Genetic diversity of Merozoite Surface Protein 1 Gene of *Plasmodium falciparum* in Thailand

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ความหลากหลายทางพันธุกรรมของยีนเมอโรซอยต์เซอร์เฟซโปรตีน 1 ของเชื้อมาลาเรียชนิดฟัลซิพารัมในประเทศไทย

น.ส.เมย์ มยาท ธู

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ในปี ค.ศ. 2030 องค์การอนามัยโลกมีเป้าหมายที่จะกำจัดโรคมาลาเรียให้หมดไป 35 ประเทศเป็นอย่างน้อย ปัจจุบันประเทศไทยเป็นประเทศที่มีพื้นที่เสี่ยงที่มีการระบาดของโรคมาลาเรียต่ำในระดับที่จะสามารถเข้าสู่ระยะการกำจัดโรคให้หมดไปได้ ในระยะ 10 ปีที่ผ่านมาพื้นที่ตามแนวชายแดนของประเทศไทยมีความชุกของโรคมาลาเรียสูง เพื่อเป็นการป้องกันไม่ให้เกิดอุบัติการณ์ของโรคมาลาเรียกลับมาการประเมินความไวต่อยาของเชื้อมาลาเรียและการพัฒนาวัคซีนมีความสำคัญ ดังนั้นความรู้พื้นฐานทางความหลากหลายทางพันธุกรรมของเชื้อมาลาเรียจึงมีความจำเป็น ยีนเมอโรซอยต์เซอร์เฟซโปรตีน 1 ซึ่งเป็นหนึ่งในยีนที่มีศักยภาพในการเป็นวัคซีนต้นแบบสามารถใช้ในการติดตามความหลากหลายทางพันธุกรรมของเชื้อมาลาเรียและยังเป็นยีนที่มีศักยภาพที่จะพัฒนาเป็นวัคซีนได้ อย่างไรก็ตามความหลากหลายของยีนเมอโรซอยต์เซอร์เฟซโปรตีน 1 บล็อก 2 ที่เกิดขึ้นในระดับสูงนั้นเป็นอุปสรรคในการออกแบบวัคซีน

ด้วยเหตุนี้การศึกษาลักษณะทางพันธุกรรมของอัลลีลที่แตกต่างกันในยีนนี้ของเชื้อในพื้นที่ระบาดของประเทศไทยจึงมีความสำคัญต่อการควบคุมโรค ตัวอย่างเลือดที่ติดเชื้อมาลาเรียชนิดฟัลซิพารัมจำนวน 236 ตัวอย่างซึ่งเก็บตัวอย่างในช่วงปี 2013-2017 จากพื้นที่ระบาดจำนวน 5 จังหวัด นำมาตรวจหาชนิดของอัลลีลของยีนเมอโรซอยต์เซอร์เฟซโปรตีน 1 บล็อก 2 ซึ่งประกอบด้วย K1 MAD20 และ RO33 ด้วยเทคนิค nested PCR ตัวอย่างดีเอ็นเอที่ใช้ในการศึกษานี้สกัดโดยใช้ Chelex-100 จากนั้นอัลลีลที่ได้จะตรวจสอบด้วยวิธีอิเล็กโทรโฟรีซิสแบบอะกาโรสเจลที่ความเข้มข้น 2.5% และทำการวิเคราะห์ขนาดของอัลลีลตัวอย่างเชื้อที่มีการติดเชื้อสายพันธุ์เดียวถูกนำไปหาลำดับนิวคลีโอไทด์ของยีนดังกล่าวด้วยวิธี direct sequencing จากนั้นนำลำดับนิวคลีโอไทด์ที่ได้มาทำการเปรียบเทียบและสร้างแผนภูมิต้นไม้

พบอัลลีลชนิด MAD20 RO33 และ K1 ของเชื้อมาลาเรียชนิดฟัลซิพารัมคิดเป็นร้อยละ 57.2 (135 จาก 236 ตัวอย่าง) 31.3 (74 จาก 236 ตัวอย่าง) และ 15.7 (37 จาก 236 ตัวอย่าง) ตามลำดับ จำนวนอัลลีลที่พบของ MAD20 RO33 และ K1 เท่ากับ 46 11 และ 16 แบบ ตามลำดับ อัลลีลของชนิด K1 ประกอบด้วยชุดของกรดอะมิโนซ้ำของ SGT จำนวนตั้งแต่ 2-7 ชุดและชุดของกรดอะมิโนซ้ำของ SGP จำนวนตั้งแต่ 2-3 ชุด นอกจากนั้นอัลลีลของชนิด MAD20 ประกอบด้วยชุดของกรดอะมิโนซ้ำของ SGG SVA และ SVT ในจำนวนที่ต่างกัน อย่างไรก็ตามอัลลีลของชนิด RO33 ที่พบทั้งหมดไม่พบชุดของกรดอะมิโนซ้ำ จากการศึกษานี้อัลลีล MAD20 เป็นชนิดที่พบมากที่สุด การติดเชื้อมาลาเรียมากกว่า 1 สายพันธุ์ในตัวอย่างเชื้อที่เก็บใน 5 จังหวัดมีค่าตั้งแต่ 1.0 ถึง 1.2 แผนภูมิต้นไม้แสดงให้เห็นถึงเชื้อมาลาเรียจาก 5 จังหวัดมีความสัมพันธ์กันทางพันธุกรรม พบความผันผวนของอัลลีลของยีนนี้ในตัวอย่างของประเทศไทยเมื่อเปรียบเทียบกับการศึกษาที่ผ่านมา ผลของความหลากหลายของยีนเมอโรซอยต์เซอร์เฟซโปรตีน 1 บล็อก 2 แสดงให้เห็นถึงการแพร่กระจายของเชื้อยังอยู่ในระดับสูง

สาขาวิชา	วิทยาศาสตร์สาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2563	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Merozoite surface protein 1, genetic diversity, Plasmodium falciparum, Thailand

May Myat Thu : Genetic diversity of Merozoite Surface Protein 1 Gene of *Plasmodium falciparum* in Thailand. Advisor: TEPANATA PUMPAIBOOL, Ph.D.

In 2030, World health organization's target is to eliminate malaria at least in 35 countries. At present, Thailand is low risk of malaria so that, it has the potential to eliminate. About 10 years ago, malaria prevalence was high along the country border areas. To prevent the recurrence in those areas, evaluation of drug susceptibility of parasites and vaccine are important. Therefore, basic knowledge on genetic diversity in malaria parasite is needed. Merozoite surface protein 1(*msp1*), one of the vaccine candidate genes, is useful for monitoring genetic diversity of the parasite and the potential gene of vaccine. However, high diversity of block 2 region in *msp1* is the barrier of vaccine design.

Therefore, in this study, genotyping of different *msp1* alleles from endemic areas of Thailand was crucial for control program. Two-hundred and thirty-six *P. falciparum*-infected blood samples collected during 2013-2017 from five endemic regions were amplified by nested PCR to detect the block 2 region of *msp1* gene; K1, MAD20 and RO33 allelic type. The DNA samples used in this study were extracted by using Chelex-100 method. These alleles were investigated with 2.5% agarose gel electrophoresis and fragment sizes were analyzed. The *msp1*, block 2 region of the isolates with mono-infection was determined by direct sequencing method. The sequences were aligned and constructed to get the phylogenetic tree.

The overall prevalence of MAD20, R033 and K1 allelic types in *P. falciparum* isolates were 57.2% (135/236) and 31.3% (74/236) and 15.7% (37/236) respectively. The number of alleles for MAD20, R033 and K1 were 46, 11 and 16 respectively. K1 type is comprised of 2-7 repetition of SGT and 2-3 repetition of SGP. Moreover, MAD20 is also comprised of different repetition of SGG, SVA and SVT. However, R033 is comprised of sequences without repetition. MAD 20 type was the most prevalent allele type found in this study. Multiplicity of infection of parasites in five provinces ranges from 1.0 to 1.2. The phylogenetic trees show that the isolates in five provinces are related each other. The fluctuation of allele is observed in Thailand by comparing with the previous studies. The high diversity of block 2 region indicates that high transmission intensity of parasites still present.

Field of Study:	Public Health Sciences	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

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List of Abbreviations

Abbreviation or Symbol	Term
A	alanine
A	adenine
bp	base pair
C-	carboxy
C	cytosine
ºC	degree Celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetraaceticacid
G	glycine
G	guanine
Hr	hour
IgG3	immunoglobulin3
K	lysine
kDa	kilo Dalton
mg	milligram
$MgCl_2$	magnesium chloride
Min	minute
mM	millimolar
N-	amino
nM	nanomolar
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Q	glutamate
S	serine
Sec	second
SNP	single nucleotide polymorphism
T	threonine
T	thymine
U	unit

List of Abbreviations

Abbreviation or Symbol	Term
UV	Ultraviolet
µl	microliter
V	valine
WHO	World Health Organization

CHAPTER I
INTRODUCTION

Backgrounds and rationales

Malaria is the parasitic disease found in many areas of the world and still remains threatening the life of people by snatches. There are many species of *Plasmodium* and their life cycle is very complex with both sexual and asexual in mosquitoes and human. The main type of clinically important cerebral malaria is caused by *P. falciparum*. Even though some areas such as Algeria in Africa, Maldives and Sri Lanka in South Asia have been recognized by World Health Organization (WHO) as malaria free, malaria mortality rate and cases are no drastically decreased in different areas around the world (1). WHO reported that there were approximate 214 million cases and 469,000 deaths of world's population in 2015, 217 million cases and 451,000 deaths in 2016 and then in 2017, 219 million cases and 435,000 deaths from this disease (2).

In Southeast Asian (SEA) region, Bangladesh, Bhutan, India, Indonesia, Myanmar, Nepal and Thailand are malaria endemic countries. There have the effective treatments and preventive measures by giving insecticide impregnated mosquito nets and repellents. In 2017, there was 0.8 malaria cases per 100,000 people in Thailand. To be free local malaria transmission by 2024, a National Malaria Elimination Strategy (NMES) was developed by Thai government. Peter Sands, executive director of the Global Fund, said that drug resistance in the Greater Mekong Sub Region becomes risks for malaria elimination efforts. Prevention is better than cure that point is also important to accommodate vaccines against malaria. The widespread antigenic polymorphism occurs in human malaria parasite, *P. falciparum* among other parasite strains /isolates. The genetic polymorphism has an effect on vaccine development against malaria and the population structure of the parasite (3).

The most prominent protein in erythrocyte invasion is merozoite surface protein-1 (MSP-1) of *P. falciparum*. The information of genetic diversity of *msp1* gene is

useful for host's immune response. The monoclonal antibodies of serum from malaria immune donors can prevent the merozoites invasion to new erythrocytes by agglutination (4). In a blood stage malaria vaccine, the merozoite surface antigens of *P. falciparum* also need to be considered. By reducing the rate of parasite growth and total parasite load, it can also reduce the severity of clinical disease and eliminate the parasite from the host (sterilizing immunity) (5). *Plasmodium falciparum* contains approximately 5,675 coding genes, among them, 352 proteins are hypothetical proteins that have not been functionally characterized. From these candidate genes, the antibody against block-2 region of PfMSP1 at the N terminal region are also associated with protection from clinical malaria (6).

The genetic diversities are different in various regions and also affect the transmission intensity and multiplicity of *P. falciparum* infections (7). The previous study in Thailand, 2014 exhibited the dominant polymorphic sizes of MSP1 and MSP2 (8). According to the target of Thai government to eliminate local malaria transmission, research on vaccine gene of *Plasmodium* species addition to the seeking of the effective treatment for malaria is needed. Sequence heterogeneity of *msp1* gene and different clonal type are needed to be considered for varied immune responses (7). The extensive genetic diversity in natural malaria parasite populations limits the effectiveness of immunity to malaria (9). Although extensive genetic polymorphisms of the *msp1* gene have been identified in *P. falciparum* isolates, MSP1 is also the potential vaccine candidate antigen among other proteins so that the genetic diversity of gene encoding protein is important to explore (10).

The diversity of *msp-1* gene from various regions are required to study for the further development of effective malaria prevention and control. The distinctive sequences in allele families of block 2 have protective immune responses (11). Both N- and C-terminal ends of the molecule are under consideration as possible vaccine candidates (12). MSP1 antibodies have been detected in people living in malaria-endemic areas. In the previous study in Ghanaian children showed that IgG3 which can pass the placenta and responses to block 2 are associated with protection from subsequent clinical malaria episodes by using panel of

immunologically well characterized recombinant antigens (6). By incorporation with the sequence repeats from major allelic types of *msp1* block 2 and potent T-cell epitopes, it elicits high-titer antibody responses and effective immunological memory (13). In this study, genetic polymorphisms for vaccine candidate gene, *msp1* of *P. falciparum* from malaria endemic areas in Thailand will be explored.

Research questions

What are the most common types of genetic variants of merozoite surface protein 1 gene (*msp1*), block 2 region of *P. falciparum* from malaria endemic areas in Thailand?

Objectives of the study

- To identity the frequencies of *msp1* gene, block 2 allelic types of *P. falciparum* collected from Tak, Kanchanaburi, Chumphon, Ranong and Yala in Thailand.
- To analyze the diversity of *msp1* gene, block 2 region of *P. falciparum* parasites and compare the data between the five endemic areas in Thailand.

Anticipated outcome

Information on the types of genetic polymorphisms of vaccine candidate gene will be useful for developing vaccination and subsequent prevention for elimination program of malaria.

Scope and Limitation Research

In this study, samples were used randomly and not specific for age, gender and ethnicity. All samples were from malaria infected patients and these samples were collected for 7 years ago so the data is not up to date since the dramatically decline in *P. falciparum* infected cases in these five endemic areas. However, this information was useful for local genetic structure of the parasites for vaccine design.

Conceptual Framework

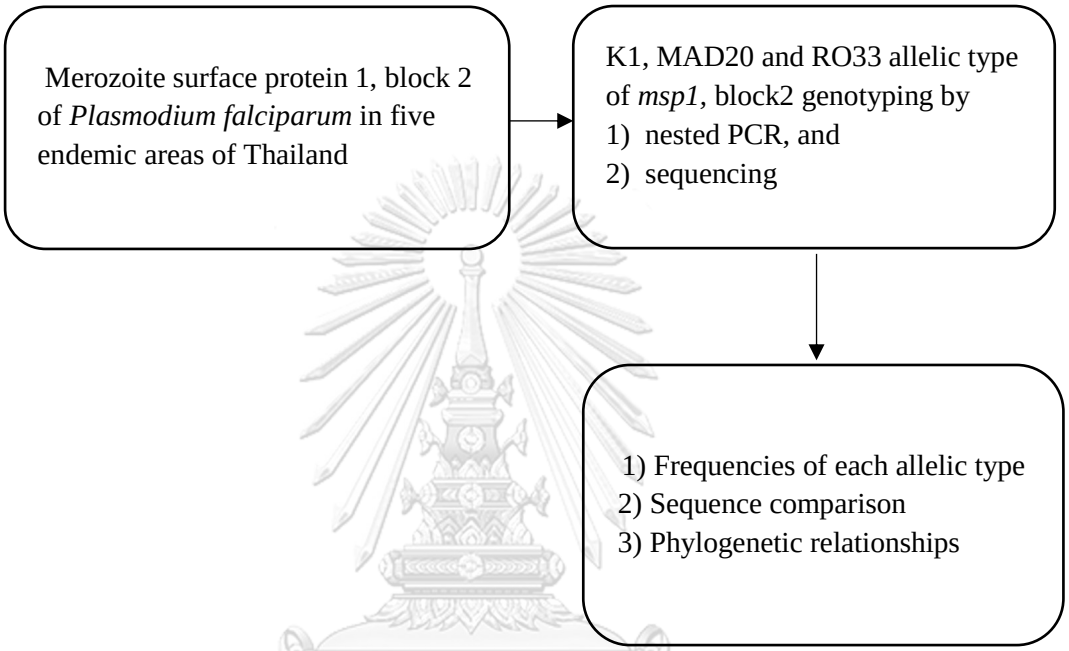


Figure 1.1 Conceptual framework

CHAPTER II

LITERATURE REVIEW

Malaria history

Over 4,000 years ago, malaria disease had been noted. Their consequence has an effect on human populations in the early period. Greece city had widely recognized by malaria in 4th century BCE (14). In November 1880, malaria parasites were discovered in the blood of patients by Alphonse Laveran, a French army surgeon stationed in Constantine, Algeria, and he was awarded the Nobel Prize for Medicine in 1907 (15). In 1897, transmission of human malaria by mosquitoes was discovered by Ronald Ross and he also received the Nobel Prize (16).

