify human malaria parasites (*Plasmodium falciparum*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. vivax*) and simian *Plasmodium* (*P. coatneyi*, *P. cynomolgi*, *P. fieldi*, *P. inui* and *P. knowlesi*) using genus-specific primers rPLU 1 and rPLU 5 for the nest 1 amplification [8], followed by species-specific primers in the nest 2 amplification [9-11]. The amplification products were analyzed using 1.5% agarose gel electrophoresis. Cloning and sequencing of *Plasmodium* 18S SSU rRNA gene fragments and Sequence editing, alignment and phylogenetic analysis were also carried out as described in [12]. Haplotype network and population genetic analysis was carried out as described in [12].

Study 3

Laboratory based experiments were conducted in three parts to evaluate the potential use of mosquitoes caught using HLC from the field for blood meal analysis. Firstly, meal preference study was conducted to investigate the possibility of the *An. cracens* (laboratory strain) to uptake second blood meal within a single gonotrophic cycle. Secondly, the rate of blood digestion in the mosquito was observed through amplification of host DNA ingested by *An. cracens* through a time course of every 12 hours for 96 hours using PCR assay for both human and monkey blood. Finally, multiple blood feeding analysis was conducted using monkey and human blood to investigate if the PCR assay was able to detect multiple blood meals. Details of the blood meal detection can be found in [13].

Study 4

All the mosquitoes detected positive for the five simian *Plasmodium* were subjected to nested PCR targeting longer fragments of the 18S SSU rRNA gene for molecular characterisation. Since SSU rRNA gene is a widely used gene marker in eukaryotic phylogeny, there is an abundance of *Plasmodium* species sequence data available [14]. Thus, molecular characterisation of the SSU rRNA gene of the simian *Plasmodium* isolated from mosquitoes will allow comparison of

sequence data from both human and macaques to better understand the transmission dynamic of the zoonotic simian malaria in this region.

Study 5

All *Anopheles* mosquitoes from the Leucosphyrus Group obtained in this study were further molecularly characterised using internal transcribed spacer 2 (*ITS2*) and mitochondrial cytochrome c oxidase subunit I (*COI*) genes. The *ITS2* gene was amplified by ITS2A and ITS2B primers [15] while LCO1490 and HCO2198 primers [16] were used for *COI* gene. DNA was extracted from the mosquitoes' legs using InstaGene Matrix (Bio-Rad, California, USA), according to the manufacturer's protocol and some were extracted using DNeasy® tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. Details of the design of the primers and the multiplex primers can be found in this reference [17].

Study 6

The study included sequences of mosquitoes collected from Peninsular Malaysia and Sarawak, while additional sequences from Sabah and Selangor were retrieved from the NCBI GenBank, providing a comprehensive representation of mosquito populations across different regions of Malaysia. *COI*, *ITS2* and combined sequences of each species were aligned using BioEdit (Version 7.2). Haplotype networks for *An. balabacensis*, *An. cracens*, *An. introlatus*, and *An. latens* based on their polymorphic sites were constructed by using the median-joining method in NETWORK version 5.0.0.1 software (Fluxus Technology LTD Suffolk, UK). Details of the methods can be obtained from [18].

Study 7

This study used vector data from various sources to create the distribution maps from 1957 to 2021. A predictive statistical model utilizing logistic regression was developed using significant environmental factors. Interpolation maps were created using the inverse distance weighted (IDW) method and overlaid with the corresponding environmental variables. Details of the methods can be found in [19].

3. LITERATURE REVIEW

Although Malaysia has successfully reached zero indigenous human malaria cases since 2018, the country faces another major challenge with zoonotic simian malaria parasites, *P. knowlesi* [20]. In recent years, as cases of human malaria were being reduced, more cases of *P. knowlesi* malaria were detected. Malaysia has reported one of the highest numbers of *P. knowlesi* cases in Southeast Asia [21] with the drastic reduction of human malaria cases. Currently, *P. knowlesi* is the predominant species being reported in Malaysia. In general, most *knowlesi* malaria cases reported in Malaysia are largely confined to Malaysian Borneo (Sabah and Sarawak). Although the burden of human malaria had decreased substantially in Malaysia due to effective malaria control program, but the presence of the fifth species, *P. knowlesi*, had caused the number of malaria cases to increase in Malaysia in the past years.

