

**MOLECULAR EPIDEMIOLOGY OF MALARIA
IN THE INTERIOR DIVISION OF SABAH,
MALAYSIA**

JOVEEN NEOH WAN FEN

**DISSERTATION SUBMITTED IN
FULFILLMENT FOR THE MASTER OF SCIENCE**



UNIVERSITI MALAYSIA SABAH
UNIVERSITI MALAYSIA SABAH

**BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH**

2011

UNIVERSITI MALAYSIA SABAH
BORANG PENGESAHAN STATUS TESIS@

JUDUL: MOLECULAR EPIDEMIOLOGY OF MALARIA IN THE INTERIOR DIVISION OF SABAH, MALAYSIA

IJAZAH: IJAZAH SARJANA SAINS (MOLEKULAR BIOLOGI)

SAYA JOVEEN NEOH WAN FEN

SESI PENGAJIAN: 2009/2010

Mengaku membenarkan (LPSM/ sarjana/Doktor Falsafah) ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:-

1. tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/)

☐

SULIT

(mengandungi maklumat yang berdjajah keselamatan atau Kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)

☐

TERHAD

(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

☒

TIDAK TERHAD

(TANDATANGAN PENULIS)

Alamat Tetap: 57, JALAN T/S 2,
TAMAN SAUJANA 2, 34600
KAMPUNG, PERAK

Tarikh: 8 OGOS 2011

Disahkan oleh

(TANDATANGAN PUSTAKAWAN)

Puan Lau Fiek Ying

Nama penyelia

Tarikh: _____

Catatan :-*potong yang tidak berkenaan.

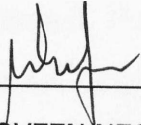
*Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali dan tempoh tesis ini perlu di kelaskan sebagai SULIT dan TERHAD.

@Tesis dimasukkan sebagai tesis bagi Ijazah Doktor Falsafah dan sarjana secara penyelidikan atau disertai bagi pengajian secara kerja kursus dan Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

AUGUST 2011


JOVEEN NEOH WAN FEN

PB20098038



UMS
UNIVERSITI MALAYSIA SABAH

CERTIFICATION

NAME : JOVEEN NEOH WAN FEN
MATRIC NO : PB2009-8038
TITTLE : MOLECULAR EPIDEMIOLOGY OF MALARIA IN
THE INTERIOR DIVISION OF SABAH, MALAYSIA
DEGREE : MASTER OF SCIENCE (MOLECULAR BIOLOGY)
VIVA DATE : 14TH JULY 2011

DECLARED BY

1. **SUPERVISOR**

MDM LAU TIEK YING



UMS
UNIVERSITI MALAYSIA SABAH

A handwritten signature in black ink, appearing to read 'Lau Tiek Ying', written over a horizontal line.

SIGNATURE

2. **CO-SUPERVISOR**

ASSOC. PROF. DR CLEMENTE MICHAEL WONG VUI LING

SIGNATURE

ACKNOWLEDGMENT

I would like to express my greatest gratitude and appreciation to my respectful supervisor, Mdm Lau Tiek Ying for her patience in supervising me, comments, suggestions, views and guidance. Thanks to my co-supervisor Assoc. Prof. Dr Clemente Michael Wong for his guidance to me.

My warmest thanks to Professor Balbir Singh from Malaria Research Centre in providing the positive controls in completing this project.

I am also grateful to Mr Erle Stanley Henry for his help in collecting the samples from each study sites. Many thanks to Thomas Lee, Lucy Chong and Rafidah from Hospital Keningau, Hospital Tambunan and Hospital Tenom; and Klinik Kesihatan Nabawan for their cooperation in samples collection.

I am also thankful and grateful to my coursemates; Sook Cheng, Cassandra and Joseph for their constant supports, help, encouragement and knowledge during laboratory work.

My deepest gratitude to the lecturer, labmates, lab assistants and staffs of Biotechnology Research Institute (BRI) who were involved directly and indirectly in completing this project.

Last but not least, I would like to express my sincere gratitude to my family for their constant encouragement and supports.