World malaria situation

In 1990, malaria deaths were around 670,000 in Sub-Saharan Africa, South-Asia, Southeast Asia, North Africa and Middle East, Oceania, Latin America and Caribbean, North America, Central Europe, Eastern Europe and Central Asia and Western Europe. The highest death rate happened in 2004 at around 930,000. These estimates from the Institute of Health Metrics and Evaluation (IHME) and are notably higher than those of the WHO. Both the IHME and WHO malaria death rate estimation 90 percent (9 in every 10 deaths) occurred in the African region (Figure 2.1). South Asia is the second one for high malaria mortality rate globally while Southeast Asia, North America and Western Europe has the few deaths by malaria.

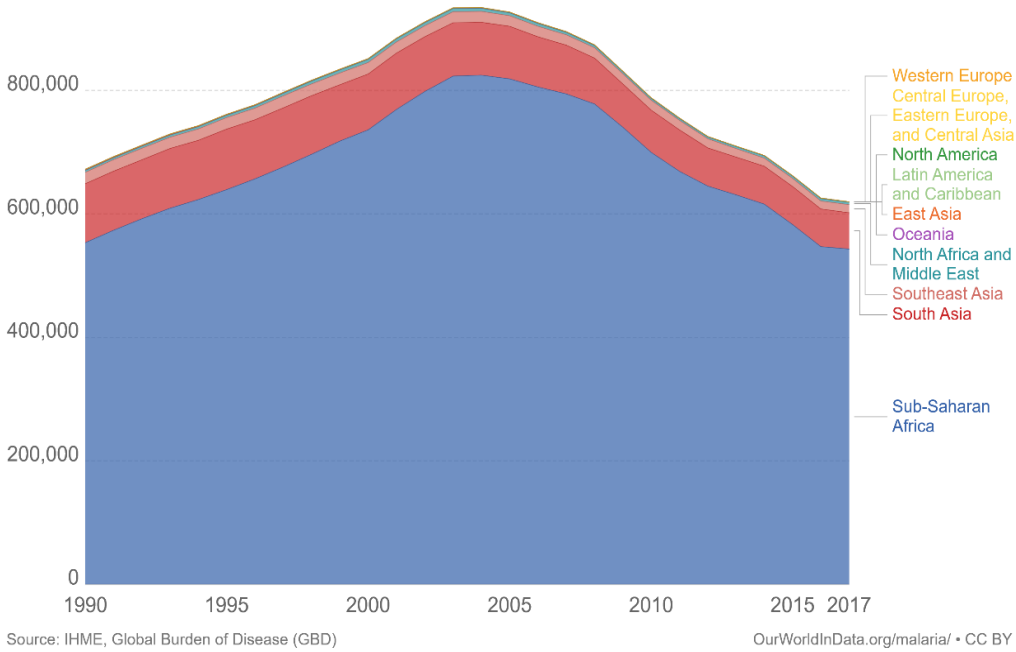
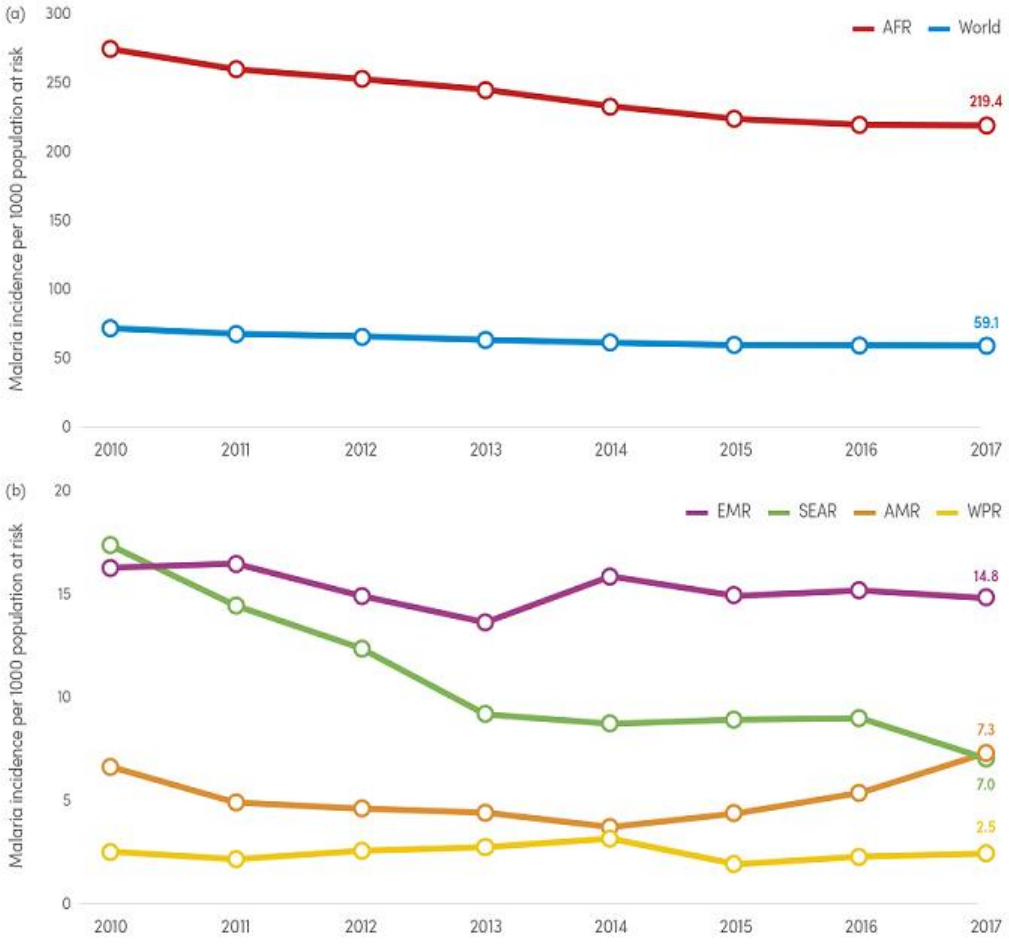


Figure 2.1 Global malaria mortality rate during 1990 – 2017 (17)

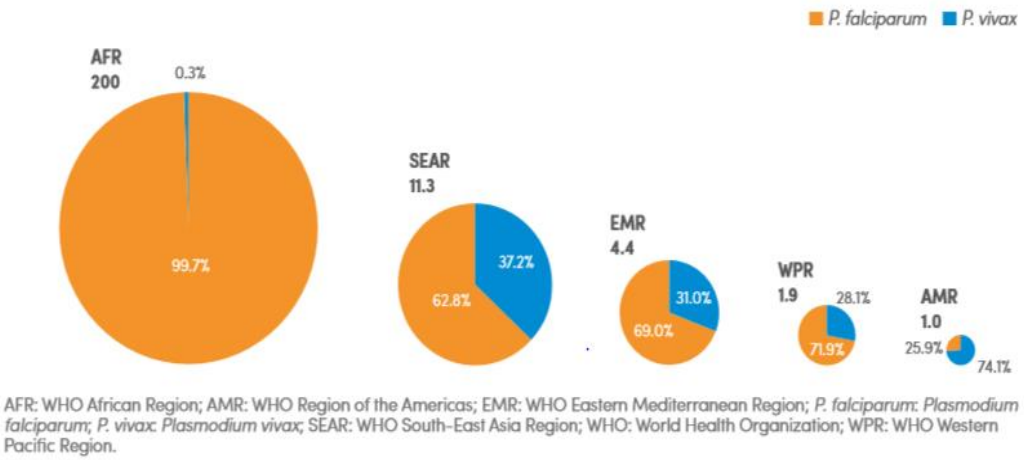
In 2017, most of the malaria cases (92%) occurred in African region, 5% in Southeast Asia region and 2% in the WHO Eastern Mediterranean region. Incidence case is fluctuated in most of the countries (Figure 2.2) (18). In African region, incident rate decreased from 278 per 1000 population in 2010 to 219 per 1,000 population in 2017. From 2010 to 2017 data, in Eastern Mediterranean region and Western Pacific region, malaria incidence per 1000 population was fluctuated but not drastically declined. In region of America, the incidence rate in 2010 was declined in 2014 however, in 2017, the incidence rate was increased again at 7 per 1000 population. It was a good situation in South East Asia region that the incidence rate is significantly decreased from approximately 18 per 1000 population in 2010 to 7.3 per 1000 population in 2017. According to 2018 WHO data, malaria is still threatening and challenging public health problem around the world (19).



AFR: WHO African Region; AMR: WHO Region of the Americas; EMR: WHO Eastern Mediterranean Region; SEAR: WHO South-East Asia Region; WHO: World Health Organization; WPR: WHO Western Pacific Region.

Figure 2.2 Global malaria incidence rate during 2010-2017 (18)

Among the human malaria parasites, *P. falciparum* and *P. vivax* are the common species in endemic regions. In 2017 estimated malaria cases in Africa region, 99.7% was caused by *P. falciparum*. In Southeast Asia, Mediterranean region and Pacific region, *P. falciparum* was the causal agent for two third of malaria cases. Except in America region, only one third was caused by *P. falciparum* (Figure 2.3) (19).



AFR: WHO African Region; AMR: WHO Region of the Americas; EMR: WHO Eastern Mediterranean Region; *P. falciparum*: *Plasmodium falciparum*; *P. vivax*: *Plasmodium vivax*; SEAR: WHO South-East Asia Region; WHO: World Health Organization; WPR: WHO Western Pacific Region.

Figure 2.3 A percentage of malaria cases caused by *P. falciparum* and *P. vivax* in each region

Global technical strategy for malaria

WHO sets up the goals 2016-2030 for malaria elimination program compared with achievements in 2015. The goal is to reduce the malaria mortality and morbidity rate by 40 percent in 2020, 75 percent in 2025 and at least 90 percent in 2030. Malaria elimination targets at least 10 countries in 2020, at least 20 countries in 2025 and at least 35 countries in 2030. And then prevent re-establishment of malaria from all countries (Table 2.1).

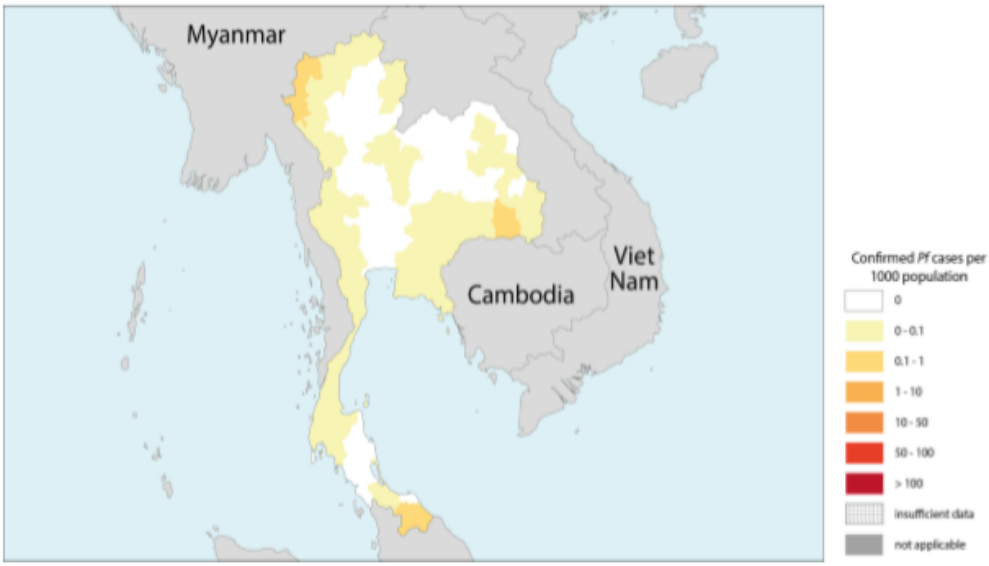
Table 2.1 Vision for malaria elimination globally **(2)**

Goals	Milestones		Targets
	2020	2025	2030
Reduce malaria mortality rates and case incidence globally compared with 2015	At least 40%	At least 75%	At least 90%
Eliminate malaria from countries in which malaria was transmitted in 2015	At least 10 countries	At least 20 countries	At least 35 countries
Prevent re-establishment of malaria in all countries that are malaria-free	Re-establishment prevented	Re-establishment prevented	Re-establishment prevented

Malaria prevalence in Thailand

Population of Thailand in 2017 was approximately 69.2 million (20). Population at risk for malaria was changed from 12.7 million, 32,480 cases and 80 deaths in 2010 to 13.1 million, 11,043 cases and 11 deaths in 2017 (2). At about 1970, the elimination program for malaria was started in Thailand. There had the effectiveness in central area. But there has still harbored for both species, *P. falciparum* and *P. vivax*, in the margin of the country. Thailand has the boundaries with Myanmar, Laos, Cambodia, Malaysia and South China Sea. These countries are also high risk for malaria and therefore the borderline region of Thailand has many ethnicity, migrant workers and geographically diverse with the favored place for different mosquito species in the mountainous area and many forests. These regions are biologically diverse region. Different types of human malaria species are also detected in that area. So these factors are challenging to control and eliminate the parasites (21).

Figure 2.4 The map of Malaria distribution in Thailand **(**22**)**



I. Epidemiological profile

Population (UN Population Division)	2017	%
High transmission (>1 case per 1000 population)	1.5M	2
Low transmission (0-1 case per 1000 population)	11.6M	17
Malaria free (0 cases)	55.9M	81
Total	69M	

Figure 2.5 Epidemiological profile for *P. falciparum* in Thailand (2)

Population with high transmission is more than one case per 1,000 population at 1.5 million and low transmission is 0-1 case per 1,000 population at 11.6 million and malaria free (zero case) is 55.9 million in Thailand. High transmission regions are located in contact with Myanmar, Cambodia and Malaysia (Figure 2.5) (2).

Biological characteristics of malaria parasite

Human malaria

Malaria is one of the ancient infectious disease and the vector is female *Anopheles* mosquitoes. It has many species causing malaria infection in human being and some other host animals: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* According to the characteristic feature of an acute fevered period, respectively noted for *P. vivax*, third time fever per day and *P. malariae* with fourth time per day. *P. ovale* and *P. vivax* cause a small chance of fatal outcome and only prevalent in northern part of Australia and the Philippines (23). *Plasmodium knowlesi* is zoonotic disease transmitted from monkeys to humans. It causes the daily fever and lethal outcome despite of the rare cases in Malaysia (24).

Among *Plasmodium* species causing human malaria, *P. falciparum* is the most fatal one (25). When a person who is infected with *P. falciparum* cannot get the right diagnosis and treatment within an unwell period, 5-50 sporozoites migrate to the liver where they multiply and are released into the blood circulation as merozoites, then invade into the red blood cells (RBSs) and develop to uninucleate ring form. After that, they mature and divide into multinucleated schizonts. When the schizont ruptures, it releases 4 – 6 merozoites to infect new red blood cells (RBCs). High parasite loads cause cytoadherence by parasitized erythrocytes and blockages of blood vessels in brain, blood-brain barrier. At that time, patient gets the cerebral malaria with seizure, unconsciousness and death. In pregnancy, sequestration also occurs in the placenta, this may cause miscarriage or low birth weight of the fetus (26).