Besides *knowlesi* malaria, Malaysia had also reported cases of other zoonotic simian malaria infections caused by *P. cynomolgi*. The first reported case of natural transmission of *P. cynomolgi* was from Hulu Terengganu, east coast of Peninsular Malaysia [22]. A 39-year-old Malay woman

was initially diagnosed with malaria caused by *P. malariae*/*P. knowlesi* through microscopic examination of the blood film. However, later when the sample was screened in Malaria & Emerging Parasitic Diseases Laboratory (MAPELAB, Spain) using the modified nested multiplex PCR [23], the amplified fragment sequence showed a highest similarity with *P. cynomolgi* sequences (99.9% similarities with *P. cynomolgi* Mulligan strain from Malaysia) [22]. Recently, in 2020, two natural cases of asymptomatic *P. inui* mono-infections were reported in Pahang among students who were undergoing training at a camp site in a forested area [24].

The differences in the species composition and abundance between different ecological niches are indeed compelling. In Kapit Sarawak, *An. latens* was predominantly found in the forested ecotype (62.3%) compared to farming area (35.6%) or area near to the longhouse (29.7%) [25-26]. In Kudat Sabah, *An. balabacensis* was found equally abundant in a wide range of ecotypes with slightly higher number near the forest fringes (22.0%), compared to other ecotypes such as oil palm plantation (20.8%), shrub bushes (19.8%) and longhouse (19.1%) [27]. A separate study on the effect of forest disturbance on the mosquito abundance had revealed that *An. balabacensis* were abundantly found in logged forest compared to virgin jungle reserve or primary forest [28]. This was mainly due to the availability of numerous larval breeding sites created by the wheel tracks left by active vehicle movements in logged areas which serve as temporary breeding grounds for the mosquitoes which are usually absent in primary forest or virgin jungle reserve [29-30].

On the other hand, in Kuala Lipis, Pahang, *An. cracens* was present in high density in fruit orchard (73.5%) followed by forested area (50.3%) and village area (38.5%) [31]. With extensive deforestation and land exploration in Malaysia over the past decades, there has been changes in the bionomic of the mosquitoes. Deforestation has caused the migration of the long-tailed macaques from forested area to farms and semi-urban areas where they usually scavenge for food sources. This factor might have triggered mosquitoes to follow the host and adapted to forest fringes and farm areas [32]. Areas with many shrubs and bushes provide plenty of refuge spaces for the *Anopheles* mosquitoes to rest before and after a blood meal, as well as shades for breeding sites with temporary water bodies after rains.

Given that effective vector control necessitates a thorough understanding of the ecology of breeding and resting habitats as well as the behaviour of the mosquito species, the advancement of a tool with such functionality and high efficiency becomes necessary. The Remote sensing (RS) and the Geographic Information Systems (GIS) are technologies that have emerged in the investigation of malaria epidemiology [33-34]. The main component of spatial technology, GIS, integrates a variety of data sets from various sources, including RS and the Global Positioning System (GPS) [35]. In fact, not only mapping the habitats but also vector densities and disease incidence predictions can be made using GIS, particularly RS [36-38]. The systematic and routine monitoring of the earth's environmental conditions is made possible by these spatial technologies (GIS and RS) [35] and has helped with risk mapping with risk factors using environmental indices, as well as determining the geospatial limits of the disease prevalence [39-40]. In the long term, it is simple to understand how disease prevalence and vector distribution are related. GIS is an information system that incorporates, stores, edits, analyses, shares and visualises spatial or geographically referenced data.

