



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR PREVALENCE, SPATIAL DISTRIBUTION AND
EPIDEMIOLOGICAL RISK FACTORS OF *Plasmodium knowlesi*
INFECTION AMONG *Macaca fascicularis* RAFFLES IN PENINSULAR
MALAYSIA**

ZARITH SURAYA BINTI SHAHAR

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By

ZARITH SURAYA BINTI SHAHAR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfillment of the Requirements for the Degree of
Master of Science**

June 2019

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

MOLECULAR PREVALENCE, SPATIAL DISTRIBUTION AND EPIDEMIOLOGICAL RISK FACTORS OF *Plasmodium knowlesi* INFECTION AMONG *Macaca fascicularis* RAFFLES IN PENINSULAR MALAYSIA

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June 2019

Chairman : Reuben Sunil Kumar Sharma, PhD, MRSB, CBiol
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Malaria caused by the haemoparasite *Plasmodium* is one of the important vector-borne tropical diseases among humans and a wide variety of animals, especially in the tropical regions where the mosquito vectors are abundant. *Plasmodium knowlesi* is a zoonotic simian malaria parasite with the Long-tailed macaques (*Macaca fascicularis*) are the natural reservoir hosts. It is now the most common and most fatal human malaria and is responsible for the increasing number of human malaria cases in Malaysia. Despite of the growing importance of zoonotic malaria, many aspects of its biology and epidemiology especially in the natural macaque hosts remains undetermined throughout Southeast Asia. The aim of this study was to ascertain the molecular prevalence, spatial distribution pattern, and epidemiological risk factors associated with *P. knowlesi* infection among Long-tailed macaques (*Macaca fascicularis*) in Peninsular Malaysia. Blood samples from 1587 Long-tailed macaques captured from 122 different locations in Peninsular Malaysia were subjected to *Plasmodium* detection by nested PCR amplification using primers targeting the 18S SSU rRNA gene. Molecular detection revealed that 48.39% of the macaques were positive for *Plasmodium* infection where *P. cynomolgi* was the most prevalent (28.92%), followed by *P. inui* (22.94%), *P. coatneyi* (14.37%) and *P. knowlesi* (13.55%). Analysis of *Plasmodium* species co-infection with *P. knowlesi* revealed a higher prevalence of multiple species infection (18.10%) compared to single *P. knowlesi* infection (9.90%). The multivariate regression analysis showed that number of *Plasmodium* species co-infection, gender of the macaques, spatial sampling zones and geographical location were the significant risk factors associated with *P. knowlesi* infection among the macaques. The spatial distribution pattern of *P. knowlesi* infection demonstrated that the highest infection occurred in the North-west zone and West-coastal

region of Peninsular Malaysia, with numerous infection hotspots having >75% infection rates. *Plasmodium knowlesi* infection among the macaques was present in all states of Peninsular Malaysia indicating the endemicity and widespread nature of the infection. The overall analysis on the influence of host factors on the prevalence of *P. knowlesi* demonstrated that the prevalence rate was higher in the adult and juvenile macaques. The macaques sampled from the forest habitat recorded the lowest infection rate (11.1%) compared to those captured from the urban (14.79%), sub-urban (14.08%) and plantation (14.67%) habitats. However, this suggested that *P. knowlesi* infection is not restricted to the macaque in forest area but is also common among macaques inhabiting the urban, sub-urban and plantation areas. Higher prevalence of infection was also noted for the macaques sampled in habitats further away from the coast (>60km) and those inhabiting the inland at higher elevations (>80m above sea level). The present study constitutes the first attempt in Southeast Asia to screen a large number of macaques and to determine the epidemiological risk factors for *P. knowlesi* infection among the sylvatic macaque hosts. It is envisaged that the findings from this study will provide a better understanding on the molecular epidemiology and spatial distribution of this zoonotic parasite in this country and facilitate further surveillance and control programs to be initiated.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Master Sains

