



UNIVERSITI PUTRA MALAYSIA

***IDENTIFICATION AND DETERMINATION OF GENETIC DIVERSITY IN
SIMIAN MALARIA PARASITES AMONG WILD LONG-TAILED MONKEY
POPULATIONS FROM VARIOUS REGIONS OF PENINSULAR
MALAYSIA***

PARASTOO KHAJEAIAN

FBSB 2015 17



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IN SIMIAN MALARIA PARASITES AMONG WILD LONG-TAILED
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PENINSULAR MALAYSIA**

By

PARASTOO KHAJEAIAN

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

March 2015

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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

**IDENTIFICATION AND DETERMINATION OF GENETIC DIVERSITY
IN SIMIAN MALARIA PARASITES AMONG WILD LONG-TAILED
MONKEY (*Macaca fascicularis*) POPULATIONS OF PENINSULAR
MALAYSIA**

By

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March 2015

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Faculty: Biotechnology and Biomolecular Sciences

Malaria still remains a major cause of mankind death globally in spite of a century of research. It is clear that, understanding and accurate measurement of malaria incidence as one of the most critical tropical diseases have significant role to control and prevent this lethal infection. Since the probability of *P. knowlesi* zoonotic transmission to humans was proven, the necessity of study on simian malaria parasites is increasingly obvious.

Simian malaria parasites are readily infectious to long-tailed (*Macaca fascicularis*) and pigtailed (*Macaca nemestrina*) monkeys. The chance of this pathogenic species switching to humans as their desired host is not ignorable due to the increase of human populations in recent years, as well as ecological alterations which are caused by pollution or deforestation. In fact macaques are preferred host for *Anopheles* mosquitoes but human may change this situation by alteration on the natural habitat of macaques and mosquitoes.

In this study, the distribution of five *Plasmodium* species namely: *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. fieldi* and *P. coatneyi* among the wild populations of *M. fascicularis* in six states in Peninsular Malaysia were determined using highly specific (nested-PCR) assays. The advantage of this method lies in its ability to detect very low numbers of parasites.

Monkey blood samples provided by the Department of Wild Life and National Parks Malaysia (PERHILITAN) were collected on Flinders Technical Association cards (FTA cards). FTA cards are strongly recommended for

collecting fluid samples for epidemiological studies particularly when sampling is being done in areas far from the main laboratory or during a long sampling trip. The prevalence of these five simian *Plasmodium* species was determined in 13 different locations of Peninsular Malaysia. Geographic distribution of the collected samples provided by PERHILITAN ranged from Northwest [Penang (island), Penang (mainland) and Jerejak Island], West (Selangor, Perak), Southwest (Negeri Sembilan), East (Pahang) and Northeastern (Kelantan) of Peninsular Malaysia. DNA was extracted from the blood spots on FTA Cards. All five simian *Plasmodium* species were successfully detected using nested PCR assay. Among the five species, *P. knowlesi* had the highest prevalence (34.3%), followed by *P. inui* (33.2%), *P. cynomolgi* (27.9%), *P. fieldi* (27.6%) and *P. coatneyi* (16.6%). Co-infections of macaques with multiple species of *Plasmodium* parasites were also observed. Kelantan had the highest prevalence rate among the states for all five simian malaria species. The incidence rate of three *Plasmodium* species which are *P. inui*, *P. fieldi* and *P. knowlesi* were higher than 50% among the samples obtained from this state.

Twenty positive DNA samples with single infection of *P. knowlesi* (12 samples) and *P. cynomolgi* (8 samples) as well as 20 uninfected monkey DNA samples were chosen to investigate the genetic diversity of these parasites using 26 different ISSR (inter simple sequence repeat) markers.

A total of 103 ISSR loci for infected samples and 95 for uninfected samples were generated. The analyses of the infected and uninfected samples using ISSR markers confirmed the efficiency of both the markers and the clustering methods. By these methods, the samples not only were separated according to their geographical distribution, but the samples were grouped into two distinct clusters according to the species of the malaria parasite.

Overall, this study shows the importance of research about malaria parasite species which are infectious to *M. fascicularis* and the necessity of preventive and control plans to decrease the chance of host-switch occurrence. This study also provides information for further investigations to design and develop diagnostic microsatellite markers for the macaques in the future.

