# COMPARATIVE STUDY BETWEEN *DHFR* AND *DHPS* GENES IN *PLASMODIUM FALCIPARUM*, *PLASMODIUM VIVAX* AND *PLASMODIUM KNOWLESI* ISOLATED FROM SABAH

**NOR AFIZAH BINTI NUIN**

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**NOR AFIZAH BINTI NUIN**

**DISSERTATION SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)**

**BIOTECHNOLOGY RESEARCH INSTITUTE**
**UNIVERSITI MALAYSIA SABAH**
**2016**

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# DECLARATION

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

9th September 2016

Nor Afizah Binti Nuin

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# CERTIFICATION

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MATRIC : **MZ1211008T**

TITLE : **COMPARATIVE STUDY BETWEEN *DHFR* AND *DHPS* GENES IN *PLASMODIUM FALCIPARUM*, *PLASMODIUM VIVAX* AND *PLASMODIUM KNOWLESI* ISOLATED FROM SABAH**

DEGREE : **MASTER OF SCIENCE (BIOTECHNOLOGY)**

VIVA DATE : **3RD AUGUST 2016**

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**DECLARED BY;**

**1. MAIN SUPERVISOR**

Lau Tiek Ying Signature

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**2. CO-SUPERVISOR**

Prof. Dr. Clemente Michael Wong Vui Ling _____

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Nor Afizah Binti Nuin
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# ABSTRACT

Malaria is one of the globally challenging parasitic infectious diseases. Treatment failure due to resistance in malaria parasites is an important factor in the effective treatment of malaria. Dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) are two genes that encode the enzymes involved in the folate pathway targeted by antifolate drugs. This study was performed to compare molecular analysis of these two genes in three most prevalent human *Plasmodium* parasites in Sabah namely *P. falciparum, P. vivax* and *P. knowlesi*. The genes *dhfr* and *dhps* were amplified and sequenced from PCR confirmed single infection isolates of *P. falciparum, P. vivax* and *P. knowlesi*. The sequences were analysed using DNASTAR and MEGA6 softwares. Among 228 samples collected, 70 of them were *P. falciparum* positive, 11 were *P. vivax*, 5 were *P. malariae* and 67 *P. knowlesi* monoinfection. DNA sequence alignment of *dhfr* gene among Sabah isolates was highly conserved in *P. falciparum* and *P. vivax* while *P. knowlesi* demonstrated polymorphisms in about 4% of the full-length *pkdhfr*. Meanwhile, the same rates were also observed in *dhps* gene of which *P. falciparum* and *P. vivax* showed less number of nucleotide polymorphisms than in *P. knowlesi*. Haplotyping analysis at positions which mutations significantly reduce antifolate drug sensitivity revealed identification of 4 *pfdhfr-pfdhps* (2.8% of A**IRN**I-S**G**KAA, 69.4% of AN**RN**I-S**G**KAA, 25% of AN**RN**I-S**G**K**G**A and 2.8% of AN**RNL**-S**GTG**A) while only 2 *pvdhfr-pvdhps* F**R**T**N**I-SAKAV (33.3%) and **LRMT**I-S**G**KAV (33.3%) type in the study areas. With respect to *P. knowlesi*, the *dhfr* orthologues haplotyping analysis showed wild-type sequence (ANSSI) at this locus. At present, there is no report in *pkdhps* gene mutation conferring resistance has been described in *P. knowlesi*. However, nucleotide polymorphisms observed in *pkdhfr* and *pkdhps* from Sabah isolates could have resulted from the selection of *P. knowlesi* populations with drug resistance alleles under continuous drug pressure indicating the possible presence of human-to-human transmission. This study also shows a remarkably high prevalence of mutations linked to drug resistance in *P. falciparum* and *P. vivax* which highlights the molecular study of polymorphisms in *dhfr* and *dhps* genes remain as a useful tool to monitor the emergence and spread of antifolate drug resistance in malaria parasites from Sabah isolates.

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# *ABSTRAK*


***Kajian perbandingan di antara gen dhfr dan dhps di dalam Plasmodium falciparum, Plasmodium vivax dan Plasmodium knowlesi pencilan dari Sabah***