ABSTRACT

MOLECULAR EPIDEMIOLOGY OF MALARIA IN THE INTERIOR DIVISION OF SABAH, MALAYSIA

Malaria is one of the important parasite transmitted diseases in Sabah, Malaysian Borneo that is covered by tropical rainforest. The objectives of this six months cross-sectional study were to accurately identify the human malaria parasites, to determine the rate of misidentification and to characterize *P. knowlesi* isolates in the interior division of Sabah based on *ssrRNA* gene using nested PCR. A total of 243 blood spot samples from patients who had requested for Blood Film for Malaria Parasite (BFMP) test were collected from four study sites namely Keningau, Tenom, Tambunan and Nabawan. There were 16.1% *P. malariae*, 10.3% *P. vivax*, 7% *P. falciparum*, 1.2% mixed infection and 65.4% negative cases based on microscopic examination. However, the result of PCR indicated that *P. knowlesi* (58.9%), *P. falciparum* (18.7%), *P. vivax* (18.7%), mixed infection (3.7%) and no *P. malariae* infection were detected in these samples. There were only 35% of 243 samples gave consistent PCR and microscopic results. The highest malaria cases found in Keningau were *P. falciparum* (36.1%) whereas the prevalence of *P. knowlesi* was higher in Tambunan (85.7%) and Tenom (77.8%). Moreover, six positive samples for *P. falciparum* and *P. knowlesi* each were detected in Nabawan. Alignment analysis between *P. knowlesi* isolates from this study and *P. knowlesi* isolates from other geographical region showed two single nucleotide polymorphisms unique to *P. knowlesi* isolates from the interior division of Sabah. Besides, isolate KN048/2010 showing significant genetic variation among *P. knowlesi* isolates from this region. Phylogenetic analysis showed that *P. knowlesi* from this study clustered with naturally-acquired *P. knowlesi* isolates in human. This study provides further evidence of the actual transmission of different *Plasmodium* species in the interior regions of Sabah.

ABSTRAK

Malaria merupakan masalah kesihatan awam yang utama di Sabah, Malaysia Borneo, kawasan yang dikelilingi oleh hutan hujan tropika. Objektif kajian enam bulan ini adalah untuk mengenalpasti kehadiran parasit malaria manusia, tahap salah pengenalpastian dan jangkitan campuran spesies Plasmodium serta mencirikan ssrRNA gene P. knowlesi yang dikumpul dari kawasan pedalaman Sabah dengan teknik Tindabalas Berantai Polimerase (PCR). Sebanyak 243 sampel daripada pesakit yang menjalani ujian Blood Film for Malaria Parasite (BFMP) dikumpul dari empat kawasan kajian iaitu, Keningau, Tambunan, Tenom dan Nabawan. Terdapat 16.1% P. malariae, 10.3 % P. vivax, 7% P. falciparum, 1.2% jangkitan bercampur dan 65.4% sampel negatif berdasarkan pemeriksaan mikroskopik. Namun demikian, keputusan PCR menunjukkan P. knowlesi (58.9%), P. falciparum (18.7%), P. vivax (18.7%), jangkitan bercampur (3.7%) dan tiada jangkitan P. malariae yang dikesan daripada sampel-sampel tersebut. Hanya 35% daripada 243 sampel kajian menunjukkan keputusan yang konsisten melalui kaedah mikroskop dan PCR. Jangkitan parasit malaria manusia yang terbanyak di Keningau adalah P. falciparum (36.1%), manakala P. knowlesi merupakan parasit Plasmodium yang terbanyak di Tambunan (85.7%) dan Tenom (77.8%). Selain itu, di Nabawan terdapat enam sampel positif untuk P. falciparum dan P. knowlesi masing-masing. Analisis antara pencilan P. knowlesi dari kajian ini dan pencilan P. knowlesi dari kawasan geografi lain menunjukkan dua "single nucleotide polymorphism" yang unik kepada pencilan P. knowlesi di kawasan kajian manakala pencilan KN048/2010 menunjukkan variasi genetik yang jelas antara P. knowlesi yang dipencilkan dari kawasan pedalaman Sabah. Analisis filogenetik menunjukkan jangkitan P. knowlesi dari kawasan kajian tergolong dalam kumpulan P. knowlesi yang dipencilkan daripada manusia. Kajian ini memberi gambaran yang lebih jelas mengenai penyebaran Plasmodium species yang berbeza di kawasan pedalaman Sabah.