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

**PENGENALPASTIAN DAN PENENTUAN KEPELBAGAIAN GENETIK
PARASIT MALARIA SIMIAN DI KALANGAN POPULASI MONYET
EKOR-PANJANG (*Macaca fascicularis*) LIAR DI SEMENANJUNG
MALAYSIA**

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Malaria masih kekal sebagai punca utama kematian manusia di seluruh dunia walaupun penyelidikan telah dijalankan selama seabad. Adalah jelas bahawa, pemahaman dan pengukuran yang lebih tepat insiden penyakit malaria yang merupakan salah satu penyakit tropika yang paling kritikal mempunyai peranan signifikan untuk mengawal dan mencegah jangkitan maut ini. Oleh kerana kebarangkalian transmisi zoonotik *P. knowlesi* kepada manusia telah terbukti, keperluan kajian mengenaipasti parasit malaria monyet semakin ketara.

Parasit malaria monyet boleh berjangkit pada kera ekor-panjang (*Macaca fascicularis*) dan kera berekor pusaran (*Macacanemestrina*). Kebarangkalian spesies patogenik ini menukar kepada manusia sebagai hos tidak boleh diabaikan disebabkan peningkatan populasi manusia dalam tahun-tahun kebelakangan ini, dan juga perubahan ekologi yang disebabkan oleh pencemaran atau penebangan hutan. Sebenarnya, monyet adalah hos utama untuk nyamuk *Anopheles*, tetapi manusia boleh mengubah keadaan ini dengan perubahan pada habitat semula jadi monyet dan nyamuk.

Dalam kajian ini, distribusi lima spesies *Plasmodium* iaitu: *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. fieldi* dan *P. coatneyi* kalangan populasi liar *M. fascicularis* di enam negeri di Semenanjung Malaysia telah ditentukan dengan menggunakan ujian PCR-berperingkat spesifik. Kelebihan kaedah ini adalah pada keupayaannya untuk mengesan bilangan parasit yang amat rendah.

Sampel darah monyet yang disediakan oleh Jabatan Hidupan Liar dan Taman Negara Malaysia (PERHILITAN) telah dikumpulkan pada kertas tapis kad FTA. Kad FTA amat disyorkan untuk mengambil sampel cecair untuk kajian epidemiologi, terutamanya apabila persampelandilakukan di kawasan-kawasan jauh dari makmal atau semasa persampelan yang lama. Penularan lima spesies *Plasmodium* monyet ini telah ditentukan di 13 lokasi berlainan di Semenanjung Malaysia. Taburan geografi sampel yang disediakan oleh PERHILITAN adalah dari kawasan Barat Laut Pulau Pinang, Pulau Pinang (tanah besar), Pulau Jerejak, Barat (Selangor, Perak), Barat Daya (Negeri Sembilan), Timur (Pahang) dan Timur Laut (Kelantan) di Semenanjung Malaysia. DNA telah diekstrak daripada darah pada kad FTA. Kesemua lima spesies *Plasmodium* monyet telah berjaya dikesan menggunakan ujian PCR berperingkat. Antara lima spesies ini, *P. knowlesi* mempunyai kadar kejadian tertinggi (34.3%), diikuti oleh *P. inui* (33.2%), *P. cynomolgi* (27.9%), *P. fieldi* (27.6%) dan *P. coatneyi* (16.6%). Jangkitan pelbagai species parasit *Plasmodium* di dalam monyet juga diperhatikan. Kelantan mempunyai kadar kejadian tertinggi di kalangan negeri-negeri untuk semua lima spesies malaria monyet. Kadar insiden tiga spesies, iaitu *P. inui*, *P. fieldi* dan *P. knowlesi* adalah lebih tinggi daripada 50% di kalangan sampel yang dikumpul daripada negeri ini.

Dua puluh DNA sampel yang positif dengan jangkitan tunggal *P. knowlesi* (12 sampel) dan *P. cynomolgi* (8 sampel) serta 20 sampel DNA monyet yang tidak dijangkiti telah dipilih untuk menyiasat kepelbagaian genetik parasit ini dengan menggunakan 26 penanda ISSR (Inter Simple Sequence Repeat) yang berbeza. Sejumlah 103 ISSR lokus bagi sampel yang dijangkiti dan 95 untuk sampel yang tidak dijangkiti telah dijana. Analisis sampel yang dijangkiti dan tidak dijangkiti menggunakan penanda ISSR telah mengesahkan kecekapan kedua-dua penanda dan kaedah kelompok. Dengan kaedah ini, sampel bukan sahaja telah diasingkan mengikut taburan geografi mereka, tetapi sampel-sampel juga dikumpulkan ke dalam dua kelompok yang nyata ssssssmengikat spesies parasit malaria.

