MUTATIONAL ANALYSIS OF *mdr1* GENE IN *Plasmodium falciparum* ISOLATES FROM SABAH

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LIST OF ABBREVIATIONS

ABC	-	ATP binding cassette
ACT	-	Artemisinin Combination Therapy
Asn	-	Asparagine
BLAST	-	The Basic Local Alignment Search Tool
Bp	-	basepairs
CIDR	-	Cystein-rich Interdomain Regions
CQ	-	Chloroquine
DBL	-	Duffy-Binding-Like
DHFR	-	Dihydrofolate reductase
DHPS	-	Dihydropteroate synthase
DNA	-	Deoxyribonucleic Acid
dNTP	-	deoxynucleotide triphosphate
DV	-	Digestive Vacuole
EtBr	-	Ethidium Bromide
FPIX	-	Ferriprotoporphyrin IX
HF	-	Halofantrine
HIV	-	Human Immunodeficiency Virus
LD	-	Linkage Disequilibrium
MEGA6	-	Molecular Evolutionary Genetic Analysis Version 6
MQ	-	mefloquine
NCBI	-	National Center For Biotechnology Information
NTS	-	N-Terminal Segment
PCR	-	Polymerase Chain Reaction
pfCRT protein	-	*Plasmodium falciparum* chloroquine resistance transporter

pfcrt gene	-	*Plasmodium falciparum* chloroquine resistance transporter
pfdhfr	-	*Plasmodium falciparum* dihydrofolate reductase gene
pfdhfr-ts synthase	-	Plasmodium falciparum dihydrofolate reductase-thymidylate
pfdhps	-	*Plasmodium falciparum dihydropteroate* synthase gene
PfEMP1	-	*Plasmodium falciparum* erythrocyte membrane protein 1
pfmdr1	-	*Plasmodium falciparum* multidrug resistance gene 1
Pgh1	-	P-glycoprotein homologue
Pgp	-	P-glycoprotein
QN	-	Quinine
RBC	-	Red Blood Cell
RCF	-	Relative Centrifugal Force
RNA	-	Ribonucleic acid
RPM	-	Revolutions per Minute
SNPs	-	Single-Nucleotide Polymorphisms
SP	-	Sulfadoxine/Pyrimethamine
ssRNA	-	small subunit ribosomal RNA
Taq	-	*Thermus aquaticus*
TDMs	-	Transmembrane Domains
Tyr	-	Tyrosine
WHO	-	World Health Organization

%	-	Percentage
µl	-	Microliter
µM	-	micromolar
kb	-	kilo basepairs
KDa	-	Kilo Dalton
Km^2	-	kilometer per square
ml	-	mililiter
mM	-	milimolar
^{O}C	-	Degree Celcius

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DECLARATIONS

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

17 June 2016

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SUPERVISOR DECLARATION

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ABSTRACT

The *Plasmodium falciparum* multi drug resistance 1 (*pfmdr1*) gene encodes P-glycoprotein homologue 1 (PGH-1) in the *Plasmodium* digestive vacuole. Point mutations at positions 86, 184, 1034, 1042 and 1246 in this gene has been previously identified to be associated with antimalarial drugs resistance. However, information regarding the mutation pattern of the *pfmdr1* from Sabah isolates have not yet been reported. This study aims to identify nucleotide mutations of *pfmdr1* gene among *P. falciparum* isolates in Sabah. Thirty-one *P. falciparum* isolates were collected from Keningau, Kota Kinabalu and Kudat, Sabah with confirmed single infection. Next, PCR amplification that covers the five point mutations (86, 184, 1034, 1042 and 1246) was carried out prior to sequencing. This study revealed 100% mutation N86Y in the studied Sabah isolates as compared to 5% prevalence in Pahang isolates was associated with chloroquine resistance (CQR). In addition, the mutation Y184F of Sabah isolates with 3.23% prevalence was linked with arthemer/lumefantrine (AL) resistance marker. Multiple amino acid sequence comparisons revealed three haplotypes namely YYSND (93.5%), YFSND (3.2%) and YYSNH (3.2%) were identified. There were two mutations which is potentially to be a chloroquine resistance marker which are *pfcrt*-K76T and *pfmdr1*-N86Y detected in this study isolates with 70.9% and 100% prevalence. However, the LD constant of both D' and r^2 were equal to zero which indicate that these two loci were not co-inherited under CQ selective pressure and independently to each other. The mutational analysis of *pfmdr1* revealed point mutations that linked to several antimalarial drugs that will be helpful in the surveillance of drug resistance in Sabah, Malaysia.