CONTENT

	PAGE
DECLARATION	ii
CERTIFICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
ABSTRAK	vi
LIST OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xii
LIST OF SYMBOLS AND ABBREVIATIONS	xv
 CHAPTER 1 : INTRODUCTION	
1.1 Background	1
1.2 Significance of Study	3
1.3 Objectives	4
 CHAPTER 2 : LITERATURE REVIEW	
2.1 Background of Malaria	5
2.2 History of Malaria	6
2.3 Discovery of Malaria Parasites	8
2.4 Classification of <i>Plasmodium</i> Species	10
2.5 Human Malaria Parasites	11
2.5.1 <i>Plasmodium falciparum</i>	11
2.5.2 <i>Plasmodium vivax</i>	12
2.5.3 <i>Plasmodium malariae</i>	13
2.5.4 <i>Plasmodium ovale</i>	14
2.5.5 Recognition of <i>P. knowlesi</i> As Fifth Human Malaria Parasite	15
2.6 Life cycle of <i>Plasmodium</i> species	19
2.6.1 Difference in Life Cycle of the <i>Plasmodium</i> Species	21
2.7 Incidence of Malaria	22
2.7.1 Incidence of Malaria in Asia	23
2.7.2 Incidence of Malaria in Malaysia	24
2.8 Detection of Human Malaria Parasite	25
2.8.1 Microscope As the 'Gold Standard'	25
2.8.2 Rapid Diagnostic Tests (RDTs)	26
2.8.3 Polymerase Chain Reaction (PCR)	26
2.9 Molecular Epidemiological Study of Malaria	28
2.10 Ribosomal RNA Genes of <i>Plasmodium</i> Species	30
 CHAPTER 3 : METHODOLOGY	
3.1 Study Sites	33
3.2 Sample Collection	34
3.3 Blood Film for Malaria Parasites	34
3.4 Extraction of Genomic DNA	35
3.5 Detection of Malaria Parasites by Nested PCR	36
3.5.1 Nest 1 <i>Plasmodium</i> Genus-specific PCR Amplification Reaction	36

3.5.2	Nest 2 Genus-specific PCR Amplification Reaction	36
3.5.3	Nest 2 Species-specific PCR Amplification	37
3.6	Gel Electrophoresis and Analysis of Nested PCR Products	38
3.7	Data Analysis of Epidemiological Finding	39
3.8	Analysis of <i>ssrRNA</i> Gene in <i>P. knowlesi</i> Isolates	39
3.8.1	Nest 2 PCR Amplification of <i>ssrRNA</i> in <i>P. knowlesi</i>	39
3.8.2	Gel Purification of PCR Amplification Products of <i>P. knowlesi</i>	40
3.9	Partial DNA Sequence Analysis of <i>ssrRNA</i> Gene in <i>P. knowlesi</i>	41
3.9.1	Computer-assisted Sequence Analysis and Assembly of <i>P. knowlesi</i> Isolates	41
3.9.2	Construction of Phylogenetic Tree	44

CHAPTER 4 : RESULTS

4.1	Study Population	46
4.2	Prevalence of <i>Plasmodium</i> Species Detected by Microscopic Examination	48
4.2.1	Prevalence of <i>Plasmodium</i> Species in Different Study Sites	49
4.2.2	Distribution of Each Species According to Geographical Areas	51
4.2.3	Prevalence of <i>Plasmodium</i> Species According to Study Population's Age Group	52
4.2.4	Prevalence of <i>Plasmodium</i> Species According to Study Population's Gender	54
4.2.5	Prevalence of <i>Plasmodium</i> Species Throughout Study Duration	55
4.3	Detection of Malaria Parasites by Nested PCR	56
4.3.1	Distribution of <i>Plasmodium</i> Infection Based on PCR Detection in the Interior Division of Sabah	63
4.3.2	Nested PCR Detection of <i>Plasmodium</i> Species Based on the Age of the Study Population	67
4.3.3	Distribution of <i>Plasmodium</i> Species Detected by Nested PCR Based on the Gender of the Study Population	69
4.3.4	<i>Plasmodium</i> species Detected by Nested PCR Throughout the Study Period	71
4.4	Comparison of the <i>Plasmodium</i> Species Detection Based on Microscopic Examination and Nested PCR	73
4.4.1	Agreement of the Methods Used in Detecting <i>Plasmodium</i> Species	74
4.5	Characterization of <i>P. knowlesi</i> Isolates Based on <i>ssrRNA</i> Gene	75
4.5.1	<i>ssrRNA</i> DNA Sequence Comparisons Between the <i>P. knowlesi</i> Isolates from the Interior Division of Sabah and Other Geographical Regions	77
4.5.2	Construction of Phylogenetic Tree	84