*Malaria merupakan salah satu penyakit berjangkit disebabkan oleh parasit yang merupakan cabaran kepada seluruh dunia. Kegagalan rawatan disebabkan oleh rintangan dalam parasit malaria merupakan factor penting untuk merawat malaria secara berkesan. Dihydrofolate reductase (dhfr) dan dihydropteroate synthase (dhps) adalah dua gen yang yang terlibat dalam laluan folat yang disasarkan oleh dadah antifolate. Kajian ini telah dijalankan untuk membandingkan analisis molekul kedua-dua gen di dalam tiga jenis parasit Plasmodium yang menjangkiti manusia yang paling lazim di Sabah iaitu P. falciparum, P. vivax dan P. knowlesi. Gen dhfr dan dhps telah diamplifikasi dan penjujukan DNA telah dibuat dari pencilan yang mempunyai jangkitan tunggal P. falciparum, P. vivax dan P. knowlesi. Jujukan-jujukan telah dianalisis menggunakan perisian DNASTAR dan MEGA6. Di antara 228 sampel yang telah diambil, 70 daripadanya adalah positif untuk P. falciparum, 11 adalah P. vivax, 5 P. malariae dan 67 P. knowlesi. Urutan penjajaran DNA bagi gen dhfr di kalangan pencilan dari Sabah menunjukkan persamaan yang tinggi di dalam P. falciparum dan P. vivax manakala P. knowlesi menunjukkan kira-kira 4% polimorfisma daripada keseluruhan gen pkdhfr. Sementara itu, kadar yang sama juga terdapat pada gen dhps di mana P. falciparum dan P. vivax menunjukkan jumlah polimorfisma yang kurang berbanding yang terdapat di dalam P. knowlesi. Analisis haplotaip pada kedudukan di mana mutasi dapat mengurangkan sensitivity dadah antifolat telah menunjukkan bahawa terdapat 4 jenis pfdhfr-pfdhps (2.8% adalah A**IRN**I-S**G**KAA, 69.4% adalah AN**RN**I-S**G**KAA, 25% adalah AN**RN**I-S**G**K**G**A dan 2.8% adalah AN**RNL**-S**G**T**G**A) manakala hanya 2 jenis pvdhfr-pvdhps iaitu F**R**T**N**I-SAKAV (33.3%) dan **LRMT**I-S**G**KAV (33.3%) yang terdapat di kawasan kajian. Berkenaan dengan P. knowlesi, analisis gen ortolog dhfr menunjukkan tiada perubahan pada haplotaip asal (ANSSI) pada lokus ini. Sehingga kini, tidak terdapat laporan mengenai mutasi pada gen dhps yang menyebabkan rintangan di dalam P. knowlesi. Walaubagaimanapun, polimorfisma nukleotida yang diperhatikan dalam gen pkdhfr dan pkdhps di kalangan pencilan dari Sabah boleh terjadi disebabkan pemilihan oleh populasi P. knowlesi terhadap alel-alel rintangan dadah di bawah tekanan dadah yang berterusan yang menunjukkan kebarangkalian terdapat pemindahan jangkitan dari manusia ke manusia. Kajian ini juga menunjukkan kelaziman mutasi yang tinggi yang dikaitkan dengan rintangan terhadap dadah di dalam P. falciparum dan P. vivax yang mana telah mengetengahkan kajian molekul polimorfisma di dalam gen dhfr dan dhps kekal sebagai kaedah yang berguna bagi*


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*memantau kemunculan dan penyebaran rintangan terhadap dadah antifolat di kalangan parasit malaria di Sabah.*

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# LIST OF SYMBOLS AND ABBREVIATIONS

| | | |
|---|---|---|
| % | - | Percentage |
| mm | - | Millimeter |
| cm | - | Centimeter |
| x | - | Times |
| ml | - | Milliliter |
| min | - | Minute |
| sec | - | Second |
| U | - | Unit |
| µl | - | Microliter |
| µM | - | Micromolar |
| $^{o}C$ | - | Degree celcius |
| V | - | Voltage |
| kb | - | Kilobase |
| bp | - | Basepair |
| M | - | Molar |
| mM | - | Milimolar |
| rpm | - | Revolution per minute |
| $MgCl_2$ | - | Magnesium chloride |
| dNTPs | - | Deoxynucleotide triphosphates |
| DNA | - | Deoxyribonucleic acid |
| *dhfr* | - | Dihydrofolate reductase |
| *dhps* | - | Dihydropteroate synthase |
| SNPs | - | Single nucleotide polymorphisms |
| Cds | - | Coding sequence |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Research

The emergence of infectious disease agents in human beings is increasingly important in public health. Malaria is one of the most challenging infectious diseases that captured most of the global attention. In most remote and underdeveloped regions of the world, malaria remains as a health problem (Cox-Singh, 1997) and become the most prevalent and most harmful blood parasitic disease of humans (White, 2004) with an estimated 214 million number of cases and 438 000 deaths by year 2015 (WHO, 2015).

Malaria is caused by single-celled organisms which are known as protozoan parasites of the genus *Plasmodium*. This protozoan parasite cannot survive in condition of less oxygen availability (Atherson, 2012). The parasite is carried by the female *Anopheles* mosquitoes which act as a vector and is transmitted by the bite of the infected mosquitoes. Five major species of *Plasmodium* that responsible for causing malaria disease in humans are *P. falciparum, P. vivax, P. malariae, P. ovale,* and *P. knowlesi* (Atherson, 2012).

Fever, shivering, cough, respiratory distress, pain in the joints, headache, watery diarrhea, vomiting and convulsions are the common symptoms of malaria infection. These symptoms can develop within six to eight days after the infection from mosquito bite (Miller *et al.*, 2002). Untreated malaria infection can quickly become a serious threat to patient's life and often leading to death (WHO, 2015).