FINDINGS

Latin square designed experiment showed HLC caught the greatest number of *Anopheles* mosquitoes (n=321) compared to HBT (n=87), Mosquito Magnet (n=58) and CDC light trap (n=13). The GLMMs analysis showed that HLC method caught significantly higher number of *Anopheles* mosquitoes compared to Mosquito Magnet ($P = 0.049$). However, there was no significant difference in mean nightly catch of *Anopheles* mosquitoes between Mosquito Magnet and the other two trapping methods; HBT ($P = 0.646$) and CDC light traps ($P = 0.197$). The mean nightly catch for both *An. introlatus* (9.33 ± 4.341) and *An. cracens* (4.00 ± 2.273) caught using HLC was higher than that of Mosquito Magnet, though the differences were not statistically significant ($P > 0.05$). This is in contrast with the mean nightly catch of *An. sinensis* (15.75 ± 5.640) and *An. maculatus* (15.78 ± 3.479) where HLC showed significantly higher number of mosquito catches compared to Mosquito Magnet ($P < 0.05$).

Our study revealed that the *Anopheles* Leucosphyrus Group mosquitoes are highly potential competent vectors, as evidenced by their high rate of parity, survival and sporozoite infections in these mosquitoes. Thus, these mosquitoes represent a risk of human infection with zoonotic simian malaria in this region. Haplotype analysis on *P. cynomolgi* and *P. inui*, found in high prevalence in the *Anopheles* mosquitoes from this study, had shown close relationship between simian *Plasmodium* from the *Anopheles* mosquitoes with its vertebrate hosts. This directly signifies the ongoing transmission between the vector, macaques, and humans. Furthermore, population genetic analysis showed significant negative values which suggest that both *Plasmodium* species are undergoing population expansion.

The laboratory-based experiment from this study revealed that mosquitoes caught using HLC had the potential to be used for blood meal analysis. Besides HLC, mosquitoes were also collected using manual aspirator and Mosquito Magnet. Overall, 47.4% of 321 field-caught *Anopheles* mosquitoes belonging to six species were positive for vertebrate host DNA in their blood meal. The most frequent blood meal source was human (45.9%) followed by wild boar (27.4%), dog (15.3%) and monkey (7.5%). Interestingly, only *Anopheles cracens* and *Anopheles introlatus* (Leucosphyrus Group) fed on monkeys. This study further confirmed that members of the Leucosphyrus Group are the predominant vectors for knowlesi malaria transmission in Peninsular Malaysia mainly due to their simio-anthropophagic feeding behavior.

The “star-like” network observed in all the four vectors suggests population expansion. Furthermore, the unimodal shape and low values of raggedness index and R_2 statistic from mismatch distribution tests along with negative values from the neutrality tests further support the population expansion of *An. balabacensis*, *An. introlatus*, *An. latens* and *An. cracens* in Malaysia. The haplotypes of *An. latens* were separated into two clusters for all three haplotype networks. The haplotype clusters contained sequences from Peninsular Malaysia separated from sequences from Malaysia Borneo. Consequently, it is unknown whether the behavioural and the capability of spreading the simian malaria parasites of these two clusters of *An. latens* mosquitoes are similar or different. Additionally, this study revealed the presence of the two genetically distinct *An. latens* clusters based on the *CO1* and *ITS2* sequences.

Correct identification of *Anopheles* species is essential in malaria vector control programmes because it influences the choice of control intervention and pesticide product. Precise species identification enables assessments of vector competence, insecticide susceptibility, and important behavioural characteristics (such as feeding and resting behaviours) by species, leading

to the design of coherent insecticide-based control strategies that can be supplemented by additional malaria elimination methodologies. This current assay will be useful for molecular identification for the scenario where sequencing was not done for the study. To our knowledge this is the first multiplex PCR designed to identify the four simian malaria vectors of the *An. Leucosphyrus* Group of mosquitoes in Malaysia. Previous studies have used the universal *ITS2 A* and *ITS2 B* primers to identify the species based on band size without sequencing. The multiplex PCR test is fast, inexpensive, specific, and easy to use. Only a small amount of material (1-2 legs) is required for identification, leaving the remainder of the body parts available for further analysis, such as sporozoite detection, blood meal analysis, population genetics, or pesticide resistance status.