Secara keseluruhannya, kajian ini menunjukkan betapa pentingnya kajian spesies parasit malaria yang berupaya untuk menjangkiti *M. fascicularis* dan keperluan pencegahan dan rancangan kawalan untuk mengurangkan peluang penularan hos berlaku. Kajian ini juga menyediakan maklumat untuk siasatan lanjut untuk mereka dan membangunkan penanda mikrosatelit diagnostik untuk monyet pada masa hadapan.



ACKNOWLEDGEMENTS

First and foremost, my gratitude goes to my truly kind and devoted supervisor Professor Dr. Soon-Guan Tan. I would never conduct this research without his continuing guidance and effective suggestions. His ever-ethical devotion to science became my inspiration to research style. I especially want to extend my heartfelt thanks and deepest gratitude to my dear co-supervisor Dr. Christina Yong who was incredibly efficient, supportive and excellent in guiding me through this novel experience. My grateful appreciation is also due to my co-supervisors Associated Professor Dr. Noorjahan Banu Mohammed Alitheen, Dr. Reuben Sharma and Mr. Jeffrine Rovie Ryan Japning who were always supportive and never withheld of providing whatever I needed.

I acknowledge The Department of Wildlife and National Parks. A special appreciation goes to Mr. Frankie Anak Thomas Sitam who assisted me through the initial steps of this study. I am certainly grateful and indebted to Dr. Alireza Valdiani for his valuable and effectual helps and ideas until the last minute of my study.

I wish to express my sincere appreciation to my close friends Maryam Roshani, Sareh Seyedi and Sonia Nikzad for their support and kind wishes.

My profound and warmest thanks and love to my husband Mohammad and my beloved sons Reza and Ilia for their love, patience, and encouragement and to my father for his constant support, love and prayers throughout my study. Last but certainly not least, I wish to express my sincere appreciation to all those who were not mentioned here that helped me to ensure the completion of my research.

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LIST OF ABBREVIATIONS

μL	Microliter
%	Percentage
°C	Centigrade Celsius
1X	One time
A	Adenine
AIDS	Acquired Immuno Defficiency Syndrom
AFLP	Amplified Fragment Length Polymerase
APS	Ammonium Persulfate
B.C	Before Christ
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
C	Cytosine
CDC	Centre for Disease Control
CQ	chloroquine
ddH2O	deionised distilled water
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium bromide
FTA	Flinders Technical Association
G	Guanine
G	Gram
ISSR	Inter Simple Sequence Repeat
Km	Kilometer
Mg	Milligram
MgCl ₂	Magnesium chloride
min	Minute
ml	Milliliter

mM	Milimolar
mm	Milimeter
MS	Microsatellites
ng	Nanogram
NCBI	National Center for Biotechnology Information
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rpm	rotation per minute
sec	Second
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
SSR	Simple Sequence Repeat
SSU rRNA	small sub-unit ribosomal ribonucleic acid
T	Thymine
TBE	Tris-Borate-EDTA
U/ μ L	Unite per microliter
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UPM	Universiti Putra Malaysia
UV	Ultraviolet
V	Volt
WHO	World Health Organization
W/V	Weight per volume
X	Times

CHAPTER 1

INTRODUCTION

1.1 General Background

In the midst of the last century it was believed to be the end of contagious diseases as a result of the development of several different drugs and vaccines (Fauci, 2001). Unfortunately this outlook has been proven wrong due to a variety of reasons, such as the failure to understand the evolutionary potential of parasites (generally described as including viruses, bacteria and eukaryotic micro- and macro parasites). For instance, visiting hospitals nowadays increases the risk of the acquisition of antibiotic-resistant infections (Peleg and Hooper, 2010). Recently, a totally drug-resistant tuberculosis has been recognized in India (Udwadia *et al.*, 2012), and the emergence of resistance to the latest frontline anti malarial drugs has been proven in many areas (Cheeseman *et al.*, 2012). Although the significant role of drugs and vaccines in the control of numerous infectious diseases are obvious, eradication has only succeeded in a few cases. It is concerning and quite surprising that the evolutionary considerations are not better integrated into biomedicine and control policies, given that infectious diseases are responsible for substantial morbidity and mortality of humans, wildlife, livestock and agricultural crops (WHO, 2013).