Life cycle of malaria

The malaria parasite survives both in human, animal host and the female *Anopheles* mosquitoes with different life stages. In the human, sporozoites is the preliminary stage of infection from the salivary glands of infected mosquito bites. Twenty to two hundred sporozoites per bite are cleared by human immune system within half an hour. They also quickly enter into the hepatocytes (liver cells) and develop as trophozoites by releasing their apical complex and surface coat off. The parasite has parasitophorous vacuole that protects from phagolysosome. Nearly ten

thousand of daughter cells, merozoites are emerged from schizont and finally merozoites are released into the human blood circulation.

In this way, they replicate within erythrocytes by feeding hemoglobin and can alter RBC's shape and rigidity. By emerging from old RBCs and invading to new RBCs, the parasites propagate in that patient. Some merozoites develop sexual differentiation into male and female gametocytes within 7-15 days. This process is called gametocytogenesis. Female *Anopheles* mosquitoes engulf the gametocytes when taking the blood (27). Gametes are become with morphological changes and next stage, ookinete forms in midgut of mosquitoes. The ookinete enters the basal epithelium of the gut wall and transforms into immotile oocyst. After several days, 10 to 11 rounds of cell division occur to form sporoblasts. Then sporoblasts pass through the meiosis produced many sporozoites. Finally, they transmigrate to and perform further development in mosquito's salivary gland and become infective to humans (Figure 2.6) (28).

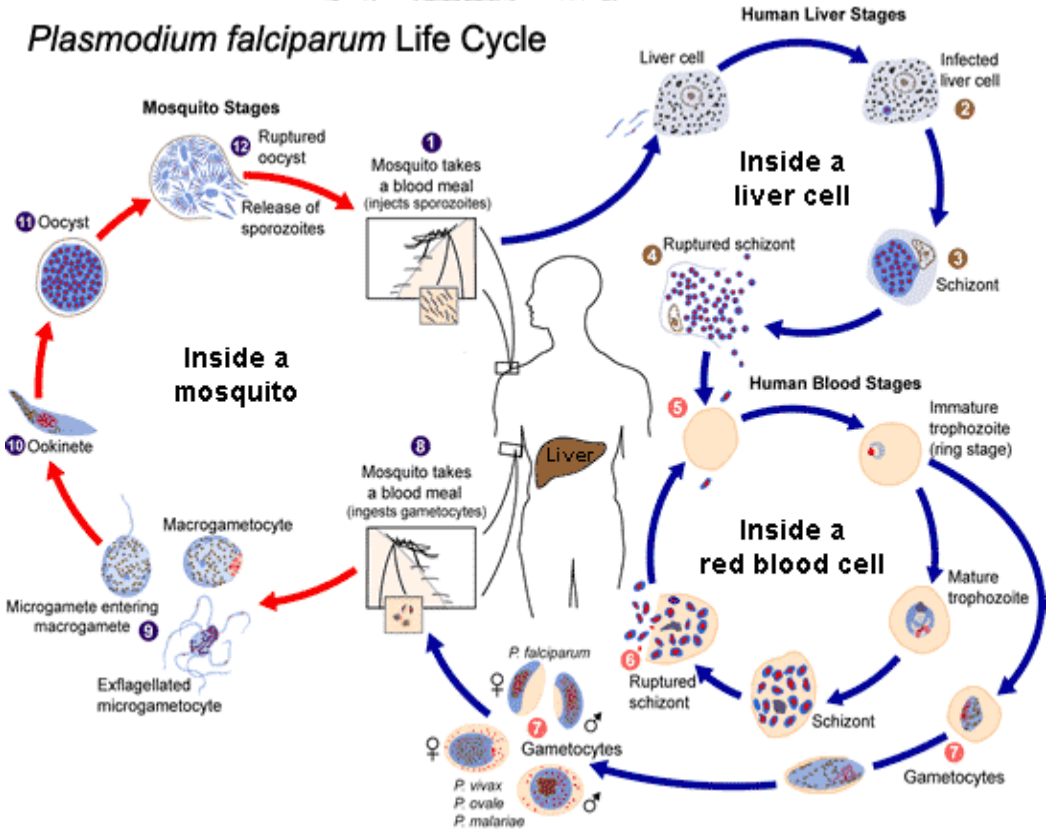


Figure 2.6 Life cycle of *Plasmodium falciparum* (28)

Morphology of *Plasmodium falciparum*

Plasmodium falciparum alters its structure with both sexual and asexual throughout the life cycle. A sporozoite is pivot-shaped and 10-15 μm long. In the liver, it becomes larger of diameter with 30-70 μm and transforms into an ovoid schizont. Each schizont produces 10,000 merozoites in the circulation. Merozoites are ovoid cells and their size is 1-1.5 μm. Only ring stage and gametocyte stage (banana shape or crescent shape) are found in peripheral blood. Period of asexual phase is 48 hours and when the red blood cell is burst and ruptured schizonts, pyrogen is released and patient feels the fever. Parasitemia in the blood may exceed 200,000 parasites per microliter of blood with maximum level (29).

Structure of Merozoite

The merozoite is like a pear-shaped and it has three organelles, rhoptries, dense granules and micronemes. Their function is binding and infiltration to new RBCs and parasitophorous vacuole formation. Erythrocyte is important to live extracellular merozoites, because they feed on haemozoin of the red blood cell (Figure 2.7).

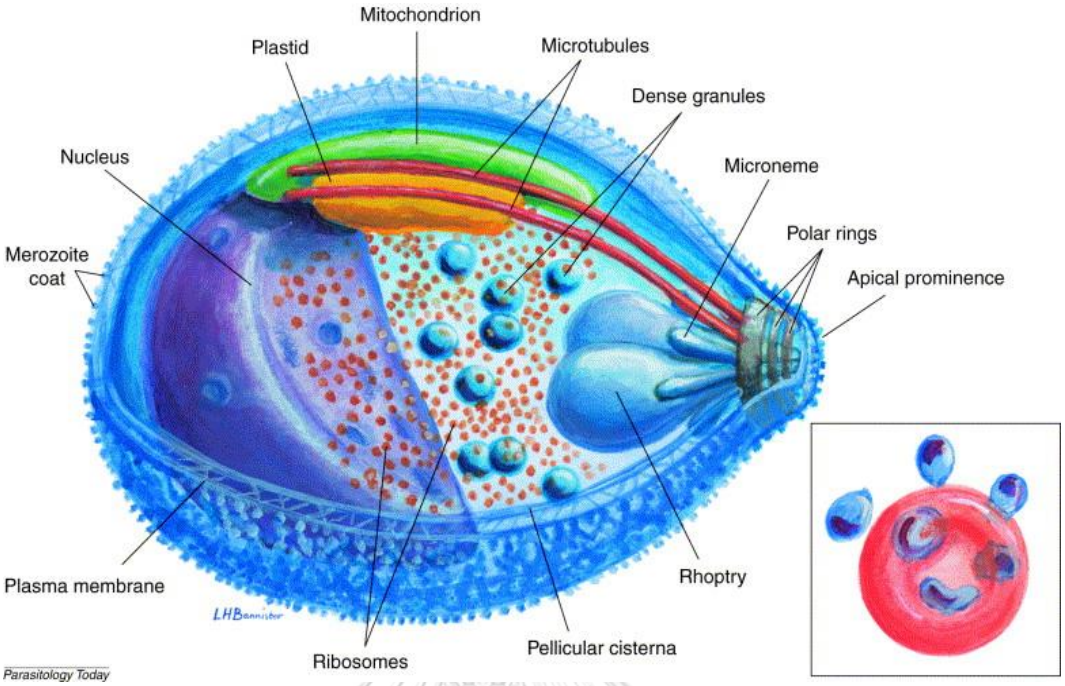


Figure 2.7 Merozoite structure of *Plasmodium falciparum* (30)

Transmission of Malaria by *Anopheles Mosquitoes*

Whenever the female *Anopheles* mosquitoes need to nurture their eggs, they take the human blood and if they are infected mosquitoes, they can transmit the infected parasites to human. *Plasmodium falciparum* are carried by most species of *Anopheles* mosquitoes, they are active only during the night. Among nearly 460 species, *Anopheles dirus* is the main vector for malaria transmission in South East Asia. There has many agricultural places and forests in rural area of Thailand. It is the place for breeding of mosquitoes (31). Among 79 species of *Anopheles* mosquitoes, generally 7 species; *Anopheles dirus*, *A. baimaii*, *A. minimus*, *A. aconitus*, *A.maculatus*, *A. sawadwongporni* and *A. pseudowillmori* are serious and common in Thailand (32).

Drug history and WHO guideline treatment for malaria

Two French chemists announced the active compound from cinchona bark as quinine in 1820. Chloroquine was formed by German chemists. Around 1970, resistance to chloroquine started throughout the world. In 1974, the new drug,

artemisinin was launched in China (33). Monotherapy with artemisinin drug had less clearance effect to parasites in Thailand-Cambodia in 2005 and at the present in the many endemic areas worldwide (34). Therefore, the last drug regimen recommended by WHO is artemisinin combination therapy (ACT).

There has the different treatment with ACT in different countries by means of WHO guideline. According to WHO 2018 report, artemether-lumefantrine (AL) is the most one in global regions. In Thailand, treatment policy was changed from artesunate and mefloquine (ASMQ) to dihydroartemisinin and piperaquine (DHA-PPQ) in 2015 (2). Now the new ACT combination, pyronaridine and artesunate has been approved by WHO in October, 2019 (35).

Treatment review for malaria in Thailand

At about 1980, in eastern part of Thailand, 'Fansidar' (pyrimethamine-sulfadoxine) treatment for *Plasmodium falciparum* was resistant and used the combination with quinine and tetracycline (36). In 1984, mefloquine had the high efficacy compared with chloroquine and Fansidar (37). From 1990-1994 study in refugee camp, mefloquine only efficacy was declined and mefloquine plus artesunate were introduced (38). In 2004, Pailin study indicated the artemisinin combination therapy drug resistance (artesunate plus mefloquine) (39). Three combinations of ACT such as artesunate-amodiaquine (AS-AQ), dihydro-artemisinin-piperaquine phosphate (DHP) and artemether-lumefantrine (AL) are still used (40).

Malaria elimination

It is the goal of malaria aspect for human being in the world. Extinction need to become in each localization and finally in the world. However, it is difficult process and depends on many factors: the weather, the environment and the ethnicity, lifestyle and the budget for control program and including the energetic movements from governments and non-governmental organizations (1).

Strategies and interventions for malaria elimination

Mosquitoes are also important in controlling malaria because they are key transporter from infected persons to another. WHO recommends the insecticide treated nets to use safely and widely to protect the mosquitoes. Indoor residual spraying is also effective to quell in combining with ITNs (41). Malaria is the longstanding disease because it has many points of view to eliminate. Right and early diagnosis and right treatment should be given not to revolve by the parasites. Some areas have the problems with ACT resistant parasites. It should be suppressed with the newest medication and the vaccine before suppressing again to malaria limited and free regions.

History about variolation and vaccination

At about 1720, variolation was used to prevent the disease in China and then England and North America. This method used the inoculation of the biomaterial from the patients to get the immunity. At approximately 1800, Edward Jenner, English physician, initiated smallpox vaccine in human that was made from cowpox disease and stopped the variolation method. Vaccine is a liquid used to energize the antibodies and improve the immunity to protect the disease and produced from the causative agent of a pathogen without pathogenicity or a synthetic structure as an epitope reacted with specific antibodies. And then, in 1891, Louis Pasteur, microbiologist, advanced the vaccination with immunity concept artificially for other infectious disease.

There are four types of vaccine –

- Live attenuated vaccine
- Inactivated vaccine
- Subunit, recombinant, polysaccharide and conjugate vaccine and
- Toxoid vaccine (42)

Malaria vaccine candidate antigens

Producing malaria vaccine is very complicated. There are many antigens concerning with different parasite stages: pre-erythrocytic and erythrocytic stage, liver stage in human and transmission blocking stage in

mosquitoes. However, they all have the limitation and different efficiency for vaccination (Figure 2.8) (43). To break the life cycle of malaria, there have many antigens tested for vaccine. To produce the high titer of antibodies, parts of the malaria parasite (antigens) has been used and tested (44). In the liver stage, circum-sporozoite protein (CSP), thrombospondin related adhesive protein (TRAP), sporozoite and liver stage antigens (SALSA), sporozoite threonine and asparagine rich protein (STARP) and liver stage antigen 1 and 3 are being tested. For blood stage antigens, there have many antigens such as merozoite surface protein 1, 2, 3, 4 and 5, erythrocyte binding antigen (EBA)-175, apical membrane antigen (AMA)-1, rhoptry associated protein (RAP)-1 and 2, acidic basic repeat antigen (ABRA), ring erythrocyte surface antigen (RESA), serine rich protein (SERP), erythrocyte membrane protein (EMP)-1, -2 and -3 and glutamate rich protein (GLURP). Pfs25, Pfs28, Pfs48/45 and Pfs230 antigens are also tested for transmission blocking vaccine (43).

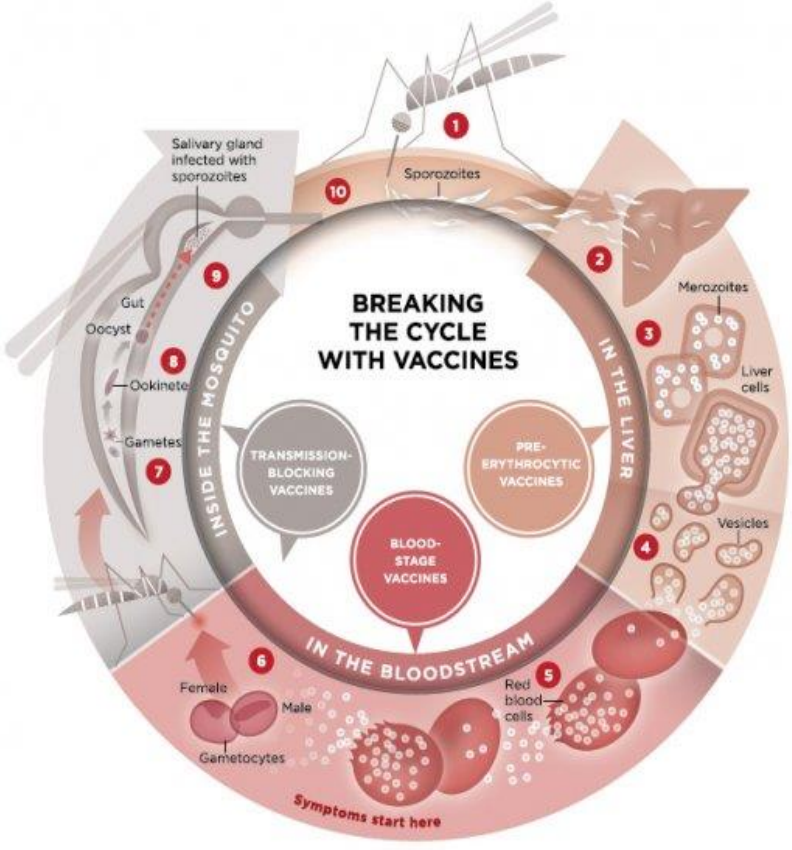


Figure 2.8 Breaking the cycle with vaccines (45)

To get the best one in the population, many studies have been done with different candidate antigens in different countries. WHO also oriented Immunization Program for malaria with roadmap for 2030 including the strategic goals;

- Developing to get license malaria vaccines and producing at least 75% efficacy for clinical malaria
- Reducing transmission and human malaria infection through mass vaccination campaigns (42). Africa's stakeholders have the positive impact on testing with malaria vaccine RTS, S/AS01 (recombinant protein fused with HBV surface antigen) in children. WHO and Ministries of Health in Africa are trying and implementing to cover long term and will be advanced and arrived in the worldwide (46).