**PREVALENS MOLEKULAR, TABURAN SPATIAL DAN FAKTOR – FAKTOR
RISIKO EPIDEMIOLOGI JANGKITAN *Plasmodium knowlesi* DALAM
Macaca fascicularis RAFFLES DI SEMENANJUNG MALAYSIA**

Oleh

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Malaria yang disebabkan oleh hemoparasit *Plasmodium* adalah satu daripada penyakit tropikal bawaan vektor yang penting dalam manusia dan pelbagai jenis haiwan terutamanya di kawasan tropika dimana vektor nyamuk adalah banyak. *Plasmodium knowlesi* merupakan parasit malaria zoonotik dengan kera (*Macaca fascicularis*) sebagai perumah semulajadi. Spesies ini kini merupakan malaria manusia yang paling biasa dijumpai dan paling berbahaya dan bertanggungjawab terhadap peningkatan jumlah kes malaria manusia di Malaysia. Walaupun kepentingan penyakit malaria zoonotik semakin meningkat, pelbagai aspek biologi dan epidemiologi terutamanya dalam perumah semulajadi kera masih tidak dapat ditentukan di seluruh Asia Tenggara. Objektif kajian ini adalah untuk mengenalpasti prevalens molekul, corak taburan spatial dan faktor risiko epidemiologi yang berkaitan dengan jangkitan *P. knowlesi* dalam kera (*Macaca fascicularis*) di Semenanjung Malaysia. Sampel darah dari 1587 ekor kera yang ditangkap dari 122 lokasi berbeza di Semenanjung Malaysia tertakluk kepada pengesanan *Plasmodium* oleh PCR tersarang menggunakan primer yang menasarkan gen 18S SSU rRNA. Pengesanan molekul menunjukkan bahawa 48.39% daripada monyet positif untuk jangkitan *Plasmodium* di mana *P. cynomolgi* adalah yang paling lazim (28.92%), diikuti oleh *P. inui* (22.94%), *P. coatneyi* (14.37%) dan *P. knowlesi* (13.55 %). Analisis jangkitan bersama *Plasmodium* dengan *P. knowlesi* mendedahkan jangkitan pelbagai spesies lebih tinggi (18.10%) daripada jangkitan spesies tunggal (9.90%). Analisis regresi multivariat menunjukkan bahawa bilangan jangkitan coinfeksi *Plasmodium*, jantina monyet, zon persampelan spasial dan lokasi geografi adalah faktor risiko yang signifikan berkaitan dengan jangkitan *P. knowlesi* di kalangan monyet. Corak taburan spasial jangkitan *P. knowlesi* menunjukkan bahawa jangkitan tertinggi terjadi di zon barat laut dan wilayah pesisir barat Semenanjung Malaysia, dengan banyak

jangkitan kawasan panas yang mempunyai kadar jangkitan > 75%. Jangkitan *P. knowlesi* di kalangan kera telah wujud di semua negeri Semenanjung Malaysia yang menunjukkan endemisiti dan jenis jangkitan yang meluas. Analisis keseluruhan mengenai pengaruh faktor perumah kepada prevalens *P. knowlesi* menunjukkan bahawa kadar prevalens adalah lebih tinggi pada kera dewasa dan kera juvana. Kera yang disampel dari habitat hutan mencatatkan kadar jangkitan terendah (11.1%) berbanding dengan yang ditangkap dari bandar (14.79%), sub-bandar (14.08%) dan habitat ladang (14.67%). Walau bagaimanapun, ini menunjukkan bahawa jangkitan *P. knowlesi* tidak terhad kepada kera di kawasan hutan tetapi juga lazim di kalangan kera yang mendiami kawasan bandar, sub-bandar dan perladangan. Prevalens yang tinggi juga dapat dilihat pada kera yang disampel dari habitat jauh dari pantai (>60km) dan yang mendiami kawasan pedalaman di ketinggian yang tinggi (>80m di atas paras laut). Kajian ini merupakan percubaan pertama di Asia Tenggara untuk menganalisis kera dalam jumlah yang besar dan menentukan faktor risiko epidemiologi untuk jangkitan *P. knowlesi* di kalangan kera liar. Data yang diperolehi daripada kajian ini akan memberikan pemahaman yang lebih baik mengenai epidemiologi molekul dan taburan spatial parasit zoonotik di negara ini dan dapat membantu program pengawasan dan kawalan yang selanjutnya.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS AND SYMBOLS

