COMPARATIVE STUDY BETWEEN DHFR AND DHPS GENES IN \textit{Plasmodium falciparum} \textit{Plasmodium vivax} AND \textit{Plasmodium knowlesi} ISOLATED FROM SABAH

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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 AIRNI-SGKAA, 69.4% of ANRNISGKAA, 25% of ANRNISGKGA and 2.8% of ANRNISGTA) while only 2 pvdhfr-pvdhps FRMTINIKAV (33.3%) and LRMTISGKAV (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.
ABSTRAK

Kajian perbandingan di antara gen dhfr dan dhps di dalam Plasmodium falciparum, Plasmodium vivax dan Plasmodium knowlesi pencilan dari 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
°C - Degree celcius
V - Voltage
kb - Kilobase
bp - Basepair
M - Molar
mM - Milimolar
rpm - Revolution per minute
MgCl₂ - Magnesium chloride
dNTPs - Deoxynucleotide triphosphates
DNA - Deoxyribonucleic acid
dhfr - Dihydrofolate reductase
dhps - Dihydropteroate synthase
SNPs - Single nucleotide polymorphisms
Cds - Coding sequence
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 resurfaced due to the
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.*, 2005).
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?

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.
CHAPTER 2

LITERATURE REVIEW

2.1 Global Incidence of Malaria
Malaria is one of the most important and major public health problem in tropical and subtropical countries in terms of morbidity and mortality in humans. National Malaria Control and Eradication Programmes was initiated in 1950 and despite the impressive results, many countries have experienced a complete failure in the eradication of malaria due to technical, operational and socio-economical difficulties, which led to the resurgence of malaria worldwide. Other than that, the control programme has also been impeded by the spread of drug resistance in parasite as well as insecticide resistance in mosquito vectors (Ballou et al., 1987) and the complex structure of genetic population of malaria parasites (Prajapati et al., 2006).

Malaria is most serious in poor countries. Populations living under impoverished conditions are highly affected. The incidence of malaria is highest especially in the forest areas which accounted for millions in Amazon regions, Central Africa, the Western Pacific and Southeast Asia (Achard et al., 2002; Mayaux et al., 2005). According to World Malaria Report 2014, there are 97 countries and territories with ongoing malaria transmission and six other countries are working to prevent reintroduction. From these, a total of 3.2 billion people are at risk of being infected with malaria and developing disease while 1.2 billion are at high risk. It has also been reported that 198 million cases with 584 000 deaths occurred worldwide in the year 2013 which represents a decrease in malaria incidence by 30% and mortality rates by 47% since year 2000 (WHO, 2014).
REFERENCES