ABSTRAK

(ANALISIS MUTASI GEN MDR1 DALAM Plasmodium falciparum DARI SABAH)

Plasmodium falciparum rintangan pelbagai dadah 1 (pfmdr1) adalah gen yang mengkodkan P-glikoprotein homolog 1 (PGH-1) dalam vakuol penghadaman Plasmodium. Titik mutasi di kedudukan 86, 184, 1034, 1042 dan 1246 dalam gen ini telah dikenal pasti mempunyai kaitan dengan rintangan ubat anti malaria. Walau bagaimanapun, maklumat mengenai pola mutasi pfmdr1 dari Sabah tidak dilaporkan lagi. Kajian ini bertujuan untuk mengenal pasti mutasi gen pfmdr1 antara P. falciparum dari Sabah. Tiga puluh satu P. falciparum yang telah disahkan positif diambil dari Keningau, Kota Kinabalu dan Kudat, Sabah. Diikuti dengan PCR yang meliputi lima titik mutasi (86, 184, 1034, 1042 dan 1246) telah dijalankan sebelum penjujukan. Kajian ini menunjukkan 100% mutasi N86Y didapati di Sabah berbanding dengan 5% kelaziman diperolehi daripada sampel Pahang telah dikaitkan dengan rintangan chloroquine (CQR). Tambahan pula, Y184F mutasi dalam sampel Sabah dengan 3.23% kelaziman dikaitkan dengan arthemer/ lumefantrine (AL) rintangan rendah. Perbandingan antara penjujukan pelbagai Asid amino mendedahkan tiga haplotaip iaitu YYSND (93.5%), YFSND (3.2%) dan YYSNH (3.2%) telah dikenal pasti. Terdapat dua mutasi yang berpotensi untuk menjadi penanda rintangan chloroquine iaitu pfcrt-K76T dan pfmdr1-N86Y yang dikesan dalam kajian ini dan masing-masing dengan 70.9% dan 100% kelaziman. Walau bagaimanapun, LD bagi kedua-dua D' dan r^2 adalah sama dengan sifar yang menunjukkan bahawa kedua-dua lokus tidak diwarisi bersama di bawah CQ tekanan terpilih dan tidak bergantung antara satu sama lain. Analisis mutasi daripada pfmdr1 mendedahkan titik mutasi yang dikaitkan dengan beberapa ubat anti malaria yang akan membantu dalam pengawasan rintangan dadah di Sabah, Malaysia.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is caused by *Plasmodium* parasites. *Plasmodium falciparum* is a parasitic protozoan which contributed to most of the malaria death cases occurring in the tropical and subtropical areas. Globally, it was estimated that 1.2 billion people are at high risk of getting malaria and Africa is the worst affected region. The latest estimation was in 2013 where 198 million cases of malaria occurred and results in 584 000 deaths around the globe (World Health Organization Malaria report, 2014). In Malaysia, 4,725 malaria cases were reported in 2012 in which the highest number of cases took place in Sabah (Ministry of Health Malaysia, 2014).

There are five *Plasmodium* species namely *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi* that cause malaria in humans. *Plasmodium vivax* and *falciparum* are responsible for the majority of malaria cases in Malaysia. In fact, *Plasmodium falciparum* has led to the most death cases from malaria around the world including Malaysia (World Health Organization Malaria report, 2014).

Several type of drugs developed from various chemical compounds are used to treat malaria including arylaminoalcohols, aminoquinolines, artemisinines, antifolates and antibiotics. Artemisinin Combination Therapy (ACT) is used worldwide as the first-line therapy for *falciparum* malaria. The ACT that is used in Sabah is either based on arthemether/lumefantrine or artesunate/mefloquine. Meanwhile, quinine is recommended to treat pregnant women with malaria (Ministry of Health Malaysia, 2014). In addition, sulfadoxine/pyrimethamine (SP) combination is also used as a

presumptive treatment by field workers to treat *P. falciparum* for patients with suspected malaria who live deep in the interior regions in which a timely microscopy result for malaria parasites may be difficult to obtain. This drug combination has also been applied as a preventive treatment in rural areas (Lau, Sylvi and William, 2013).

Unfortunately, the effectiveness of the drug have been challenged by the *Plasmodium* parasite's ability to resist treatment. *Plasmodium falciparum* multi drug resistance 1 gene (*pfmdr1*) have been reported to be associated with several antimalarial drugs resistance. This gene encodes the P-glycoprotein homologue 1 (Pgh-1) which is located at the membrane of the parasite's digestive vacuole (Sanchez, Rotmann, Stein and Lanzer, 2008). A Recent study by Reiling and Rohrbach (2015), proposed that the alteration of drug susceptibility which includes mefloquine, halofantrine and quinine are related to one or more mutations at five amino acid positions of the *mdr1* gene (N86, Y184, S1034, N1042 and D1246).

