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

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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.

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LIST OF SYMBOLS AND ABBREVIATIONS

/	or
mm	millimeter
%	percentage
cm	centimeter
x	times
ml	mililitre
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/µl 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.

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.

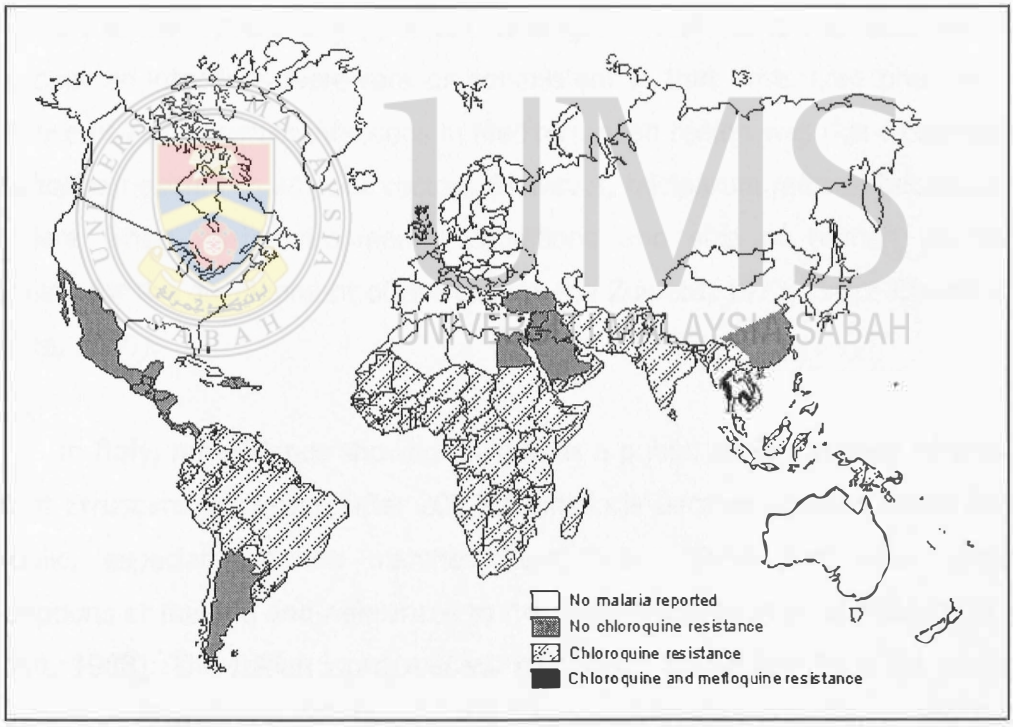

Figure 2.1: Worldwide malaria endemic zones.
Source: Canada Communicable Disease Report March 2000 (CRRD)

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