The fast evolution of parasites makes them important to understand from the applied perspectives; which also makes them very interesting and helpful models for studying basic evolutionary processes. Several factors which make parasites capable of fast evolution: short generation times, large population sizes, considerable genetic and phenotypic variation that may be generated through sexual reproduction and variable mutation rates (Poulin, 2007; Stearns and Koella, 2007). Monitoring studies of malaria parasites among the long- tailed macaques (*Macaca fascicularis*) in Malaysia revealed that this species have the maximum prevalence of malaria compared to the other macaque species (Eyles, 1963). Long-tailed macaques (*M. fascicularis*) (Raffles, 1821) are generally distributed in mainland and insular Southeast Asia from 21°N to 10°S latitude and from 92°E to 126°E longitude, covering the Philippines, Indonesia, Malaysia, Cambodia, Mauritius, Vietnam, Thailand, Myanmar, Singapore and Timor-Leste (Groves, 2001). After human and rhesus macaques, the long- tailed macaques have the third most wide-ranging geographical spread among primates (Fooden, 1995).

They used to live in the forests and near human habitations. Previous studies have found that this species of macaque is the natural host for human *Plasmodium knowlesi* infection (Jeslyn *et al.*, 2011). It is certainly necessary to determine the prevalence of simian malaria parasites in local macaques so that the risk of potential malaria hooknoses may be recognized. It is known that *P. knowlesi* infection could be severe and fatal in humans (Cox-Singh *et al.*, 2010; Galinski and Barnwell, 2009) while some other species of simian malaria parasites such as *Plasmodium cynomolgi* and *Plasmodium inui*, have now been proven to be infectious to humans too (Galinski and Barnwell, 2009).

The record of the locally acquired *P. knowlesi* cases and the subsequent detection of *P. knowlesi* parasites in samples of local wild long-tailed macaques show a potential risk of zoonotic transmission of malaria infection in Malaysia (Tan *et al.*, 2008). However, there is a need to screen for simian malaria parasites in more substantial populations of macaques, as only a small sample of macaques were tested formerly for the presence of this parasite. For a much better understanding on the prevalence of malaria disease in local macaques, it is preferable to collect samples from various geographical locations. This is to enabling the evaluation of the risk of zoonotic transmission of simian malaria parasites to the human population from the long tail monkey. Recent achievements in molecular methods, especially the polymerase chain reaction (PCR) enabled scientists to genotype malaria parasites directly from the patient's blood samples without any prior *in vitro* culture needed. Molecular markers are efficient tools for genetic analyses, taxonomic classification, studying the phylogenetic relationship, as well as prognostic studies in different taxa (Valdiani *et al.*, 2014). The inter-simple sequence repeats (ISSRs) can be useful and beneficial in this era.

1.2 Hypothesis

The long-tailed macaques are the biggest population of non-human primates in Malaysia. In addition to *P. knowlesi*, this species of macaques is also harboring *P. cynomolgi*, *P. inui*, *P. fieldi* and *P. coatneyi* (Coatney *et al.*, 1971). However to-date, there has been no reports on the prevalence of simian malaria in peninsular Malaysia. Macaques as the natural hosts of *P. knowlesi* inhabit in various public nature parks near to human population. Since most of the surveillance studies regard natural incidence of simian malaria had been conducted in Kapit Division of Sarawak, East Malaysian on the Borneo Island (Lee *et al.*, 2011), This study will provide evidence to show the presence and ongoing transmission of malaria parasites among local macaques and answer the question if there is a need to be alarmed for the risk of the general population acquiring zoonotic malaria. In this thesis study of evolutionary

biology with tools from molecular biology will allow us to study macaque malaria parasites at both evolutionary and ecological aspects.

1.3 Main objective

The general objective of this project was to determine the prevalence of simian malaria parasites in the long-tailed macaques of Peninsular Malaysia.

1.4 Specific objective

2. To determine the incidence of five malaria parasites namely *P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. fieldi* and *P. coatneyi* in samples of long- tailed macaque (*M. fascicularis*) from Peninsular Malaysia.
3. To assess the genetic diversity using inter simple sequence repeat (ISSR)
4. To compare the sensitivity and accuracy of direct nested - PCR method with standard nested – PCR method.

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ISSRs: Neglected DNA Markers for Molecular Dissection of *Plasmodium* Species in Long-tailed Macaque (*Macaca fascicularis*) Parastoo Khajeaian¹, Soon Guan Tan¹, Alireza Valdiani^{2*}, Christina Yong Seok Yien³, Mohammad Zareian Jahromi⁴, Noorjahan Banu Alitheen¹, Jeffrine Japning Rovie-Rian⁵ *BioMed Research International* (Under Review)



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