Malaria had been eliminated or effectively suppressed in many parts of the world. However, in poor countries in the tropics, malaria has resurged due to the

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lack of enforcement as well as the failure of the global eradication campaign in year 1960s (White, 2004).

## 1.2 Antimalarials Resistance: Facing the Problem

In the past four decades, the global resurrection of malaria has been contributed mainly by the development of resistance (Marsh, 1998). Antimalarial resistance is becoming an increasingly important factor in the effective treatment of malaria. Chloroquine is highly resistant in *P. falciparum* in most of the areas that were affected by malaria. Widespread of sulphadoxine-pyrimethamine resistance and their rapid development were also reported while resistance in mefloquine was found only in areas where it has been used extensively such as Thailand, Cambodia and Vietnam (Nosten *et al.*, 2000).

Treatment failure due to resistance in malaria parasite with chloroquine has already become a pressing problem in malaria management since many years ago (Greenberg *et al.*, 1989; Trape *et al.*, 1998) which best explains the increase number of malaria mortality among children in eastern and southern Africa (Korenromp *et al.*, 2003). Similar results will likely happen with the increasing resistance to antifolates drug such as sulphadoxine-pyrimethamine where it has been used as the first-line treatment of malaria disease after chloroquine (Gregson and Plowe, 2005). In East Malaysia, chloroquine is no longer used as the first line treatment for uncomplicated *P. falciparum* due to high prevalence of the *pfcrt* K76T mutants among *P. falciparum* isolates from Sabah. The treatment was then replaced by sulphadoxine-pyrimethamine and followed by artemisinin combinational therapy (ACTs) recently (Cox-Singh *et al.*, 2003; Nor Azrina *et al.*, 2011; Atroosh *et al.*, 2012).

Resistance to sulphadoxine-pyrimethamine has been reported to be associated with specific mutations at dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes. These two genes encode the enzymes involved in the folate pathway targeted by existing antifolate drugs (Bzik *et al.*,

1987; Brooks *et al*., 1994; Triglia and Cowman, 1994). Both genes have been cloned and sequenced, and the discoveries of mutations in these genes have been determined to be associated with resistance against antifolate drugs (Peterson *et al*., 1988). Interruption of folate synthesis by these two genes inhibitors will cause decreasing levels of fully reduced tetrahydrofolate which is an important cofactor in biosynthetic pathways of purine, pyrimidine, and amino acid (Ferone, 1977).

## 1.3 Research Question

Studies to determine the prevalence of molecular markers for antimalarial drug resistance in Sabah already started more than ten years ago. Given that the effectiveness of widely used antimalarials such as sulphadoxine-pyrimethamine is not promising, a study to characterize the drug related polymorphism in malaria parasites in Sabah would be timely. There are two research questions that will need to be answered by this study:

i. Do mutations at both loci *dhfr* and *dhps* exist in *Plasmodium* parasites isolated from Sabah population?
ii. Do mutations conferring resistance in *P. knowlesi* orthologues of the *P. falciparum* and *P. vivax* genes exist?
iii. Are there novel mutations detected at both *dhfr* and *dhps* genes in *Plasmodium* parasites isolated from Sabah population?

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## 1.4 Hypothesis of the Study

Mutations in the gene *dhfr* and *dhps* that confers to the antimalarial drug resistance are present in the *Plasmodium* isolates across Sabah.

## 1.5 Objectives of the Study

This study aimed to achieve the following objectives:

i. To characterize *dhfr* and *dhps* genes in *P. falciparum, P. vivax* and *P. knowlesi* from Sabah isolates.
ii. To identify single nucleotide polymorphisms in *P. knowlesi* orthologues of the *P. falciparum* and *P. vivax* genes associated with antimalarial drug resistance.
iii. To detect novel mutations of antimalarial drug resistance markers in *dhfr* and *dhps* genes of *P. falciparum, P. vivax* and *P. knowlesi* isolates in the stated areas of Sabah.

## 1.6 Significance of the Study

Previous studies have been carried out to determine the prevalence of molecular markers for antimalarial drug resistance in *Plasmodium falciparum* Sabah isolates (Cox-Singh *et al*., 2001; Nor Azrina *et* al., 2011; Noor Rain *et al*., 2013; Lau *et al*., 2013). Understanding the genetic basis of drug resistance is essential for implementing rational measures to overcome the increase number of resistance in the widely used antimalarials. Genetic information can be used for early detection of resistance loci as well as for future monitoring of drug resistant malaria. Hence, genotyping genetic loci associated with drug resistance genes are a potentially useful epidemiological tool in conjunction with the conventional *in-vitro* and *in-vivo* drug-sensitivity assessments. Findings from this study may modulate drug resistance or response in *P. vivax*, *P. knowlesi*, and reassess polymorphisms in *P. falciparum* in Sabah. The prevalence data of various mutations in this study will provide the first time information on polymorphisms on *P. knowlesi* orthologous genes associated with resistance in antifolate drug particularly the sulphadoxine-pyrimethamine.

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