CHAPTER 5 : DISCUSSION

5.1	Study Sites	89
5.2	Sample Collection	89

5.3	Extraction of Genomic DNA	89
5.4	Detection of Malaria Parasites by Nested PCR	90
5.5	Prevention of Contamination While Performing Nested PCR	90
5.6	Prevalence of <i>Plasmodium</i> Species in the Interior Division of Sabah	91
5.6.1	Prevalence of <i>Plasmodium</i> Species in Different Study Sites	94
5.6.2	Prevalence of <i>Plasmodium</i> Species According to Study Population's Age	96
5.6.3	Prevalence of <i>Plasmodium</i> Species According to Study Population's Gender	97
5.6.4	Prevalence of <i>Plasmodium</i> Species Throughout Study Period	97
5.7	Comparison of the Malaria Detection Using Microscope and Nested PCR	100
5.8	Characterization of <i>P. knowlesi</i> Isolates Based on <i>ssrRNA</i> Gene	101
5.8.1	Genetic Variation for <i>ssrRNA</i> DNA Sequence of <i>P. knowlesi</i> Isolates	102
5.8.2	Analysis of Phylogenetic Tree	104
CHAPTER 6 : CONCLUSION		106
REFERENCES		108
APPENDIXES		117



UMS
UNIVERSITI MALAYSIA SABAH

LIST OF TABLES

	Page
Table 2.1 Summary of the characteristics of infection with five human <i>Plasmodium</i> species	22
Table 3.1 <i>Plasmodium</i> genus-specific ssrRNA primers	37
Table 3.2 <i>Plasmodium</i> species-specific ssrRNA primers	38
Table 3.4 Complete ssrRNA DNA sequences of <i>P. knowlesi</i> strains from GenBank used in the alignments	43
Table 4.1 Gender of the study population	47
Table 4.2 Samples distribution in four study sites	47
Table 4.3 Sample collection period (2010)	47
Table 4.4 Distribution of samples based on age group	47
Table 4.5 Samples distribution according to patients' races	48
Table 4.6 Diagnosis of <i>Plasmodium</i> species based on microscopic examination	48
Table 4.7 Number of samples collected in the four study sites	49
Table 4.8 Distribution of <i>Plasmodium</i> species based on microscopic examination according to age group	54
Table 4.9 Correlation test between the collection period and number of samples collected	56
Table 4.10 Detection of malaria parasite based on PCR and microscopic examination	63
Table 4.11 Correlation test for positive cases and the collection period	73
Table 4.12 Comparison of the <i>Plasmodium</i> species detected by nested PCR and samples by microscopic examination	74
Table 4.13 The interpretation of the Kappa's coefficient	74
Table 4.14 Cohen's Kappa coefficient test	75
Table 4.15 Sequences of <i>P. knowlesi</i> isolates from interior division of Sabah, Malaysian Borneo	76

Table 4.16	DNA polymorphism of <i>ssrRNA</i> gene of <i>P. knowlesi</i> isolates from the interior division of Sabah with reference to <i>P. knowlesi</i> strain from Sarawak, DQ350255	81
Table 4.17	<i>ssrRNA</i> gene sequence of <i>P. knowlesi</i> from GenBank used for phylogenetic tree	88



UMS
UNIVERSITI MALAYSIA SABAH

LIST OF FIGURES

	Page
Figure 2.1	Worldwide malaria endemic zones 6
Figure 2.2	Ring-form trophozoites of <i>P. falciparum</i> 12
Figure 2.3	Gametocytes of <i>P. falciparum</i> 12
Figure 2.4	Growing trophozoites of <i>P. vivax</i> 13
Figure 2.5	Band-form trophozoites of <i>P. malariae</i> 14
Figure 2.6	Trophozoites of <i>P. ovale</i> 15
Figure 2.7	Ring form trophozoites of <i>P. knowlesi</i> 17
Figure 2.8	Mature schizonts of <i>P. knowlesi</i> 18
Figure 2.9	Gametocyte of <i>P. knowlesi</i> in thin blood film 18
Figure 2.10	Overview of the life cycle of <i>Plasmodium</i> species 20
Figure 2.11	Distribution of malaria in Asia 24
Figure 3.1	Study sites 33
Figure 3.2	Collection of blood spot sample on chromatography paper 34
Figure 3.3	Partial sequence of <i>ssrRNA</i> gene of <i>P. knowlesi</i> 40
Figure 4.1	Proportion of samples collected from Tambunan 49
Figure 4.2	Distribution of microscopic diagnosed samples collected from Nabawan 50
Figure 4.3	Distribution of <i>Plasmodium</i> species based on microscopic diagnosis in Keningau 50
Figure 4.4	Distribution of <i>Plasmodium</i> species based on microscopic diagnosis in Tenom 51
Figure 4.5	Distribution of each <i>Plasmodium</i> species according to study sites 52
Figure 4.6	Histogram showing the proportion of the samples collected based on their age group 53
Figure 4.7	Distribution of <i>Plasmodium</i> species based on microscopic examination according to gender 55
Figure 4.8	Samples collection throughout the study duration (2010) 55