Merozoite surface protein 2

The merozoite surface protein 2 (*msp2*) gene is the potential vaccine candidate antigen of asexual blood stage of *P. falciparum*. It is located on chromosome 2 of *P. falciparum* and is the protein coding gene having 1065 nucleotides. N terminal and C terminal end are conserved regions in spite of the central regions are high polymorphism with tandem repeat sequences. It's function is unable to express accurately. However, it connects with merozoite surface membrane by glycosylphosphatidylinositol anchors (GPI-anchors). The protein size is about 30 kDa and comprised of two families (3D7 and FC27 alleles) (47). MSP2 is more polymorphic than MSP1 and Glurp of *P. falciparum.* 3D7 type is more dominant than FC27 in MSP2 gene of *P. falciparum* (48). Antibodies response to each allelic types has lower than the antibodies of recombinant protein.

Glutamate-rich protein

Glutamate-rich protein of *P. falciparum* is also the surface antigen. This gene is present on chromosome 10 including 4,812 nucleotides. It has R0 (N-terminal non-repeated region with amino acid 27-500), R1 (Central region with amino acid 500-705) and R2 (C-terminal repeated region with 705-1,178). It is related with merozoite release from schizont and invaded to red blood cells in human circulation. Therefore, the antibodies to Glurp antigen can interfere the merozoite invasion to

human's erythrocytes. The entire sequence of this protein has many glutamate amino acid so that it is called glutamate rich protein. R2 portion has the immune-dominant T and B cell recognition site (49). The immunogenicity from antibody dependent cellular inhibition (ADCI) to R2 is higher than R0 (50).

Circumsporozoite (CS) protein

This circumsporozoite protein (CPS) is located on the surface of sporozoite that is found in mosquitoes as well as the first stage of infection in human. It is located on chromosome 3 of *P. falciparum* including 1,552 nucleotides. It has three segments; amino terminal region, central repeat region and carboxyl region. Region I between amino and central segments is viable to enter in salivary glands of mosquito and region II between central segment and carboxy end is to bind with liver cells of human (51). It is the highly conserved structure with cysteine rich C-terminal end. RTS/S recombinant CSP antibody by T-cell epitopes has the high protection to the pre-erythrocytic parasites (51).

Merozoite Surface Protein 1

Merozoite surface protein 1 (*msp1*) gene of *P. falciparum* has 6,711 nucleotides encoded for 2237 amino acids and total 17 blocks and is located on the chromosome number 9. They are polymorphic with single nucleotide polymorphisms (SNPs) and repeated nucleotides. Among 17 blocks, block 1, 3, 5, 12 and 17, are conserved while block 7, 9, 11, 13 and 15 are semi-conserved and block 2, 4, 6, 8, 10, 14 and 16 are variable block.

Block 2 is the only atypical portion including 3 major types of alleles i.e., K1, MAD20 and R033 and all the left blocks have only two alleles like K1 and MAD20 (Figure 2.9). Therefore, Block 2 antigen has the significant immunity response and protection from clinical malaria (6). That is the potential for the development of efficient vaccine (52). Repeat motif sequences of MAD20 and K1 alleles can produce the specific antibodies incorporating with T-cell epitopes for long lasting responses (11).

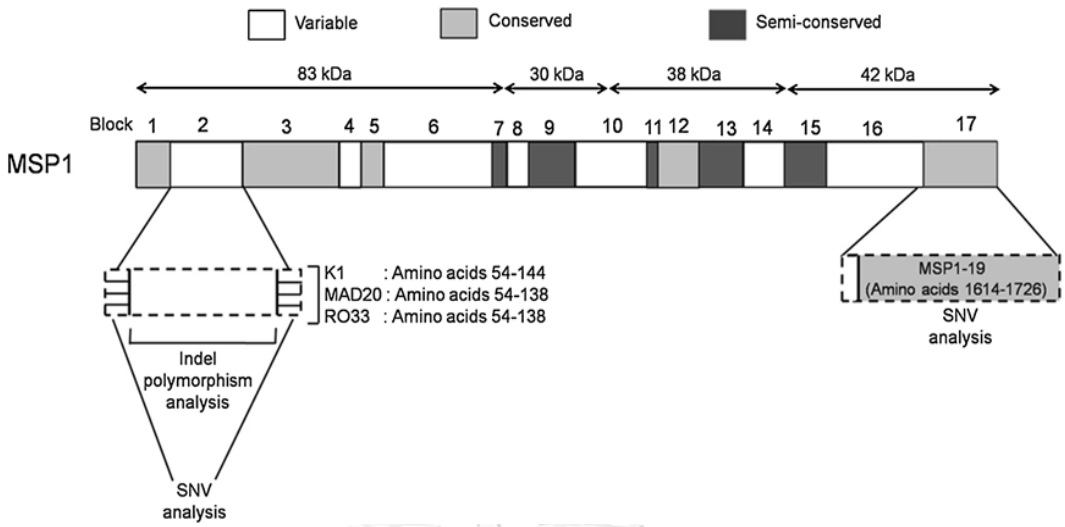


Figure 2.9 Schematic representation of *P. falciparum* merozoite surface protein 1(53)

Diversity of merozoite surface protein 1, N- terminal Block 2 region of *P. falciparum*

The parasite genetic structures are diverse to resist the different drugs for their survival. That is one of the reasons for difficulties of parasite control and development of vaccines (54). In 2012, Akter and collegues showed the result for diversity of *msp1*, block 2 antigen which implies the multiplicity of infection (MOI) and also the treatment outcome of chloroquine. In 2004, PCR adjustment (PCR correction to differentiate recrudescence and reinfection) calculated by Kaplan-Meier analysis for sulphadoxine-pyrimethamine is 11.7%, in 2005, PCR adjustment for ACT is 5.7% and in 2008, for azithromycin plus artesunate is 5.4%. Before treatment, MOI for K1 and MAD20 were 1.5 and MOI for R033 was 1.6 but after treatment, MOI for K1 and MAD20 were 1.6 and MOI for R033 was 1.5. Chloroquine resistant allele frequencies were decreased and found that reinfection rather than recrudescence infection. (Table 2.2) (55). MOI is the total fragments divided by the positive PCR samples. If the higher MOI, that means more diversity of the genotype. High diversity occurs in high transmission areas due to sexual intragenic recombination. High heterogenicity causes the more fitness and fitness has also influenced by allele frequencies.

Table 2.2 The multiplicity of infection and different alleles of *msp1* block 2 of *Plasmodium falciparum* detected before and after treatment (55)

MSP1 Block 2 alleles	Number of alleles	Pre -treatment MOI	Post-treatment MOI
K1	6	1.5	1.6
MAD20	8	1.5	1.6
R033	4	1.6	1.5

It has the main role for immunogenicity by analyzing their different types of alleles and their frequencies. K1 and MAD20 are more diverse allele families than R033 in many regions. And then R033 has no significant association for antibodies (6, 56). The sequence of block 2 has unusual amino acid compositions due to intrinsically unstructured protein domains (57). And then balancing selection determines the level of dynamics for immunity to MSP1, block 2 antigens. From one of the previous studies from 2007 to 2010, China-Myanmar border area, K1 and MAD20 allele frequencies were high in 2007 and 2008 but that frequencies were declined in 2009. For R033 allele frequency, it was significantly increased in 2009. Moreover, MOI was the highest in 2009 and the greatest level of multiclonal infection was found in 2009 (Figure 2.10) (58).

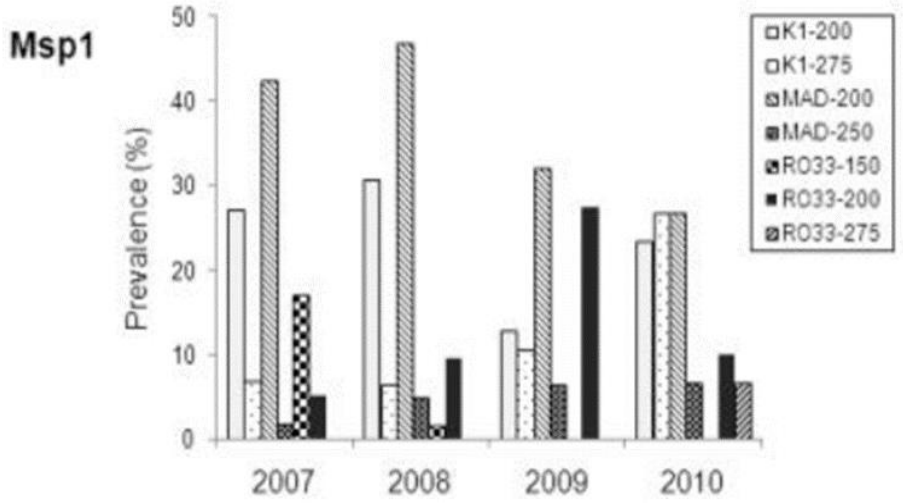


Figure 2.10 Prevalence of *P. falciparum msp1* alleles from northeastern Myanmar (58)

If frequencies of allelic diversities can be monitored regularly, it has the high efficiency for modern changes of malaria epidemiology and vaccine development for control program. In Myanmar, 99 samples from 115 blood samples from *P. falciparum* infected patients had successfully genotyped and sequenced for block 2 region of *msp1* gene between 2013-2015. Twenty-eight different allele types of K1, MAD20 and R033 had been found. K1 and MAD20 were highly diverse with different compositions of tripeptide repeat motifs (SAQ, SGT, SGA, and SGP for K1 type, and SVA, SGG, SKG, and SVT for MAD20 type) and R033 allele type was less polymorphic (Figure 2.11) (59).

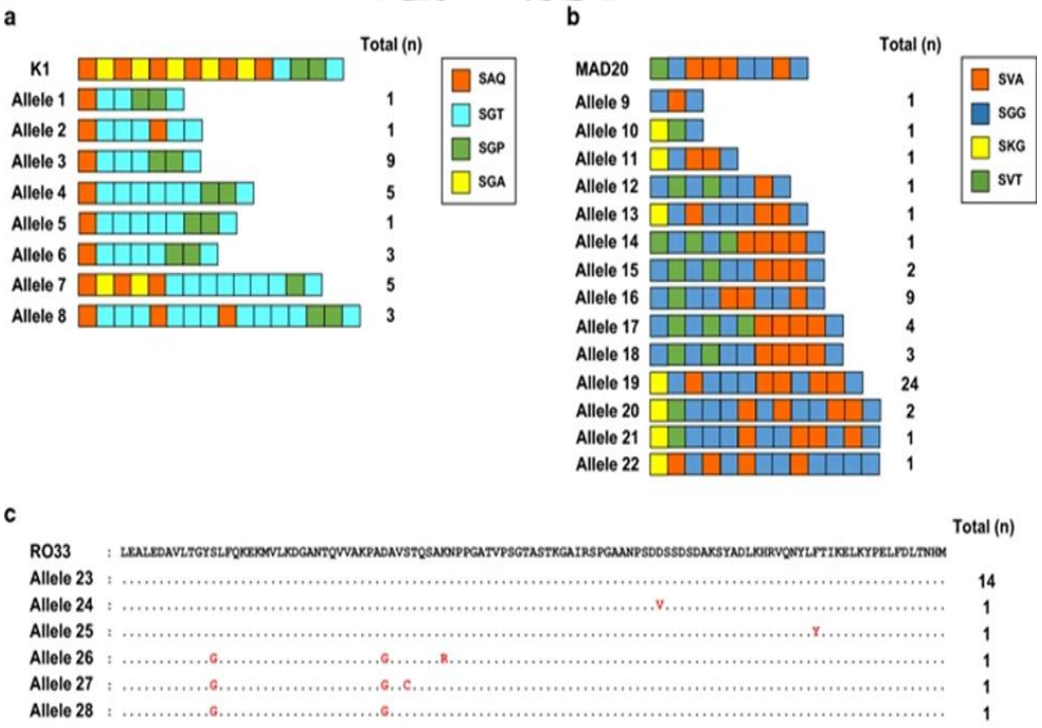


Figure 2.11 Schematic structures of the polymorphic patterns in Myanmar *Pf*MSP1block2 (59)

For local elimination, many studies also did the genetic profiles for many antigens of *P. falciparum* including *msp1* gene (60). In 1997 northwest Colombia, simple infection with only MAD20 allele type was presented and mixed infection with MAD20-1 + MAD20-2, K1 + MAD20-1, RO33 + MAD20-1,

MAD20-1 + MAD20-2 + K1, MAD20-1 + MAD20-2+RO33, MAD20-1+K1+RO33 and MAD20-1 + MAD20-2 + K1 + RO33. No infection occurred in only MAD20 allele type but mixed infection with high allelic diversity causes the possibility of cross-fertilization and meiotic recombination in the mosquito vector increases (61). For *msp1* block 2 study in Gabon, their results showed that three types of alleles frequencies in different percentage, K1- 52.5%, MAD20- 32.5% and RO33- 15%. Appearance and disappearance of alleles imply the drug efficacy and recrudescence by drug resistance or reinfection (62).

In Zambia, K1 like repeat sequence (SAQSGA) of *msp1* block 2 antigen had tested with monoclonal antibodies. The immunogenicity of human sera from endemic area. was high. To design the specific epitopes as a vaccine, sequence diversity is useful to construct the polyvalent hybrid protein together with MAD20 and R033 sequence and potent T-cell epitope for high immunity (13). In India, there was the dynamic of allele frequencies in 2005 and 2009. Prevalence of K1 type was higher than other two alleles in 2005 but in the latter, R033 was the highest allele. To induce the high titer immunity, high polymorphic surface proteins are potential for vaccine candidate antigens. There has the relationship between the natural evolution of the ancestral sequences and vaccine development. Moreover, these result indicated that high transmission areas have more diversity of the sequences due to the fact that there has the high recombination rate in the vector (63).

Allele frequencies of K1, MAD20 and R033 in *msp1*, block 2 region of *P. falciparum* in Thailand are dynamic. In 1993, *msp1* block 2 was found that variant with 4 Kl-type, 5 MAD20-type and only 1 R033 type in malaria clinic at the village of Borai, Trat Province in Thailand (64). In 1998, block 2 of MSP1 had total 9 alleles for K1, MAD20 and R033 in Thailand – Myanmar border, Karen refugee camp (65) and then from 2008 and 2014, 6 types of K1, 13 types of MAD20 and 2 types of R033 were found in those endemic areas (i.e., Ranong, Chumphon, Surat Thani and Yala) in Thailand - Malaysia (66-68). At Thai Myanmar border, allele distribution are also different in Ranong, Kanchanaburi and Tak. Allele frequencies of parasite isolates depends on temporal, geographical, vector population, human

immunity and drug treatment (69). The isolates with same allelic pattern is related with good treatment outcome. K1 and MAD20 alleles have different repeated sequences and has the potential to form the new malaria antigen. Failure in treatment outcome is associated with high frequencies of R033. But K1 and MAD20 are not neutral and associated with human immune response.

Detection method for malaria parasite

Laboratory diagnosis approach to detect and identify *Plasmodium* species can be considered into primary screening, secondary screening and definitive DNA analysis by using molecular techniques.

Screening test

Microscopic test

This is one technique for screening test using micorscope. *Plasmodium* infections can be screened from peripheral blood samples with Giemsa stained slides under the microscope. It involves thin and thick blood films well prepared and well staining blood film and skillful direct visualization of the parasite under the high objective lens of the ordinary microscope. Ring form of trophozoites (2.5-2.9 μm) and crescent shape or banana shape of gametocytes (two third of red blood cell) are seen in thin and thick blood film for *P. falciparum* infection (70). For species identification, thin blood film has the good quality than thick blood film. But it has the limitation if the parasite density is lower than 75 per microliter of the blood. Thick blood film is used for parasite density quantitatively (71).