μL	microliter
μM	micromolar
μm	micrometer
ACT	artemisinin combination therapy
AIDS	Acquired Immune Deficiency Syndrome
AKI	Acute Kidney Injury
AL	artemether-lemefantrine
ARDS	Acute Respiratory Distress Syndrome
ASMQ	artesunate-mefloquine
bp	basepair
CI	Confidence interval
cm	centimeter
CQ	chloroquine
csp	circumsporozoite protein
dL	deciliter
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
EDTA	ethylenediaminetetracetic acid
g	gram
HIV	Human Immunodeficiency Virus
IUCN	International Union for Conservation of Nature
kb	kilobase

km	kilometer
MASL	meters above seal level
mg	miligram
MgCl ₂	Magnesium chloride
min	minute
ml	milliliter
mM	milimolar
ng	nanogram
nm	nanometer
°C	degree celcius
OR	Odds ratio
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
sec	second
SSU rRNA	small-subunit ribosomal RNA
TAE	Tris-acetic acid-EDTA
UV	ultraviolet
V	voltage

CHAPTER 1

INTRODUCTION

Parasitic haemoprotozoan of the genus *Plasmodium* are known as the causative agents of malaria in humans and animals. Malaria is considered to be one of the most important parasitic disease in the world, and remains a serious public health problem in many tropical countries (Prasad, 2010; Binns and Low, 2015; Abat *et al.*, 2016). Malaria is known as the big three along with HIV/AIDS and tuberculosis because of their detrimental impact on human health and wellbeing (Bourzac, 2014; Abat *et al.*, 2016). Approximately 216 million cases and 445,000 deaths due to malaria occurred globally in 2016, an increase of five million cases from 2015 [World Health Organization (WHO), 2017]. The global burden of malaria is worsen by the unavailability of effective vaccines (Bourzac, 2014; Coelho *et al.*, 2017), the paucity of reliable anti-parasitic drugs (Andrews *et al.*, 2014), and the emergence of resistance to the pre-existing anti-malarial drugs (Horn and Duraisingh, 2014; Blasco *et al.*, 2017) and insecticides (Quiñones *et al.*, 2015; Alout *et al.*, 2017).

Malaria is a very important parasitic disease in Malaysia, despite a gradual decline in the incidence and mortality rate since the early 1990's due to ongoing control programs introduced back in 1960 [Singh and Cox-Singh, 2001; Nissapatorn *et al.*, 2007; Ministry of Health Malaysia (MOH), 2014]. The disease has been endemic in the country and the major populations at risk include the aborigines (Orang Asli), rural people, farmers and forest workers (Alias *et al.*, 2014). The prevalence of malaria is higher in Sabah and Sarawak compared to Peninsular Malaysia, with *Plasmodium falciparum*, *P. vivax* and *P. malariae* being the predominant in humans (Singh and Cox-Singh, 2001; WHO, 2013, 2014). Collectively, these species of *Plasmodium* accounted for 1092 and 606 indigenous cases in 2013 and 2014, respectively (WHO, 2015). However, the prevalence estimates of malaria in Malaysia is largely based on traditional microscopy diagnosis of confirmed cases from government clinics and hospitals, thus the actual prevalence of the disease may well be an underestimate (Singh and Cox-Singh, 2001). At the global level, Malaysia is one of the three countries in Western Pacific region that is on course of malaria elimination by 2020 (MOH, 2014; WHO, 2017).