		Page
Figure 4.9	A 2.5% agarose gel showing PCR products using primer pair rPLU3 and rPLU4	57
Figure 4.10	A 2.5% agarose gel showing the PCR products following nest 2 PCR using the species-specific primer pair Pmk8 and Pmk9	58
Figure 4.11	A 2.5% agarose gel showing detection of <i>P. malariae</i> using primer pair rMAL1 and rMAL2	59
Figure 4.12	A 2.5% agarose gel illustrates nest 2 PCR amplification with the primer pair rVIV1 and rVIV2	60
Figure 4.13	A 2.5% agarose gel shows the nest 2 PCR products with primer pair rFAL1 and rFAL2 when visualized under ultraviolet transillumination	61
Figure 4.14	Nest 2 PCR amplification using primer pair rOVA1 and rOVA2 on 2.5% agarose gel	62
Figure 4.15	PCR detection of <i>Plasmodium</i> species in the interior division of Sabah	63
Figure 4.16	Distribution of malaria cases in the interior division of Sabah	65
Figure 4.17	Distribution of each <i>Plasmodium</i> species in four study sites bases on nested PCR	66
Figure 4.18	Distribution of positive malaria cases based on the age group of the study population	67
Figure 4.19	Distribution of <i>Plasmodium</i> species infection by PCR based on the age groups	69
Figure 4.20	<i>Plasmodium</i> species detected from the male population	70
Figure 4.21	<i>Plasmodium</i> species detected from the female population	70
Figure 4.22	Nested PCR detection of <i>Plasmodium</i> species throughout the collection period	72
Figure 4.23	A 1.5% agarose gel showing gel purified products following amplification using primer pair Pmk8 and rPLU5	75
Figure 4.24	Phylogenetic tree based on the 27 ssrRNA gene sequences of <i>P. knowlesi</i> produced by the maximum likelihood method	85
Figure 4.25	Phylogenetic tree based on the ssrRNA gene sequences of <i>P. knowlesi</i> produced by the maximum likelihood method	86

Figure 4.26	Phylogenetic tree based on the <i>ssrRNA</i> gene sequences of <i>P. knowlesi</i> produced by the neighbor-joining method	87
Figure 5.1	Annual rainfalls in Malaysia	99



LIST OF SYMBOLS AND ABBREVIATIONS

/	or
mm	millimeter
%	percentage
cm	centimeter
x	times
ml	millilitre
min	minute
μl	microlitre
μM	micromolar
μg	microgram
°C	degree celcius
V	voltage
kb	kilobase
M	molar
mM	milimolar
rpm	revolution per minute
bp	basepair
MgCl ₂	magnesium chloride
dNTP	deoxynucleotide triphosphate
B.C	Before Century
%	Percentage
A	Adenosine
G	Guanine
DNA	Deoxyribonucleic acid
r	Ribosomal
RNA	Ribonucleic acid
ssr	Small subunit ribosomal

CHAPTER 1

INTRODUCTION

1.1 Background

Malaria is one of the most important infectious diseases in the world. It is a tropical disease caused by parasites of the genus *Plasmodium*. In 2006, there were around 250 million cases of malaria which resulted in almost one million deaths (World Malaria Report 2009). Malaria remains a health problem especially in underdeveloped and remote regions of the world (Cox-Singh, 1997). Moreover, in most of the countries of South East Asia, malaria remains a serious public health problem (Vythilingam, 2005). In remote areas of Malaysia, malaria is the most common vector-borne parasitic disease (Singh & Cox-Singh, 2001). However, huge reduction in malaria cases has been achieved from the malaria control programme which was established since 1967 in Malaysia (Abdulelah *et al.*, 2010).

Four human *Plasmodium* parasites have been well-recognized; namely *Plasmodium falciparum*, *P. malariae*, *P. vivax* and *P. ovale* to cause malaria worldwide. Certain malaria-endemic regions may have mixed infections where two or more of these species are involved (Zimmerman, 2004). Recently, *P. knowlesi*, a simian malaria parasite which incidence was rare in humans has been defined as the "fifth human malaria species" following its discovery in humans in Malaysian Borneo (White, 2008). Naturally acquired *P. knowlesi* infection in humans was found to be widely spread in Malaysian Borneo and in the state of Pahang in West Malaysia from previous studies (Cox-Singh *et al.*, 2008). *Plasmodium knowlesi* was first isolated in 1931 from a long-tailed macaque and has a relatively broad range of host extending to humans (Knowles & Gupta, 1932).