Rapid diagnostic tests (RDTs)

Immunochromatography method is useful for screening in field without any special apparatus. Small amount of blood needs to be used and the result can be read within short period. The principle of test is to detect parasites' protein i.e. histidine rich protein-2 (HRPII) and parasite-specific lactate dehydrogenase (pLDH). In the device, there has nitrocellulose surface (immobile phase) coated with labelled specific antibodies (monoclonal antibody) for control and test for antigen detection of the sample. HRPII antigen is found in asexual and

early gametocyte stage of *P. falciparum.* pLDH enzyme is essential for glycolytic pathway and pan-specific for *Plasmodium* species. The weakness is false negative for *P.vivax* and other species when using the test kit with HRPII protein (72, 73).

Definitive diagnosis

Being compared with microscopy and RDT, definitive diagnosis methods are sensitive and high throughput methods to detect malaria parasites in low-transmission settings and low parasite density are needed. Although microscopy is gold standard method, it is labor-intensive and inaccurate. Moreover, RDTs is convenience and easy to use but there has limitations in specificity, sensitivity and species identification. To overcome, PCR-based methods are better for improved sensitivity and specificity especially in low parasite count and mixed infected samples. In addition, different samples including dried blood spots can be used in PCR methods. Dried blood spot sample collection is suitable and easy for malaria screening and long- term storage (74).

Loop-Mediated Isothermal Amplification (LAMP)

It is another PCR based test to detect the parasite with high sensitivity and specificity using specific primers. This method has no denaturation step, specific primer can anneal the complementary sequence of double strand and is able to amplify few to 10 copies of target DNA within 30 min. The results can be easily determined by colour changes. This method is feasible to use in field because of the usage of simple apparatus. LAMP, RDT and thin blood film can detect the parasite with 100 % sensitivity. But LAMP method has the limitation because of the usage of two to three specific primers (75).

Nested Polymerase Chain Reaction (PCR)

It is one of the PCR techniques performed the amplification with high sensitivity and specificity. In traditional PCR, single pair of primer is needed to amplify the product. However, in nested PCR, two pairs of primers (inner and outer primers) have to use to do the amplification. The product size produced by outer primers is longer than that produced by inner primers. The outer product has the

high sensitivity and inner product has the high specificity. Therefore, this method is very useful to differentiate the specific allelic types of the gene, the gene changes before and after treatment and to get the differential diagnosis of reinfection and relapse of drug resistant species. Moreover, the samples used for this technique are DNA isolates extracted from dried blood spots and fresh blood and are feasible to detect the small amounts of parasites. Therefore, this method can overcome the limitations of other detection methods. The disadvantage is time consuming and protracted to avoid the contamination (76).

Genotyping by sequencing

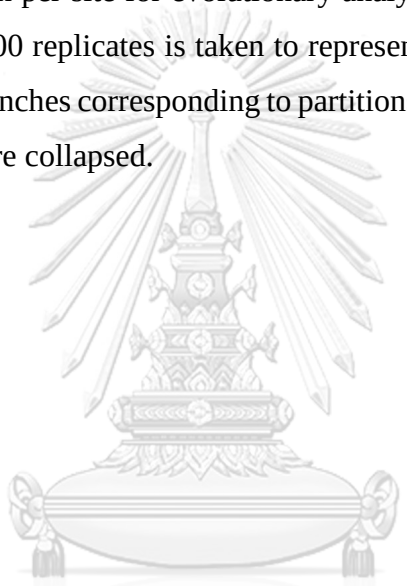
Genotyping is the process of determining the variants of the gene and differences in the genetic make-up (genotype) of an individual by examining the individual's DNA sequences with biological assays and comparing it to reference sequences. Sequencing is the technique to determine the order of the nucleotide bases along a DNA strand compared with reference genome.

In 1977, Sanger invented the chain termination method for sequencing DNA. In this method, the polymerization is stopped at the dideoxy nucleotides (ddNTPs) without 3' hydroxyl group which is needed for phosphodiester bond. These nucleotides (ddATP, ddGTP, ddCTP and ddTTP) are labelled with radio-active ^{32}P or fluorescent dyes. The mixture is fragmented in polyacrylamide gel. The emitted light is detected at different wavelengths. In the reagent, there has additional one, dideoxy nucleotide (unlike in PCR), for chain termination at the hydroxy end (77).

Phylogenetic analysis

Maximum likelihood phylogenetic tree is used for evolutionary analysis of different strains and their distant with multiple alignments (78). It can give the knowledge for sequencing information of vaccine design (79) and imply for hypothetical sequence of ancestors. By constructing the genetic tree, all people can know and monitor easily the relationship of different strains and recombination rate of the parasites and can know the information for diagnosis and treatment up to the vaccine by viewing the tree (80). Multiple alignments were performed with the

Muscle algorithm (version 1.83) (81) to construct Phylogenetic tree with Tamura and Nei method for nucleotide substitution method. It is easy to use with version 1 to 10. The latest version is MEGAX and was invented by Prof. Dr Masatoshi Nei and his student Sudhir Kumar and Koichiro Tamura. Evolutionary distances were calculated using the Kimura two-parameter model. Bootstrap values were calculated on the basis of 1,000 replications. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model for nucleotide substitution by transition and transversion. It calculates the frequencies of nucleotide substitution per site for evolutionary analysis. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.



CHAPTER III
RESEARCH METHODOLOGY

Sampling

Study population and sample size calculation

The parasitized blood samples were collected during 2013-2017 from the malaria patients who sought for malaria diagnosis at malaria clinics in Tha Song Yang district, Tak province, Sai Yok district, Kanchanaburi province, Kra Buri district, Tha Sae district, Chumphon Ranong province, where bodering with Myanmar, and Mueang district, Yala province bodering with Malaysia (Figure 3.1). These provinces had high prevalence of malaria cases.

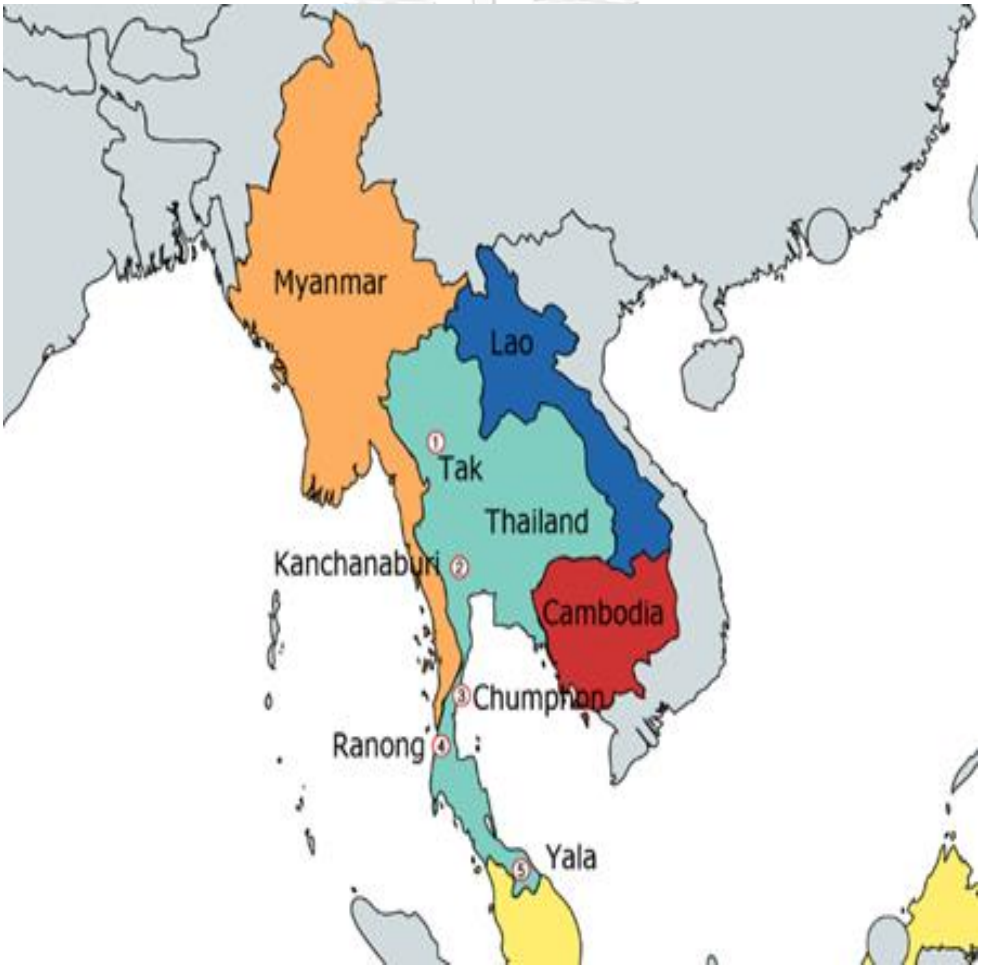


Figure 3.1 Study sites in Thailand. This map shows the area of sample collection in Tak, Kanchanaburi, Chumphon, Ranong and Yala province. The map was modified from website https://mapchart.net/asia.html

The sample size calculation used the formula for estimating the population proportion as following

$$n = \frac{Z^2 P (1-P)}{e^2}$$

$$n = \frac{(1.96)^2(0.6)(0.4)}{(0.15)^2}$$

$n = 40.9$

Where, Z = confidence level = 1.96 (95%CI)

P = Genetic diversity of *P. falciparum* in Thailand = 0.6 (82)

e = margin of error = 0.15

The sample size was 41 samples per province. An additional 20% samples were added thus samples size was 50 samples per province.

Study design

This study was the cross-sectional study design aimed to describe genetic diversity of *msp- 1* gene of *P. falciparum* in malaria endemic areas of Thailand.

Malaria samples used in this study

The dry blood samples of patients who diagnosed as *P. falciparum* infection by malaria clinic staff using microscopic test and parasite species also confirmed by nested PCR technique (83) were used in this study. These dried blood samples were collected during 2013-2017 from the patients who aged 18 and above, had no experience of malaria infection within 28 days, no severe symptoms observed, and signed consent to the study before drug administration. The parasitized blood samples from fingertip were absorbed onto Whatman filter paper and dried at the room temperature then kept in the plastic bag. Two hundred and thirty-six *Plasmodium falciparum* blood samples without patients' information were used to analyzed. Twenty-eight samples were from Tha Song Yang district of Tak province, 45 samples from Sai Yok district of Kanchanaburi province, 21 samples from Tha Sae distict of Chumphon province, 44 samples from Kra Buri district of Ranong province and 98 samples from Mueang Yala district of Yala province respectively (Table 3.1)

Table 3.1 areas, codes and number of *P. falciparum* samples

Studyareas (provinces)	**Code of materials**	**Number of samples**
Tak (T)	T 227, 228, 229, 230, 231,232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254	28 samples
Kanchanaburi (K)	K 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471,472, 473, 474, 475, 476	45 samples
Chumphon (CHU)	CHU 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21	21 samples
Ranong (RN)	RN 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194	44 samples

Table 3.1 Sutdy areas, codes and number of *P. falciparum* samples (continued)

Study areas (provinces)	**Code of materials**	**Number of samples**
Yala (YA) 2013	YA 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117	53 samples
Yala (YA) 2014	YA 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162	45 samples

DNA extraction

Dried blood spots were cut in a small piece and incubated with 0.5% saponin in phosphate-buffered saline (PBS) at 4°C for overnight. Fifty microliters of Chelex-100 resin and 150 µ1 water were mixed together in microcentrifuge tube and heated to 95°C in a heat block. After treated blood spots with 0.5% saponin solution, the blood spots were washed with PBS for twice. Then, the spots were placed in the preheated Chelex solution, vortex at high speed for 30 seconds and placed it in the heat block at 95°C for

10 minutes with a brief vortex in between and after the incubation. After that Chelex resin were separated by centrifugation at 10,000 rpm for 5 minutes, the supernatant was recovered, centrifuged again under the same conditions, and the supernatant was pipetted and collected into a new tube. Supernatants were used for the further experiment immediately or stored at -20°C (84).

Molecular Techniques for detection of MSP1 gene diversity

***msp1* block 2 allelic type detection**

The three allelic types of *msp1* block 2, K1, MAD20 and RO33 were determined by the nested PCR technique using published primers as shown in following Table 3.2 (54)

Table 3.2 Primers for amplication of *P. falciparum* block 2, *msp*1 gene in nested PCR

	Primer	Sequence (5' – 3')	Tm (°C)	Amplicon sizes (bp)
Outer Primer	Forward	AAGCTTTAGAAGATGCAGTATTGAC	62.5	400-450
	Reverse	ATTCATTAATTTCTTCATATCCATC	56.9	
K1	Forward	GAAATTACTACAAAAGGTGCAAGTG	62.0	100-225
	Reverse	AGATGAAGTATTTGAACGAGGTAAAGTG	64.4	
MAD20	Forward	AAATGAAGGAACAAGTGGAACAGCTGTTAC	68.6	100-250
	Reverse	ATCTGAAGGATTTGTACGTCTTGAATTACC	66.2	
R033	Forward	TAAAGGATGGAGCAAATACTCAAGTTGTTG	66.7	100-230
	Reverse	CATCTGAAGGATTTGCAGCACCTGGAGATC	71.4	

The first PCR was performed with 0.8 Unit *Taq* DNA polymerase (GoTaq®, Promega, USA), 200 µM each deoxyribose nucleotide triphosphate (dNTP), 75 nM of each conserved primer, 2.0 mM $MgCl_2$, 1 µl DNA and nuclease free water upto total 25 µ1 using an Applied Biosystems Veriti™ 96-Well Thermal Cycler. After pre-denaturation at 94 °C for 5 minutes, for primary denaturation, 35 cycles at 94°C for 30 sec, annealing at 58 °C for 1 minute, and extension at 72°C for 1 minute was performed and followed by the final extension at 72°C for 10 minutes. In the second PCR, the same condition and program for PCR amplification were used. The products were determined on a 2.5% agarose gel electrophoresis (85). Two and a half grams of agarose powder was added into 100 ml of Tris-borate EDTA (TBE) buffer and boiled it until melt. Agarose solution was poured into the mold and be cool until solidify. An agarose gel was placed in the electrophoresis chamber filled with TBE buffer. The PCR samples, negative control, positive control with each specific allelic types and DNA ladder (BBI life sciences) was loaded in the wells and the electrophoresis was started run with 80-120 voltage about 1 hour. After electrophoresis, the gel was stained with ethidium bromide and the band was observed with UV transilluminator and photographed with gel documentation. The band size was estimated using Syngene software.

DNA Sequencing

One hundred and twenty- five field isolates, which successfully amplified for three allelic types, were selected for *msp1* gene sequencing. The block 2 of MSP1 gene was amplified using primers shown in Table 3.3.

Table 3.3 The primers for sequencing of K1, MAD20 and R033 type of block 2, *msp1* gene

	Primer	Sequence (5’ – 3’)	Tm (°C)
K1	Forward	5’-GAAATTACTACAAAAGGTGCAAGTG -3’	62.0
	Reverse	5’-AGATGAAGTATTTGAACGAGGTAAAGTG-3’	64.4
MAD20	Forward	5’-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3’	68.6
	Reverse	5’-ATCTGAAGGATTTGTACGTCTTGAATTACC-3’	66.2
R033	Forward	5’-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3’	66.7
	Reverse	5’-CATCTGAAGGATTTGCAGCACCTGGAGATC-3’	71.4

After performing the allelic type detection, the samples with only one allelic type were performed for PCR again with the high-fidelity DNA polymerase (Pfu DNA polymerase, Promega, USA) with the same primer pair for each allelic type. After that 2 µl of PCR product was mixed with 5 µl of Exo SAP-IT^{TM} PCR product cleanup (Thermo Fisher Scientific, USA) to remove the primers and other dNTPs. The sequencing reaction was done separately for forward and reverse strand with BigDye Terminator v1.1/v3.1 Cycle Sequencing Kits (Thermo Fisher Scientific, USA) together with each primer, sequencing buffer and deionized water up to 10 µl in appendix 2. The thermal cycler program is different for forward and reverse reaction. The program was 96°C for 1min, 25 cycles of 96°C for 10 sec, 50 °C for 5 sec, 60 °C for 4 min for forward strand. For reverse strand, the same program except annealing temperature at 45 °C 5 sec was performed. After amplification,

the product was cleaned up by using ethanol and EDTA (appendix 3). For capillary electrophoresis, 10 µl of Hi-Di formamide was added to each sample pellet and then heated at 95°C for 1 minute before loading in a machine. The products were continued for electrophoresis in 3700 ABI Genetic analyzer to determine the precise order of nucleotides.