Using GIS, this study has outlined the distribution of the *Anopheles* Leucosphyrus Group of mosquitoes in Malaysia. The information gathered from previous literature and current field data is crucial for identifying and managing malaria vectors. The study also suggests this approach could help plan and advance vector management strategies. The results show significant geographic variation in the distribution of *Anopheles*, providing an opportunity to effectively target prevention efforts where they are needed most, particularly with limited resources. Despite limited data in some parts of the country, the interpolation map estimates vector abundance. High vector abundances are observed in Sarawak's southwestern region, Pahang's northern region, and Sabah's northwestern region. Environmental variables such as forest cover, deforestation, elevation, water bodies, and temperature can significantly influence vector abundance. This study found that vector abundance was lower in areas with both high elevation and low temperatures. The high number of vectors noted in the IDW interpolation map was thus influenced by factors such as forest loss, forest cover, elevation, and water bodies and temperature. The predicted high-risk zones for *knowlesi* malaria are mainly around the Titiwangsa range and central-northern region of Peninsular Malaysia. The same pattern was noticed from the predictive vector map from this study. The association between the predictive vector and human case maps would further support the model's accuracy and reliability.

4. CONCLUSION

This study revealed that human landing catch (HLC) remains the best trapping method for catching *Anopheles* mosquitoes for vector surveillance. However, the comparable ability of Mosquito Magnet to catch some of the *Anopheles* species makes it an ethical and safer alternative. Mosquito Magnet, which is less labour intensive can be effectively used to study the vectors of zoonotic simian malaria especially *An. cracens* and *An. introlatus* which are the vectors of *knowlesi* malaria in Peninsular Malaysia. Mosquito Magnet may offer a novel solution to some of the issues associated with the conventional trapping methods. This longitudinal study revealed that the *Anopheles* Leucosphyrus Group mosquitoes, namely *An. cracens*, *An. introlatus* and *An. latens* deemed to be highly competent vectors, as evidenced by its high rate of parity, survival and sporozoite infections in these mosquitoes. However, the relatively low man biting rate of these species of mosquitoes in most of the study locations might be one of the reasons for low number of zoonotic malaria cases reported around the study locations. Nevertheless, these mosquitoes will continue to represent a risk of human infection with zoonotic simian malaria due to its high sporozoite rate. With the presence of competent vectors and a large macaque reservoir, malaria elimination in Malaysia will be greatly challenged. This is especially with the possible emergence of other potential zoonotic simian malaria caused by *P. inui* and *P. cynomolgi*, besides the current increase in *knowlesi* malaria cases. However, extensive entomological studies and *Plasmodium* screening on the vectors in Peninsular Malaysia especially

targeting northern states are still needed where less *Anopheles* samples were collected from this current study.

Besides, the bionomics of the vectors from this study displayed that the current malaria vector control strategies in Malaysia which heavily relied on long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) might not be sufficient to protect against outdoor, early-biting vectors of zoonotic simian *Plasmodium*. New tools are needed for the control of the vectors. The *Anopheles* mosquitoes from the Leucosphyrus Group which take blood meals from both host type, human and macaques, could serve as bridge vectors for *P. knowlesi* and potentially other zoonotic simian *Plasmodium*. This is the first such study in Malaysia. With the rapid changes in the landscape and the potential emergence of other zoonotic simian malaria, blood meal analysis would be instrumental in monitoring the hematophagous behavior of the local *Anopheles* mosquitoes.