In human hosts, different malaria parasite species differ greatly in responses to antimalarial drugs, transmission potential and their nature of immunity (Liu *et al.*, 1998). Accurate diagnosis of *Plasmodium* species infection is important for proper management, disease control and treatment. *Plasmodium falciparum*, a

malignant *Plasmodium* species results in high mortality and caused more than one million deaths each year worldwide, especially in Africa (World Malaria Report, 2005). Therefore, differentiating *P. falciparum* from the other species of *Plasmodium* is essential in effective control and treatment of this disease. Besides, based on microscopic examination, *P. knowlesi* has been commonly misdiagnosed as *P. malariae* infection which is benign. This is due to the similar morphological appearance of these two parasites microscopically. As opposed to *P. malariae* infection, *P. knowlesi* infection can be fatal and hence it is not benign (Cox-Singh *et al.*, 2008).

Epidemiology is defined as the study of the genotypes and expression of the pathogen with its relation to the occurrence of infection and disease in human populations (Conway, 2007). Molecular information detected within individuals will be incorporated in the study of molecular epidemiology studies (Conway, 2007). Epidemiological studies on molecular identification of malaria parasite species are important for proper management and control of the disease. Previously, a high incidence of *P. malariae* infection had been reported in Malaysian Borneo (Conway, 2007). However, a molecular epidemiological study conducted in the Kapit District of Sarawak showed no *P. malariae* infection but *P. knowlesi* infection was detected by PCR (Singh *et al.*, 2004). Therefore, accurate diagnosis of *Plasmodium* species is crucial as wrong diagnosis could result in wrong treatment of a potentially fatal disease.

Routine microscopic examination is the primary, cheapest and commonly used method which has been considered as the "gold standard" for the diagnosis of *Plasmodium* parasite infection. Standard malaria diagnosis is done based on Giemsa-stained thick and thin blood films. It is simple, rapid and cost effective in maintenance and can be easily applied in the field (Singh, 1997). However, a well-trained microscopist is needed for accurate diagnosis due to the small size and morphological similarity of *Plasmodium* species. Nevertheless, this method is also prone to misdiagnosis especially in the cases of mixed infections and low level parasitemia (Genc *et al.*, 2010). Besides, microscopic examination is also labour intensive in epidemiology studies especially in studies involving large samples which

need to be diagnosed in a short period of time. Therefore, molecular techniques have been developed for more sensitive, specific and rapid diagnosis and detection of malaria parasites.

The polymerase chain reaction (PCR) is one of the molecular techniques that have been widely used for malarial detection in epidemiological studies. PCR-based assays have better sensitivity in parasite diagnosis and are more specific especially for the detection of mixed infections (Singh, 1997). Then it has been consistently shown to be powerful tools for the diagnosis of malaria. The most sensitive PCR-based assays are nested-PCR and real-time PCR (Boonma *et al.*, 2007). These techniques are able to detect as low as 1 parasite/ μ l of blood (Boonma *et al.*, 2007). For diagnosis of low-level parasitemia and correct diagnosis of malaria parasite species, nested-PCR has been reported to be more useful compared to microscopic examination (Aslan *et al.*, 2007). Real-time PCR provides higher sensitivity, specificity and quantification. Although PCR-based technique is very sensitive and specific, it is however more expensive and not as rapid as microscopy. Nested PCR based on the amplification of the genus- or species-specific small subunit ribosomal RNA gene (ssrRNA) was used to detect the presence of *Plasmodium* species parasite in patient samples in this study.

1.2 Significance of Study

Previous epidemiological study has been carried out in 15 administrative districts in Sabah, Malaysian Borneo in 2005 in detecting *P. knowlesi* infection from microscopy-confirmed archival *P. malariae* blood films (Cox-Singh *et al.*, 2008). Since then, no epidemiological study has been conducted in Sabah and hence this molecular epidemiology study would be timely. Actual prevalence of different malaria infections in the interior divisions of Sabah obtained by using sensitive nested PCR for the detection of *Plasmodium* species would provide a clearer picture and better insights into the actual situation of this disease in Sabah. Epidemiology findings from this study could assist the Department of Health Sabah in accessing the risk of knowlesi malaria. Moreover, these findings could also be used to provide institute appropriate guidelines in the proper management and treatment of the disease.

1.3 Objectives

The objectives of this study were

- To use sensitive and specific nested-PCR methods to accurately identify the human malarial parasites in the interior division of Sabah.
- To compare the PCR findings with the microscopic examination results in order to determine the rate of misidentification as well as mix-infection of *Plasmodium* species.
- To characterize *P. knowlesi* isolates in the interior division of Sabah based on the *ssrRNA* gene.