Sequence alignment

Raw DNA sequence data files from field isolates were manually edited to exclude signal noises. Sequences were assembled and aligned using the Bio edit software. Nucleotide (nt) sequence alignments for phylogenetic analyses were generated using Muscle alignment (86). To compare the sequence identity, NCBI BLAST analysis was performed with all test sequences. MEGAX tool was used to perform multiple sequence alignment and to translate DNA sequences into amino acid codes. Allele specific sequence motifs were used to search *msp1* block 2 sequences to assign family types.

Data Analysis

Descriptive analysis i.e., frequency was used to describe allele frequency of each allele types in each province. The genetic diversity of the MSP1 gene, block 2 region of *P. falciparum* from Thailand was analyzed by molecular evolutionary genetic analysis (MEGAX) software version (1.0.5). MEGAX is the computer software with statistical analysis for evolution and phylogenetic tree. In this program, the nucleotide sequence substitution for each site was calculated statistically by Tamura and Nei 1993 method for gene flow and evolutionary relationship by using the reference sequence to construct phylogenetic tree (87).

Phylogenetic analyses were conducted using MEGAX version 10.0.5 (88). The reference sequences from *Plasmodium* species retrieved from the GenBank database were included in the phylogenetic analyses. Phylogenetic analyses were also conducted with nucleotide sequence alignments of the *msp1* gene from *P. falciparum*. Phylogenetic trees were generated using the Maximum likelihood algorithm on Tamura and Nei parameter distances derived from first, second and

third codon positions (89). The reliability of the internal nodes of the trees was assessed by the bootstrap method after 1000 replicas.

Ethical consideration

Thesis study was reviewed and approved by the Ethical Review Committee for Research Involving Human Research Participants, Health Science Group Chulalongkorn University (COA No. 072/2020).

CHAPTER IV
RESULTS

Optimization of polymerase chain reaction

In this study, the nested polymerase chain reaction was used for amplification the MSP1 block 2 region on chromosome 9 using the primers from previous study of Naly Khaminsou et. al., (90).

Firstly, the *P. falciparum* clone samples, T9/94RC17, K1CB1, 3D7, HB3, and G112CB1.1, which were *in vitro* grown in the laboratory and spotted the parasitized blood sample on the Whatman filter paper were used. The DNA of these clones were extracted by using Chelex resin extraction method. MAD20 clones possess different *msp1* block 2 allelic type i.e. T9/94RC17 and HB3 possess MAD20 type while K1CB1, 3D7 and G112CB1.1 possess K1 type. They were used for trying out the amplification condition along with reference paper (91).

After running agarose gel electrophoresis and staining with ethidium bromide, the PCR product bands detected from all each sample showed the products from primary round PCR (the upper bands) while the band for K1 type was found from K1CB1, 3D7 and G112CB1.1 sample as shown in lane 3, 4, and 6 in Figure 4.1, and the band for MAD20 type was found from HB3 and T9/94RC17 sample as shown in lane 3 and lane 5 in Figure 4.2 respectively. There was no specific PCR product found from the amplification using R033 allelic specific primers (lane 9-14).

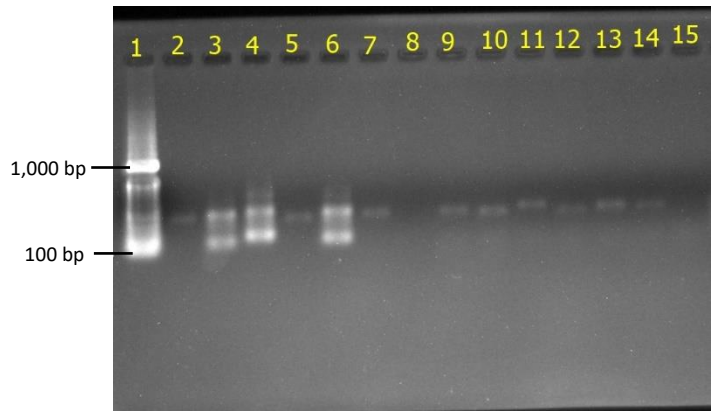


Figure 4.1 PCR products for K1 and R033 allele types. Lane 1 is 100 bp DNA ladder, Lane 2-8 are K1 type amplification, lane 9-15 are R033 type amplification. Lane 2 and 9, T9/94RC17 extracted by Chelex method used as a template while lane 3 and 10, K1CB1; Lane 4 and 11, 3D7; Lane 5 and 12, HB3; Lane 6 and 13, G112CB1.1; and lane 7 and 14, T9/94RC17 extracted by phenol-chloroform method were used as template respectively. Lane 8 and 15 are negative control for K1 and R033 respectively.

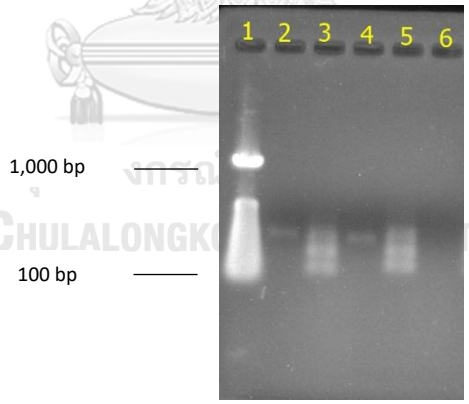


Figure 4.2 PCR products for MAD20 allele type. Different clones were used as template; 3D7 (lane 2), HB3 (lane 3), G112CB1.1(lane 4) and T9/94RC17 (lane 5) running along with 100 bp DNA ladder (lane 1) and negative control (lane 6).

To get the control samples for R033 allelic type, field samples were amplified together with control samples for three allelic specific primers. Fourteen field samples from Yala province coded YA 118 to YA 131 were amplified with three specific

primers. There was no PCR product band observed for K1 type amplification (Figure 4.3 a). Almost all sample presented MAD 20 type (Figure 4.3b) except YA123 sample revealed only R033 type (Figure 4.3c, lane 6). Thus, YA123 was used as R033 type control for further optimization. Furthermore, YA123 was mono-infected sample determined by using microsatellite marker.

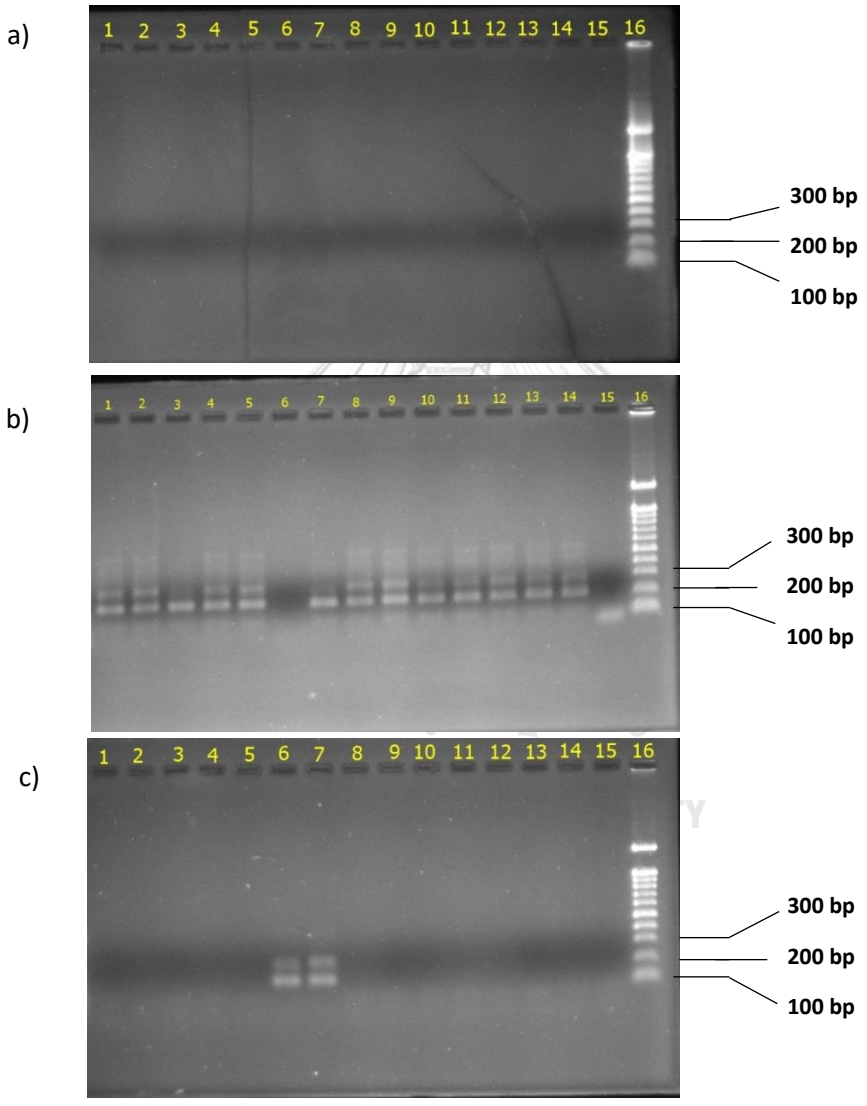


Figure 4.3 Gel electrophoresis result for amplification of field samples. K1 type (a), MAD20 type (b) and R033 (c). Lane 1 to lane 14 is field samples (YA 118 to YA131), lane 15 is negative control and lane 16 is 100 bp ladder.

This experiment, diluted DNA template at 1 in 10 and 1 in 100 of control samples; 3D7, HB3 and YA123 and four different concentrations of $MgCl_2$; 1.5, 2.0, 2.5 and 4.0 mM for primary and secondary PCR reaction were used for amplification. The result showed non-intense bands (Figure 4.4) when comparing with products amplified from undiluted template in previous experiment, thus further optimization undiluted DNA template was used.

Because of the non-specific product obtained from the published protocol, the optimization of components in the reaction was performed. The components in the PCR reaction should be optimized were amount of DNA template and $MgCl_2$ concentration. In figure 4.4, 1 in 10 and 1in 100 dilution of DNA template of control samples; 3D7 for K1, HB3 for MAD20 and YA123 for R033 allelic type and 1.5, 2.0, 2.5 and 4.0 mM for primary and secondary PCR reaction were done.

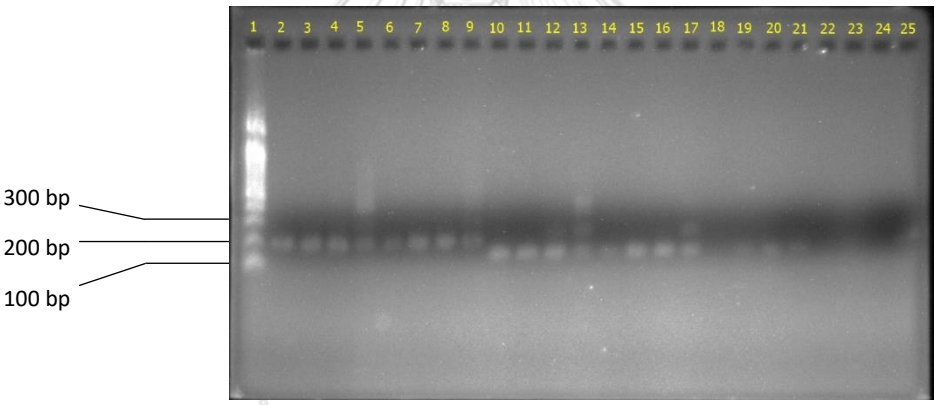


Figure 4.4 Gel electrophoresis result for amplification of control samples. The concentration of $MgCl_2$ was varied at 1.5, 2.0, 2.5 and 4.0 mM with 1 in 10 dilution of template for K1 (lane 2-5), for MAD20 (lane 10-13) and for R033 (lane 28-21) and also with 1 in 100 dilution of template for k1 (lane 6-9), for MAD20 (lane 14-17) and for R033 (lane 22-25). The products were run comparing with 100 bp DNA ladder (lane 1).

After choosing 1 µl of undiluted DNA as the optimal template concentration, the $MgCl_2$ was varied from 1.5, 2.0, 2.5 and 4.0 mM for primary and secondary PCR reaction using factorial design. In this way, the 4 reactions for primary PCR and 16 reactions for secondary PCR together with negative control were performed. For K1 type, 3D7 clone was used for optimization, the result of using 1.5 mM

$MgCl_2$ in the primary reaction showed specific PCR product band for every concentration of $MgCl_2$ varied from 1.5 to 4.0 mM while in the reaction with 2.0 mM $MgCl_2$ in the primary reaction revealed the specific band product when the secondary reaction contained 1.5 to 2.5 mM $MgCl_2$. Non-specific band was found when the concentration of $MgCl_2$ in the secondary reaction reached at 4.0 mM. $MgCl_2$ is the cofactor of Taq polymerase enzyme with optimal conditions, however high $MgCl_2$ causes the result with high amplification for non-specific band (92). For the PCR reaction containing 2.5 mM $MgCl_2$ in the primary reaction, only two conditions, 1.5 and 2.0 mM $MgCl_2$ in the secondary reaction provided specific band. There were non-specific band observed in all conditions when using 4.0 mM $MgCl_2$ in the primary reaction (Figure 4.5 a).

For MAD20 type (Figure 4.5 b), HB3 was used for optimization. The condition of $MgCl_2$ at 1.5 and 2.0 mM in the primary reaction with 1.5 and 2.0 mM $MgCl_2$ in the secondary reaction provided a good specific band (lane 2-3 and 6-7, respectively) except at 4.0 mM $MgCl_2$, no PCR product was found (lane 5) but more intense band was observed in the 2.0 mM $MgCl_2$ in the primary and secondary reaction.

Finally, the optimization of PCR reaction for R033 also performed by using YA123 isolate (Figure 4.5 c). The specific band product was obtained from the condition of 1.5 mM $MgCl_2$ in the primary reaction and at 1.5, 2.0, and 2.5 mM $MgCl_2$ in the secondary reaction (lane 2-4) At the higher concentration of $MgCl_2$ in the primary reaction, the specific band was found in the condition of 1.5, 2.0, and 2.5 mM $MgCl_2$ in the secondary reaction with 2.0 mM $MgCl_2$ in the primary reaction (lane 6-8) and this specific band was also found in the condition of 1.5, and 2.0 mM $MgCl_2$ in the secondary reaction with 2.5 mM $MgCl_2$ in the primary reaction (lane 10-11). The non-specific bands were still found in every $MgCl_2$ concentration when using the 4.0 mM $MgCl_2$ in the primary reaction (lane 14-17).

From those results, the best reaction was 2.0 mM $MgCl_2$ in both PCR for amplification of field samples. Its contents were 2.0 mM of $MgCl_2$, 200 µM of each deoxy nucleoside triphosphate, 75 nM of each primer and 0.8 U of Taq DNA polymerase, 1 µl of DNA template in 25 µl reaction. The program for amplification with outer primer pairs and inner primers was as following, denaturation at 94°C

for 4 minutes, 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute and final extension step at 72°C for 10 minutes.