In spite of concerted efforts to eliminate this deadly disease, the country now faces a new and significant challenge as there is an increasing number of cases in humans caused by the zoonotic simian malaria parasite *P. knowlesi* over the past few years (WHO, 2015, 2017). *Plasmodium knowlesi* is now considered as the most common form of human malaria in the country where in 2012 and 2014, it accounted for 38% and 81% of total reported cases, respectively (MOH, 2014;

WHO, 2015). In the past, the prevalence of *P. knowlesi* in humans was underestimated and microscopically misdiagnosed as *P. malariae* until molecular tool to distinguish between both species became available (Singh and Cox-Singh, 2001; Singh *et al.*, 2004). In addition, *P. knowlesi* has become the most important *Plasmodium* species in Southeast Asia where naturally acquired cases involving locals and tourist have been reported in almost all member countries except Timor Leste (Barber *et al.*, 2017).

Plasmodium knowlesi is a zoonotic simian malaria parasite with its natural host including Asian macaques (*Macaca fascicularis* and *M. nemestrina*) and Leaf monkeys (*Presbytis femoralis*, formerly known as *P. melalophos*) (Coatney *et al.*, 1971; Collins, 2012). *Plasmodium knowlesi* was first isolated and described in early 1930's, and subsequently researchers in India successfully inoculated the parasites into humans in 1932 (Knowles and Gupta, 1932). The first natural infection in humans was reported in Peninsular Malaysia in 1965 (Chin *et al.*, 1965). The consensus that zoonotic malaria was extremely rare in humans was challenged when a large foci of human infection was detected in Kapit, Sarawak in 2004 using nested polymerase chain reaction (PCR) (Singh *et al.*, 2004). The transmission of *P. knowlesi* is primarily zoonotic and is transferred from the macaque reservoir host to humans through the bites of forest dwelling *Anopheles* mosquitoes (Vythilingam, 2012; WHO, 2015). It is still uncertain if human to human transmission occurs in nature, although the possibility of host switching cannot be ruled out at present [Vythilingam, 2012; WHO Regional Office for the Western Pacific (WPRO), 2017].

Due to the increasing destruction of forests for development in many Southeast Asian countries, troops of wild *M. fascicularis* have been displaced from their natural habitat and now co-existed with humans in urban and sub-urban areas. Many wild macaques now subsists in villages, plantations and orchards, recreational parks, and even housing estates, where they are often in close contact with humans. This narrows the transmission interface and increases the risk of zoonotic simian malaria in humans. In spite of the apparent public health importance of zoonotic malaria, many aspects of its biology and epidemiology still remains undetermined in the region. Existing reports in Malaysia have detected *P. knowlesi* in macaques only in Sabah, Sarawak, Selangor and Pahang where emphasis was placed on prevalence, and only a small number of macaques were sampled (Vythilingam *et al.*, 2008; Lee *et al.*, 2011; Akter *et al.*, 2015; Divis *et al.*, 2015; Muehlenbein *et al.*, 2015). Thus, it is important to ascertain the levels of *P. knowlesi* infections in the major primate reservoir hosts from various locations to better understand the epidemiology, prevalence and spatial distribution of this zoonotic parasite in the country. The present study was therefore undertaken to determine the molecular epidemiology and spatial distribution of *P. knowlesi* infection among *M. fascicularis* in Peninsular Malaysia with the following specific objectives:

- a) To ascertain the molecular prevalence of *Plasmodium knowlesi* infection among Long-tailed macaques (*Macaca fascicularis*) in Peninsular Malaysia, and its co-infection status with other simian *Plasmodium* species.
- b) To determine the spatial distribution patterns of *Plasmodium knowlesi* infection in Long-tailed macaques (*Macaca fascicularis*) in Peninsular Malaysia and to identify the hotspots of infection in the country.
- c) To determine the epidemiological risk factors associated with *Plasmodium knowlesi* infection in Long-tailed macaques (*Macaca fascicularis*) in the country.



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