UMS
UNIVERSITI MALAYSIA SABAH

CHAPTER 2

LITERATURE REVIEW

2.1 Background of Malaria

Malaria is an ancient disease that remains a serious public health problem in the world. There are an estimated 247 million cases of malaria occurring yearly with approximately 85% of the cases found in Africa south of Sahara and caused about 881,000 deaths. About 91% of the death caused by malaria was reported in young children as malaria affected mostly children under the age of five years old. Besides, it also poses a serious risk to pregnant women and infants as well as a common cause of miscarriage (Basic Malaria Microscopy, WHO, 2010).

In brief, malaria is a disease caused by the protozoan parasites of the phylum Apicomplexan, namely *Plasmodium* parasite which infects human's red blood cells. It is transmitted by the bite of infected female *Anopheles* mosquitoes. Previously, four common *Plasmodium* species have been well recognized to cause malaria in human, namely *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. Recently, the fifth *Plasmodium* species in human has been identified to cause naturally acquired human malaria, *P. knowlesi* (White, 2008). Among five human malaria parasites, *P. falciparum* is the most dangerous as re-infection can be life-threatening and cause death if it is not recognized and properly treated.

Socially, malaria may cause poverty. It is estimated that malaria cause approximately more than US\$ 12 billion per year globally (Basic Malaria Microscopy, WHO, 2010). Malaria is most serious in poor countries. Population living under impoverished conditions are highly affected (Malaria, 1998). In rural areas, incidence of malaria is highest when the need of agriculture work is greatest. Areas with concentrated populations like the workers at construction sites are commonly attacked by malaria (Malaria, 1998). Community with many ill members affected by malaria will result in the absence of work and school. According to Mharakurwa and Mugochi (1994), school absenteeism is around 28% in area where malaria is

endemic and high drug resistance. Nevertheless, education is affected. Besides, heavy spending on treatment, reduction of crops production and family income were also caused by the repeated attacks of malaria in the endemic areas (Malaria, 1998).

Unstable incidence of malaria and their geographical distribution are caused by drug resistance at the high prevalence areas, expansion of malaria into areas at higher elevation as well as the widespread availability of fake medicines. Moreover, different kinds of population mobility and deforestation for development also cause instability in the transmission of malaria (Basic Malaria Microscopy, WHO, 2010). Figure 2.1 shows the worldwide distribution of malaria and reported drug resistance.

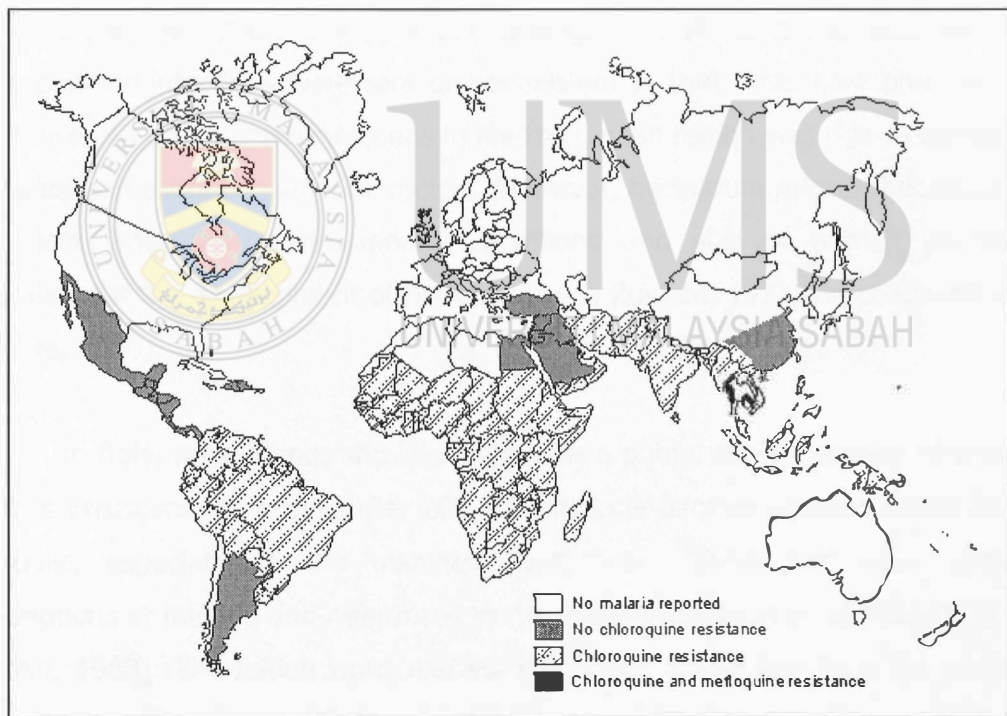


Figure 2.1: Worldwide malaria endemic zones.