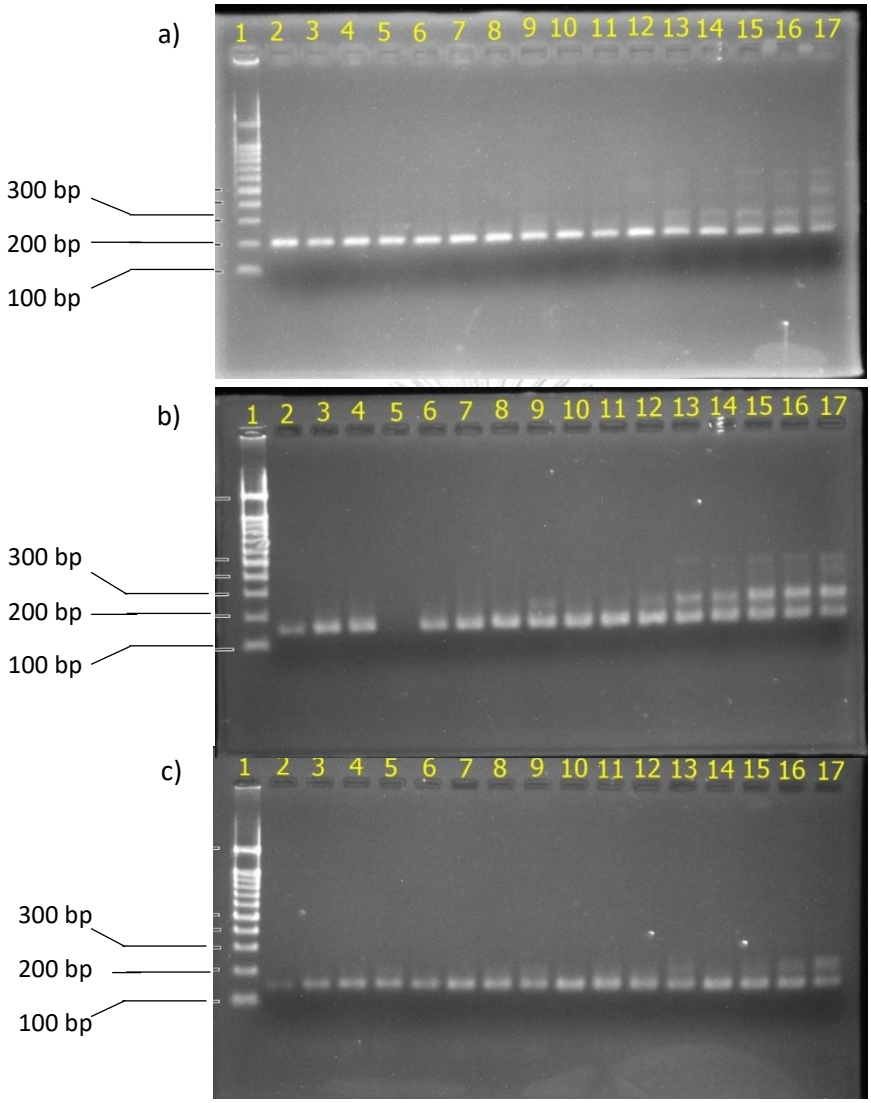


Figure 4.5 Optimization of nested PCR condition y varying $MgCl_2$ concentration The result of K1 (a), MAD20 (b) and R033 allelic type (c) were shown. Lane 1 is 100 bp DNA ladder, Lane 2-5 is 1.5 mM $MgCl_2$ in Primary PCR (Lane 2 = 1.5 mM, Lane 3 = 2.0 mM, Lane 4 = 2.5 mM, Lane 5 = 4 mM in Secondary PCR), Lane 6-9 are 2.0 mM $MgCl_2$ in Primary PCR (Lane 6 = 1.5 mM, Lane 7 = 2.0 mM, Lane 8 = 2.5 mM, Lane 9 = 4 mM in Secondary PCR), Lane 10-13 are 2.5 mM $MgCl_2$ in Primary PCR (Lane 10= 1.5 mM, Lane 11 = 2.0 mM, Lane 12 = 2.5 mM, Lane 13 = 4 mM in Secondary PCR), Lane 14-17 are 4.0 mM $MgCl_2$ in Primary PCR (Lane 14= 1.5 mM, Lane 15 = 2.0 mM, Lane 16 = 2.5 mM, Lane 17 = 4 mM in Secondary PCR)

Genotyping of human malaria parasite, *Plasmodium falciparum* merozoite surface protein 1 gene, block 2 of parasitized blood samples

Two-hundred and thirty-six (236) field samples from Tak, Kanchanaburi, Chumphon, Ranong, and Yala province were genotyped with the specific primers for *msp1* block 2 allelic type identification. The allele type, length of allele, and infection with single or multiple clones of *P. falciparum* were expressed in (Table 4.1)**.** Out of 236 DNA samples, 11 samples were PCR negative and 225 samples were successfully genotyped with specific primers (Table 3.2). Among 225 samples, 17 samples (7.5 %) were multiple infections (Table 4.3); MAD20+MAD20 (2 isolates), R033+R033 (1 isolate), K1+MAD20 (3 isolates), K1+R033 (4 isolates), MAD20+R033 (4 isolates), MAD20+MAD20+MAD20 (1 isolate) and K1+MAD20+R033 (2 isolates) and 208 samples (92.5%) were mono-infection. The most common allele family was MAD20 with 57.2% of total fragments. The R033 family was found in 31.3%, while K1 was found in 15.7% of total allele fragments. The multiplicity of infection (MOI) was calculated by the total number of PCR fragments for each allelic type divided by the total number of isolates (93). Multiplicity of infection (MOI) of Kanchanaburi province was highest (1.20) and Ranong (1.16), Chumphon (1.04) and Tak (1.00) and Yala (1.02) (Table 4.4).

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala

Isolates	Allele type	Allele length (bp)	Infectious Status
T 227	MAD20	159	Single
T 228	MAD20	163	Single
T 229	-	-	Negative
T 230	MAD20	164	Single
T 231	MAD20	163	Single
T 232	MAD20	166	Single
T 233	K1	160	Single
T 234	RO33	136	Single
T 235	MAD20	168	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continued)

Isolates	Allele type	Allele length (bp)	Infectious Status
T 236	MAD20	117	Single
T 237	K1	119	Single
T 238	MAD20	124	Single
T 239	MAD20	130	Single
T 240	MAD20	130	Single
T 241	MAD20	145	Single
T 242	K1	120	Single
T 243	MAD20	145	Single
T 244	MAD20	152	Single
T 245	MAD20	140	Single
T 246	K1	140	Single
T 247	K1	140	Single
T 248	K1	142	Single
T 249	MAD20	159	Single
T 250	MAD20	162	Single
T 251	MAD20	171	Single
T 252	-	-	Negative
T 253	MAD20	175	Single
T 254	MAD20	175	Single
K 428	K1+ MAD20	130/140	Mixed
K 429	RO33	96	Single
K 430	MAD20	147	Single
K 431	K1+ RO33	110/104	Mixed
K 432	RO33	104	Single
K 433	K1	112	Single
K 434	K1	136	Single
K 435	K1+RO33	114/106	Mixed
K 436	MAD20	145	Single
K 437	K1	114	Single
K 438	K1	121	Single
K 439	RO33	114	Single
K 440	K1	157	Single
K 441	MAD20	176	Single
K 442	K1+ MAD20+RO33	138/155/134	Mixed

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continued)

Isolates	Allele type	Allele length (bp)	Infectious Status
K 443	K1+ MAD20+RO33	131/188/105	Mixed
K 444	MAD20+RO33	195/105	Mixed
K 445	K1	122	Single
K 446	K1	133	Single
K 447	RO33	113	Single
K 448	RO33	117	Single
K 449	MAD20	187	Single
K 450	K1+RO33	138/124	Mixed
K 451	MAD20+RO33	174/126	Mixed
K 452	MAD20	203	Single
K 453	MAD20	163	Single
K 454	MAD20	208	Single
K 455	RO33	137	Single
K 456	RO33	137	Single
K 457	MAD20	177	Single
K 462	K1	126	Single
K 463	RO33	124	Single
K 464	K1	130	Single
K 465	K1+ MAD20	128/113	Mixed
K 466	K1	128	Single
K 467	MAD20	160	Single
K 468	K1	124	Single
K 469	K1	124	Single
K 470	K1	124	Single
K 471	MAD20	155	Single
K 472	MAD20	185	Single
K 473	MAD20	145	Single
K 474	MAD20	142	Single
K 475	K1	126	Single
K 476	K1	128	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala. (continue)

Isolates	Allele type	Allele length (bp)	Infectious Status
CHU 1	RO33	148	Single
CHU 2	MAD20	168	Single
CHU 3	MAD20	168	Single
CHU 4	MAD20	172	Single
CHU 5	RO33	133	Single
CHU 6	RO33	131	Single
CHU 7	RO33	129	Single
CHU 8	RO33	131	Single
CHU 9	RO33	156	Single
CHU 10	RO33	131	Single
CHU 11	MAD20	172	Single
CHU 12	MAD20	172	Single
CHU 13	MAD20	165	Single
CHU 14	RO33	147	Single
CHU 15	RO33	152	Single
CHU 16	MAD20	170	Single
CHU 17	K1	134	Single
CHU 18	RO33	128	Single
CHU 19	K1+RO33	138/128	Mixed
CHU 20	K1	128	Single
CHU 21	MAD20	161	Single
RN 151	MAD20	122	Single
RN 152	MAD20	135	Single
RN 153	K1	130	Single
RN 154	K1+ MAD20	137/141	Mixed
RN 155	MAD20	118	Single
RN 156	MAD20	150	Single
RN 157	MAD20	213/180/152	Mixed
RN 158	MAD20	187/156	Mixed
RN 159	MAD20	154	Single
RN 160	MAD20	156	Single
RN 161	MAD20	192	Single
RN 162	MAD20	169	Single
RN 163	MAD20	171	Single
RN 164	MAD20	162	Single
RN 165	MAD20	143	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continue)

Isolates	Allele type	Allele length	Infectious Status
RN 166	RO33	120	Single
RN 167	RO33	118	Single
RN 168	MAD20	146	Single
RN 169	MAD20	158	Single
RN 170	MAD20	158	Single
RN 171	MAD20	156	Single
RN 172	K1	97	Single
RN 173	MAD20	142	Single
RN 174	MAD20	161	Single
RN 175	K1	116	Single
RN 176	MAD20	218	Single
RN 177	RO33	133	Single
RN 178	MAD20	259/200	Mixed
RN 179	MAD20	167	Single
RN 180	MAD20	163	Single
RN 181	RO33	137	Single
RN 182	RO33	137	Single
RN 183	RO33	135	Single
RN 184	K1	125	Single
RN 185	K1	125	Single
RN 186	MAD20+RO33	160/147	Mixed
RN 187	RO33	147	Single
RN 188	RO33	150	Single
RN 189	RO33	150	Single
RN 190	RO33	152	Single
RN 191	-	-	Negative
RN 192	MAD20	137	Single
RN 193	RO33	148	Single
RN 194	RO33	151	Single
YA 65	RO33	135	Single
YA 66	RO33	135	Single
YA 67	MAD20	185	Single
YA 68	MAD20	187	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continue)

Isolates	Allele type	Allele length (bp)	Infectious Status
YA 69	MAD20	192	Single
YA 70	MAD20	195	Single
YA 71	MAD20	200	Single
YA 72	MAD20	202	Single
YA 73	RO33	145	Single
YA 74	RO33	145	Single
YA 75	-	-	Negative
YA 76	MAD20	168	Single
YA 77	-	-	Negative
YA 78	MAD20	168	Single
YA 79	RO33	169	Single
YA 80	-	-	Negative
YA 81	-	-	Negative
YA 82	-	-	Negative
YA 83	-	-	Negative
YA 84	MAD20	183	Single
YA 85	-	-	Negative
YA 86	RO33	154	Single
YA 87	MAD20	203	Single
YA 88	RO33	158	Single
YA 89	MAD20	208	Single
YA 90	MAD20	208	Single
YA 91	RO33	157	Single
YA 92	MAD20	203	Single
YA 93	MAD20	205	Single
YA 94	-	-	Negative
YA 95	MAD20	198	Single
YA 96	MAD20	195	Single
YA 97	RO33	109	Single
YA 98	MAD20	171	Single
YA 99	MAD20	198	Single
YA 100	MAD20	200	Single
YA 101	MAD20	198	Single
YA 102	MAD20	191	Single
YA 103	MAD20	200	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continue)

Isolates	Allele type	Allele length	Infectious Status
YA 104	RO33	124	Single
YA 105	MAD20	204	Single
YA 106	RO33	171	Single
YA 107	MAD20	186	Single
YA 108	RO33	171	Single
YA 109	RO33	171	Single
YA 110	MAD20	207	Single
YA 111	MAD20	195	Single
YA 112	MAD20	191	Single
YA 113	MAD20	193	Single
YA 114	MAD20	198	Single
YA 115	RO33	157	Single
YA 116	MAD20	200	Single
YA 117	MAD20	205	Single
YA 118	MAD20	130	Single
YA 119	MAD20	136	Single
YA 120	MAD20	130	Single
YA 121	MAD20	144	Single
YA 122	MAD20	134	Single
YA 123	RO33	121	Single
YA 124	RO33+MAD20	119/134	Mixed
YA 125	MAD20	138	Single
YA 126	MAD20	142	Single
YA 127	MAD20	146	Single
YA 128	MAD20	152	Single
YA 129	MAD20	156	Single
YA 130	MAD20	160	Single
YA 131	MAD20	167	Single
YA 132	MAD20	180	Single
YA 133	RO33	107	Single
YA 134	MAD20	157	Single
YA 135	RO33	102	Single
YA 136	MAD20	152	Single
YA 137	MAD20	152	Single
YA 138	MAD20	160	Single

Table 4.1 *Msp1* allelic types and its length, infectious status of the field isolates. T stand for Tak, K stand for Kanchanaburi, CHU stand for Chumphon, RN stand for Ranong, and YA stand for Yala (continue)

Isolates	Allele type	Allele length	Infectious Status
YA 139	MAD20	157	Single
YA 140	RO33	107	Single
YA 141	RO33	114	Single
YA 142	MAD20	165	Single
YA 143	MAD20	170	Single
YA 144	MAD20	168	Single
YA 145	RO33	118	Single
YA 146	RO33	125	Single
YA 147	RO33	134	Single
YA 148	RO33	100	Single
YA 149	RO33	103	Single
YA 150	RO33	106	Single
YA 151	RO33	109	Single
YA 152	MAD20	138	Single
YA 153	RO33	113	Single
YA 154	MAD20	152	Single
YA 155	MAD20	157	Single
YA 156	RO33	120	Single
YA 157	RO33	125	Single
YA 158	RO33	125	Single
YA 159	MAD20	188	Single
YA 160	RO33	127	Single
YA 161	RO33	107/135	Mixed
YA 162	MAD20	216	Single

Table 4.2 Successfully genotyped samples for each parasite population

	Total samples	**PCR (+)ve samples**	**PCR (-)ve samples**
Tak	28	26	2
Kanchanaburi	45	45	0
Chumphon	21	21	0
Ranong	44	43	1
Yala	98	90	8
Total	236	225	11

Table 4.3 Number of mono-infection and multi-infection isolate samples in each province

Province	**Mono-infection (n)**	**Multi-infection (n)**
Tak	26	0
Kanchanaburi	36	9
Chumphon	20	1
Ranong	38	5
Yala	88	2
Total	208	17

Table 4.4 Summarization of Genotyping results and multiplicity of infection in each province

Allele	Tak	Kanchana-buri	Chumphon	Ranong	Yala	Total
K1	6	15	2	5		**28**
MAD20	19	13	8	22	57	**119**
R033	1	8	10	12	31	**62**
MAD20+MAD20				2		**2**
R033+R033					1	**1**
K1+MAD20		2		1		**3**
K1+R033		3	1			**4**
MAD20+R033		2		1	1	**4**
MAD20+MAD20+ MAD20				1		**1**
K1+MAD20+ R033		2				**2**
Negative	2			1	8	**11**
Total	28	45	21	44	98	**236**
Total K1	6	22	3	6	0	**37**
Total MAD20	19	19	8	31	58	**135**
Total R033	1	15	11	13	34	**74**
Multiclonal isolates		9	1	5	2	**15**
MOI	1.00	1.20	1.04	1.16	1.02	**1.08**

The variation of allelic size of each family was found. The K1 allelic family possessed allele size ranging from 100-160 bp, while the MAD20 and R033 allele sizes ranging from 110-220 bp and 100-170 bp, respectively (Figure 4.6). The MAD20 allelic family is the most diverse one with 12 different alleles, compared to 8 alleles in the R033 family while the K1 family only 6 allele were found. Three allelic families of *msp1*, block 2 were found almost in all provinces with different proportions with exception for samples from Yala province, no K1 allele type existed in Yala parasite population (Figure 4.7). MAD20 was a dominant allele type in Tak (19/26, 73.1%), Ranong (22/43, 51.1%) and Yala (57/90, 63.3%) parasite populations while K1 type was dominant in Kanchanaburi parasite population (22/45, 48.9%). In Chumphon population, majority of allele type was R033 (11/21, 52.4%).