With constant microevolutionary processes, there is potential for both *P. inui* and *P. cynomolgi* to emerge and spread as a major public health problem, following the similar trend of *P. knowlesi*, as the four classical human malaras are reduced, and environmental change brings human into increased contact with simian malaria. Thus, with the presence of competent vectors and high prevalence of these zoonotic simian *Plasmodium* in the *Anopheles* mosquitoes as shown in this study, might possess a great challenge in malaria elimination program in Malaysia. Therefore, the early recognition and containment of transmission of these zoonotic simian malaria among humans should be given high priority.

This study provides a quick, fast, specific, and effective multiplex PCR test for detecting the four Leucosphyrus Groups that are known to be simian malaria vectors. This reliable identification technique will enable a wide variety of investigations on the Leucosphyrus Group of species. Furthermore, multiplex PCR can aid in identification if sequencing technology is unavailable. Correct species identification is critical in all downstream works concerning the species in question, especially for malaria vector control programmes.

The application of GIS and RS approaches in data storage, mapping, and analysis for developing vector maps was also demonstrated in this study. A further benefit of this approach is that it enables us to model the spatial distribution of the simian malaria vectors in relation to environmental variables derived from RS satellite data and known to affect their distribution patterns. This allows a better understanding of the vector habitats. Identifying endemic areas offers valuable information on developing the vector control intervention more precisely and effectively.

In conclusion, besides *P. knowlesi*, other simian *Plasmodium* such as *P. cynomolgi* and *P. inui* may soon be a public health concern in Malaysia and other neighbouring countries as the natural environment of the hosts and vectors are disturbed, as well as the evolution of these *Plasmodium* parasites itself could drive the transmission of this emerging zoonotic disease. With extensive deforestation that drastically changes the landscape coupled with climatic changes may have an impact on these new zoonotic *Plasmodium* transmission dynamics as an emerging cause of malaria in humans. More research utilising molecular and multidisciplinary techniques are needed to detect these simian *Plasmodium* parasites in humans, vectors and natural hosts if human infections with *P. cynomolgi* and *P. inui* do become public health concerns in future. The dynamic of zoonotic simian malaria transmission is indeed complex. Thus, gaining a better understanding on the interaction between environment, mosquito vectors, macaques hosts, human malaria disease and demography will aid in the implementation of effective control

measures in a rapidly changing environment. It is believed that the simian malaria might emerge extensively when human malaria is eradicated in future. As these simian *Plasmodium* parasites can successfully adapt to a wide range of hosts in tropical and sub-tropical regions worldwide, they could evade control measures that do not account for the animal reservoir. The importance of early finding and containment of transmission of these simian malaria among humans in the event of a complete emergence into the human population should not be taken lightly. Since there are already evidences of simian malarias other than *P. knowlesi* being transmitted to humans, proactive measures are needed to prevent escalated risk of infection in the future.