Source: Canada Communicable Disease Report March 2000 (CRRD)

2.2 History of Malaria

The word malaria was introduced by Horace Walpole in 1740 and comes from the Italian *mal'aria* which means spoiled air (Russell, 1955). Previously, malaria fever was known to be caused by miasmas rising from the swamps. This idea was

persisted for over 2500 years. There are ancient references reported that malaria occurred about 2700 B.C. in a Chinese document, 2000 B.C. from clay tablets from Mesopotamia, 1570 BC from Egyptian papyri and as far as sixth century B.C. from Hindu texts (Cox, 2010).

At the end of the sixth century B.C., there had been references of malaria being described as intermittent fevers in the Greek poems, writings of Aristophanes (445-385 B.C.), Aristotle (384-322 B.C.), Plato (428-347 B.C) and Sophocles (496-406 B.C.). Moreover, physician Hippocrates (460 - 370 B.C.) had clearly discussed the quartan and tertian fevers. By the fifth century B.C., it was no doubt that *P. malariae* and *P. vivax* were present in Greece. Hippocrates believed that the intermittent fevers were caused by disturbances in the body's humors due to drinking water from stagnant marshes. However, there was no record on severe, malignant tertian fevers in Hippocratic writings. Therefore, it was assumed that, *P. falciparum* infections were rare or nonexistent in that time. Late presence and incidence of *P. falciparum* infections in Mediterranean region was due to indigenous *Anopheline* mosquitoes as poor vectors. However, falciparum malaria became more prevalent when the environmental conditions and size of human population adequate for the development of the vectors (de Zulueta, 1973; Bruce-Chwatt & de Zulueta, 1980).

In Italy, no evidence showed malaria as a public health problem among the ancient Etruscans. However, after 200 B.C., malaria became obvious in the Roman Republic, especially in the marshes near Ostia. There had been accurate descriptions of malaria and references to marshes as the sources of malaria (Bruce-Chwatt, 1988). The Italian word *mal'aria* mentioned above rise from the condition described as "Roman fever". Malaria was uneven distributed in Greece and Rome. Eventually, its endemicity fluctuates in cyclical manner (Boyd, 1949).

Malaria reached far west to Spain and in the east in Poland and Russia by the 12th century. In Eastern Europe, intermittent fevers were reported to be common in 15th century (Bruce-Chwatt, 1988).

In the 14th, 15th and 16th centuries, seasonal fevers called as argues were common in England. References of agues were present in the writings of Chaucer (1340-1400) and Shakespeare (1564-1616). Malaria was prevalent in England from 17th and 18th century where cases were imported from soldiers and sailors returning from India and Africa (Bruce-Chwatt, 1988).

In the New World, no malaria was recorded before the arrival of European explorers and colonist. Therefore, it was assumed that *P. malariae* and *P. vivax* were brought to the Americas in post-Columbian times. Transportation of African slaves later introduced falciparum malaria (Bruce-Chwatt, 1988). By early 1800's malaria was distributed worldwide (Malaria, 1998).

2.3 Discovery of Malaria Parasites

In 1880, Charles Louis Alphonse Laveran was the first person to discover the parasites in the blood of patients infected with malaria. The parasitic protozoan discovered was known as *Oscillaria malariae*. By 1890, the protozoan parasite that caused malaria was found to invade and multiply in the red blood cells. Later on, Laveran was awarded Nobel Prize for Medicine in 1907. After all the confusions and studies done, there were three species were identified; benign tertian (*Haemamoeba vivax*), malignant tertian (*Laverania malariae*) and quartan (*Haemomoeba malariae*) malaria. In 1897, William MacCallum observed the flagellated structures/bodies fused with the non-motile bodies to form a vermicule (now known as ookinete) when examining the blood of crows infected with *Haemoproteus columbae* which is closely related to malaria parasites. It was suggested that the sexual stages of the parasite were found (Cox, 2010).

In the study of the transmission of the parasite among human, Ronald Ross was the first to show that malaria parasite was transmitted by the bite of infected mosquitoes in 1897 when he was working in India. Ross discovered that culicine mosquitoes transmitted *P. relictum*, the avian malaria parasite. He suggested that it might also be the same for human malaria parasites. Ross classified the mosquitoes as grey (culicine), brindled and dappled-winged mosquitoes (anophelines). The 'dappled-wing' mosquitoes were found to contain pigmented bodies which was