Interestingly, the mutations found in *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) and also in *pfmdr1* have been implicated in chloroquine (CQ) resistance among malarial parasites (Das Sutar, Gupta, Ranjit, Kar and Das, 2011). The haplotypes found in both genes are believed to have association in conferring chloroquine resistance among *P. falciparum* population (Ibraheem, Abd Majid, Mohd Noor, Mohd Sidek and Basir, 2014). In addition, the mutations found at position 76 of *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) and also at position 86 of *pfmdr1* have been implicated in chloroquine (CQ) resistance among malarial parasites (Das Sutar et al., 2011). Linkage disequilibrium between *pfcrt*-T76 and *pfmdr1*-Y86 in the parasite population revealed that they are co-transmitted under drug selection and hence supports the role of the *pfmdr1*-Y86 allele for being resistant only in the presence of the *pfcrt*-T76 allele.

1.2 Problem statement

Artemisinin combination therapy (ACT) such as arthemether/lumefantrine and artesunate/mefloquine are used as a first line therapy for *falciparum* malaria in Sabah and quinine is recommended to treat pregnant women with malaria. However, it is

undeniable that chloroquine is cheaper than other current antimalarial drugs such as mefloquine, halofantrine and ACT (Philips, 2001). Mutations pattern in *pfmdr1* gene will be helpful to reveal whether the *P. falciparum* isolates from Sabah is still resistance to chloroquine treatment or not.

As acknowledged in several studies, mutations in specific codons of *pfmdr1* gene have a role in the alteration of anti-malarial drug susceptibility and also believed to have a correlation with mutations that occur in *pfcrt* gene which are responsible for malarial parasites' resistance to chloroquine treatment.

A previous study done by Tan, Lau, William and Prabakaran (2014), have discovered several point mutations that formed several haplotypes (SVMNT, CVMNK, CVIET and SVMNT) related to chloroquine resistance and sensitivity. These mutations is useful to be associated with mutations found in the *pfmdr1* gene that is amplified from the same samples by associating the mutations from both genes as both of these polymorphisms are related with chloroquine resistance marker. The information regarding the mutations' patterns of the *pfmdr1* as well as the relationship between *pfcrt* and *pfmdr1* genes isolated from Sabah are not reported yet. Hence, this study is important to determine the potential of antimalarial drug resistance in Sabah.

1.3 Objectives of the study:

The objectives of the study are:

1. To identify point mutations at the *pfmdr1* gene in *Plasmodium falciparum* isolates collected from patience from hospitals in Kota Kinabalu, Kudat and Keningau, Sabah.
2. To compare the mutations of *pfmdr1* isolates in Kota Kinabalu, Kudat and Keningau, with the isolates from other geographical regions.
3. To associate mutation of *pfmdr1* and *pfcrt* among *Plasmodium falciparum* isolates in Sabah.

1.4 Significance of study

This project will determine mutation of the *pfmdr1* gene in *Plasmodium falciparum* isolates of Sabah as well as its association with mutations found in the *pfcrt* gene amplified from the same samples of previous study done by Tan *et al.* (2014). It will be helpful to investigate whether the mutation in the *pfmdr1* gene is correlated with the resistance or susceptibility against a specific antimalarial drug such as quinine and also its relationship with *pfcrt* gene mutations which functions as a chloroquine resistance marker. Importantly, this research will be useful in understanding of mutations pattern in *pfmdr1* which will affect the efficacy of antimalarial drug treatment uses for malaria in Sabah.

CHAPTER 2

LITERATURE REVIEW

2.1 History of malaria

Malaria is a dangerous disease caused by the parasite *Plasmodium spp.* and *Anopheline* mosquito as the vector of the parasite. Malaria-caused parasite was first identified by Charles Louis Alphonse Laveran in 1880. He seen several transparent, mobile filaments emerging from a clear spherical body observed within drop of blood collected from one of the patients. Initially, Laveran named it as *Oscillaria malariae* or known as a *Plasmodium falciparum* (Sherman, 1998).

In 1882, Italian scientist Ettore Marchiafava, Angelo Celli and Corrado Tomassi-Crudeli discovered a second *Plasmodium* parasite that they named it as *Plasmodium malariae*. This parasite is non-pigmented and has an ameboid movement character. This was followed by the discovery of Camillo Golgi six years of Leveran's findings, where he found that the asexual development and reproduction by multiple fission of the parasites led to *P. malariae* and *P. vivax* fevers. He showed that the rupture of red blood cell and parasites' liberation occurred at the onset of malarial fever (Sherman, 1998).

In 1897, Ronald Ross have succeed demonstrated that malaria disease is transmitted by mosquito. He discovered that the stomach of an *Anopheles* mosquito which have ingested the infected blood consist of clear and circular outline with a black pigment cluster in it that is known as malaria pigment. On the next day, he dissected the *Anopheles* mosquito that had fed on the same patients and found that the parasite inside this mosquito have a larger body size and carried the malaria