ACHIEVEMENT

i) Name of articles/ manuscripts/ books published

1. Low,VL., Wong,ML., Liew, JWK., Pramasivan, S.,Jeyaprakasam, NK., and Vythilingam,I. (2019). Gender beyond male and female: Occurrence of a gynandromorph in the Japanese encephalitis vector *Culex sitiens* (Diptera: Culicidae). *Acta Tropica*, 201, 1-3
2. Wong ML, Liew JW, Wong WK, Pramasivan S, Hassan NM, Sulaiman WY, Jeyaprakasam NK, Leong CS, Low VL, Vythilingam I. Natural Wolbachia infection in field-collected *Anopheles* and other mosquito species from Malaysia. *Parasites & Vectors*. 2020 Dec;13(1):1-5.
3. Jeyaprakasam NK, Liew JW, Low VL, Wan-Sulaiman WY, Vythilingam I. *Plasmodium knowlesi* infecting humans in Southeast Asia: What's Next? *PLOS Neglected Tropical Diseases*. 2020 Dec 31;14(12):e0008900.
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5. Vythilingam, I., Chua, T.H., Liew, J.W.K., Manin, B.O and Ferguson, H., 2021. The vectors of *Plasmodium knowlesi* and other simian malarias Southeast Asia: challenges in malaria elimination. *Advances in Parasitology* 2021;113:131-189.
6. Pramasivan, S., Ngui, R., Jeyaprakasam, N.K., Liew, J.W.K., Low, V.L., Mohamed Hassan, N., Wan Sulaiman, W.Y., Jaraee, R., Abdul Rahman, R., Jelip, J. and Vythilingam, I. Spatial distribution of *Plasmodium knowlesi* cases and their vectors in Johor, Malaysia: in light of human malaria elimination. *Malaria Journal*, 2021 Dec;20(1):1-2.
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8. Sandhya Pramasivan, Jonathan Wee Kent Liew, Nantha Kumar Jeyaprakasam, Van Lun Low, Romano Ngui, and Indra Vythilingam. Multiplex PCR Assay for the Identification of Four Species of the *Anopheles Leucosphyrus* Sub-Group in Malaysia. *Insects* 2022; 13:95
9. Pramasivan S, Low VL, Jeyaprakasam NK, Liew JW, Ngui R, Vythilingam I. Cryptic Diversity and Demographic Expansion of *Plasmodium knowlesi* Malaria Vectors in Malaysia. *Genes*. 2023 Jun 28;14(7):1369.
10. Jeyaprakasam NK, Low VL, Pramasivan S, Liew JW, Wan-Sulaiman WY, Vythilingam I. High transmission efficiency of the simian malaria vectors and population expansion of their parasites *Plasmodium cynomolgi* and *Plasmodium inui*. *PLoS Neglected Tropical Diseases*. 2023 Jun 29;17(6):e0011438.
11. Pramasivan S, Ngui R, Jeyaprakasam NK, Low VL, Liew JW, Vythilingam I. Spatial analyses of *Plasmodium knowlesi* vectors with reference to control interventions in Malaysia. *Parasites & Vectors*. 2023 Oct 9;16(1):355.

ii) **Title of Paper presentations (international/ local)**

- **Simian malaria, an emerging threat to malaria elimination.** 16 International Conference of Medical Arthropodology 2023. 13-15 December, Calicut, India. Invited Speaker
- **Zoonoses a threat to malaria elimination.** Asia Pacific Conference on Mosquito and Vector control (AMV) 2023. 27-30 November 2023. Chiang Mai, Thailand. Invited Speaker
- **Current status of simian malaria and its vectors in Southeast Asia.** International vector borne disease conference 2023. 21-23 November 2023 National University of Singapore. Invited Speaker
- **Vectors of simian malaria the way forward.** 16th International Congress of Physiological Anthropology (ICPA2023). 7-8 September 2023. Universiti Sabah Malaysia. Invited Speaker
- **Is zoonotic malaria transmission a threat to malaria elimination in Southeast Asia?** One World Health Congress 7-11 November 2022. Singapore. Temasek Foundation Pinnacle series. Invited speaker
- **Spatial distribution and incrimination of vectors in relation to zoonotic transmission of malaria in Malaysia” and “In need of new surveillance toolbox for knowlesi malaria vector.** 20th International Congress of Tropical medicine and malaria, Bangkok Thailand. **24-28 October 2022.** Invited speaker
- **Plasmodium knowlesi and forest goers: what’s next?** APMEN TECH talks. Forest Goers and residual malaria addressing the challenges. 9 June 2020. Invited speaker
- **Plasmodium knowlesi malaria from monkeys to humans: What’s next?** Singapore Malaria Network meeting: Feb 20-21st, 2020. Invited speaker

iii) **Human Capital Development**

Two students completed their PhD and graduated in November 2023. One of the students (Nantha Kumar Jeyaprakasam) passed with distinction and is now lecturer in Universiti Kebangsaan Malaysia.

iv) **Awards/ Others**

International Conference of Medical Parasitology and Entomology. 17 August 2022. Poster presentation. Blood meal analysis: Insight into blood feeding preference of *Anopheles* vectors of simian malaria. First prize to Nantha Kumar Jeyaprakasam PhD student.

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