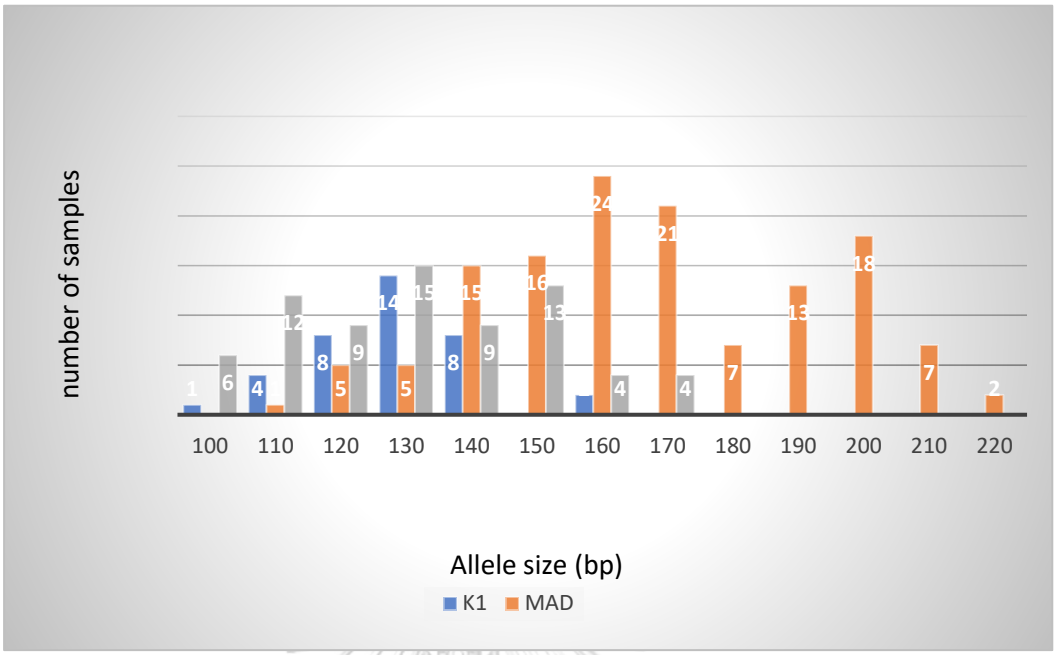


Figure 4.6 *msp1* Block 2 allelic size of *Plasmodium falciparum* isolates

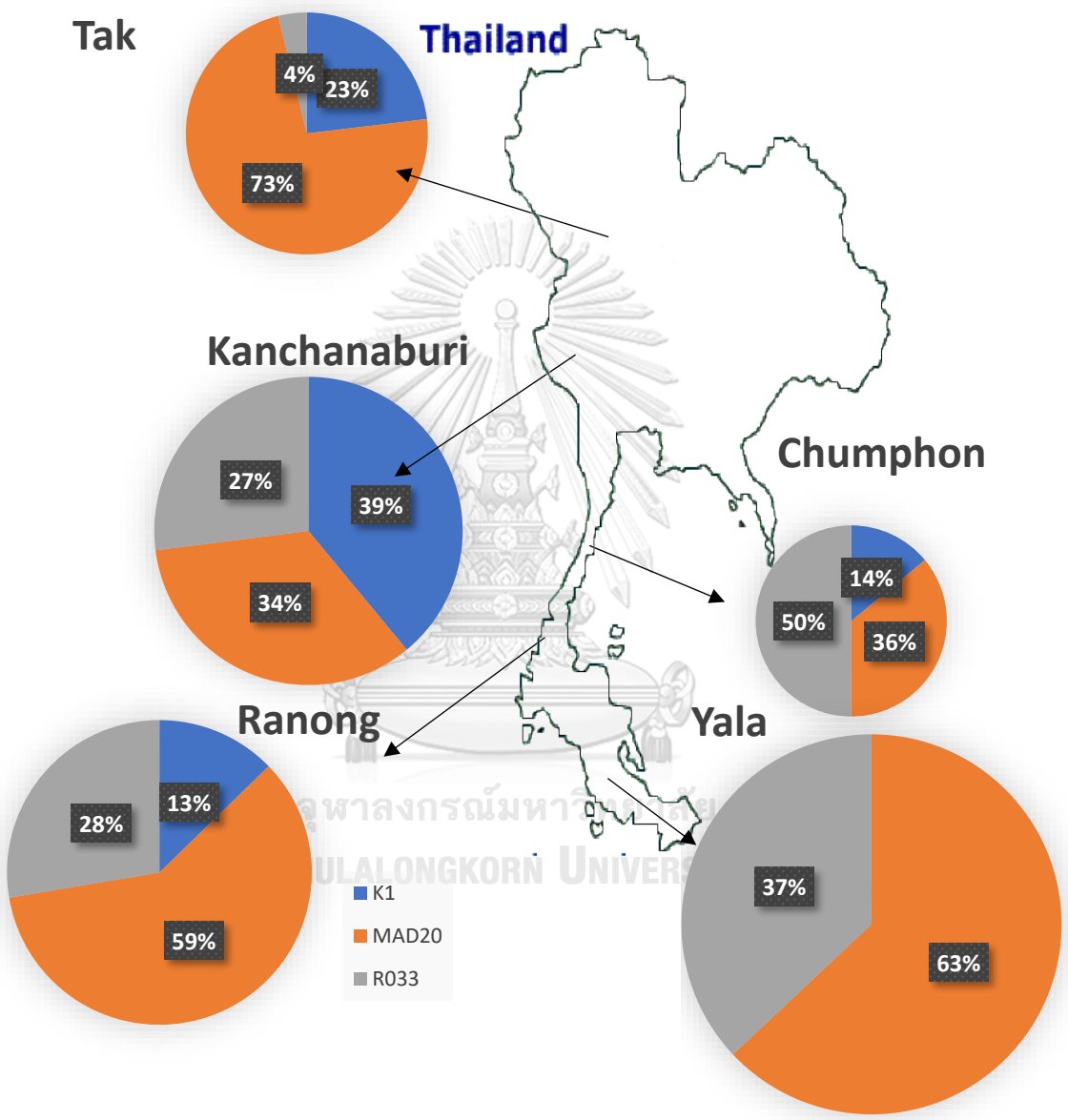


Figure 4.7 Distribution of Allelic frequencies of K1, MAD20 and R033 in 5 provinces.

Amino acid sequences of Block 2 region of *msp1* gene from field single genotype isolates

After identifying the allele type of *P. falciparum* samples from 5 provinces, 209 samples from all provinces were mono infected samples. From these PCR products, MAD20 allelic type product is the most samples. Yala samples were from 2013 and 2014 and the sequencing samples used were from 2013. By doing the sequencing with each specific primer for each allelic type sequence, 24 samples from Kanchanaburi, 13 samples from Chumphon, 33 samples from Ranong, 23 samples from Tak and 32 samples from Yala were used for sequencing.

Amino acids sequences of K1 type isolates

Twenty-two DNA sequences of K1 types were translated to amino acid sequences with MEGAX software. Sixteen haplotypes of K1 types were found in this study. Monopeptide, dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide and heptapeptide of SGT and monopeptide, dipeptide and tripeptide of SGP were found.

```
K470        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTSSGASP
            PADASDSDA
K476        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTSSGASP
            PADASDSDAKS
K446        EITTKGASAQSGTSGTSGTSGTSGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSSI
K445        EITTKGASAQSGTSGTSGTSGTSGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSSL
CHU17       EITTKGASAQSGTSGTSGTSGTSGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSS*
CHU20       EITTKGASAQ---SGTSGTSGTSGTSGTSGPSGPSGPSGTSPSSRSNTLPRSNTSSL
K462        EITTKGASAQ---------SGTSGTSGTSGTSGP------SPSSRSNTLPRSNTSSI
K464        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTSSI
K475        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRS
T242        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTS
T247        EITPKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTS
T248        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTSS
K466        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPFSRSNTLPRSNTSSL
RN172       EITTKGASAQ---------SGTSGTSGTSGPSGPSGPSGTSPSSRSNTLPRSNTSS
T237        EITTKGASAQ---------SGTSGTSGTSGTSGPSGTSGTSPSSRSNTLPRSNTSSL
K469        EITTKGASAQ---------SGTSGTSGTSGTSGP---SGTSPSSRSNTLPRSNTSSI
RN184       EITTKGASAQ----------SGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSS
RN185       EITTKGASAQ----------SGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSSI
RN153       EITTKGASAQ---SGTSGTSGTSGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSS
RN175       EITTKGASAQ---SGTSGTSGTSGTSGTSGTSGPSGPSGTSPITRSNTLPRSNTSS
T233        EITTKGASAQ---SGTSGTSGTSGTSGTSGTSGPSGPSGTSPSSRSNTLPRSNTSSI
T246        EITTKGASAQ----------------SGTSGTSGTSGTSPSSRSNTLPRSNTSS*
```

Figure 4.8 Amino acid sequence alignment of *msp1* block 2 of K1 types of field isolates. Asterisk (*) is site targeted amino acid recombination predictor

Repetition of SGT (in the left site)

Repetition of SGP (in the middle)

Repetition of SGT (in the right site)

Amino acid sequences of MAD20 type isolates

Seventy-seven DNA sequences of MAD20 types from 5 provinces of Thailand were translated to amino acid sequences with MEGAX software. Forty-six haplotypes of MAD20 type were found in this study. SGGSGG, SVASGG repeated sequences are mostly found. Some sequences are presented with SVTSGG. NEGTSGTAVTTSTPG is started in the 5’ end and they are ended with SGNSRRTNPSDN and SGNSRRTNPSNY in the 3’ end.

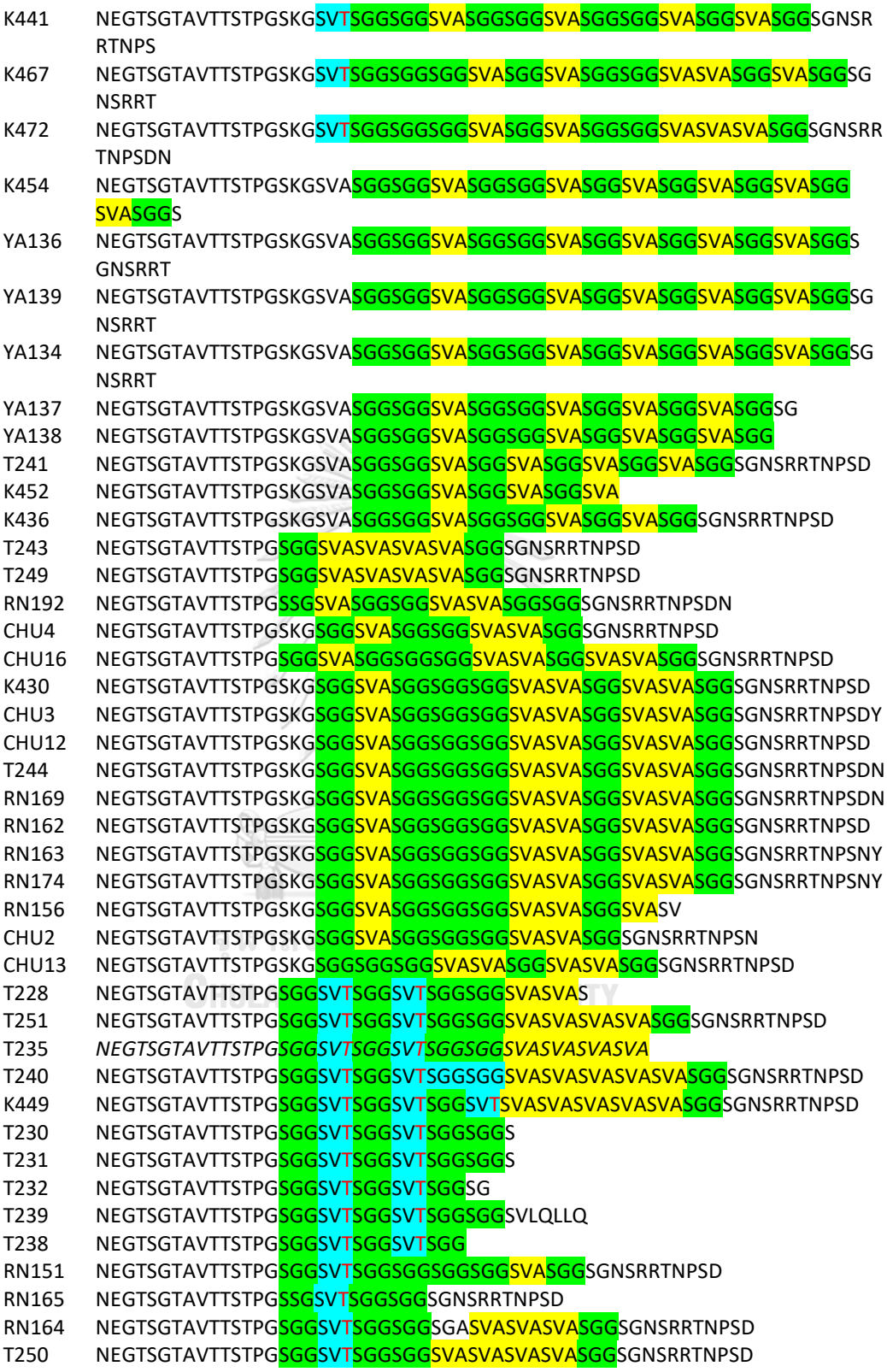


Figure 4.9 Amino acid sequence alignment of *msp1* block 2 of MAD20 types of field isolates. SGG SVA SVT.

Figure 4.9 Amino acid sequence alignment of *msp1* block 2 of MAD20 types of field isolates. SGG SVA SVT (continued).

Amino acid sequences of R033 type isolates

Twenty-eight DNA sequences of R033 types (26 sequences from 5 provinces of Thailand and 2 identical sequences from Gen Bank) were translated to amino acid sequences with MEGAX software. Accession numbers for reference sequences are HM153228 and MG092933. Eleven haplotypes of R033 were found in this study. No repeated peptide in all haplotypes except in CHU 15 was found.

CHU18	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
RN166	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
RN181	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
YA66	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
YA65	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
YA74	LTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
K455	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGS PGAANPSD
YA86	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGSP
YA88	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGSPGAANPSDE
CHU1	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIGSPGAANPSDE
K429	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSPGAANPSDE
K432	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSPGAANPSDE
K448	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSPGAANPSD
RN177	LKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSPGAANPSDE

Figure 4.10 Amino acid sequence alignment of *msp1* block 2 of R033 types of field isolates and references from GenBank @. Asterisk (*) is site targeted amino acid recombination predictor.

T234	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASY*RCYRI SRCCKSFR*
RN188	VLTGYSLFQKEKMVLKDGANSQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSP GAANPSD
RN194	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGANSTFRYCKY*RCYKISR CCKSFR*
YA73	VLTGYSLFQKEKMVLKDGANTKVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSPG AANPSD-
K463	LTGYSLFQKEKMVLKDGANTQVVAKPADAVSTQSAKNPPGATVPSGTASTKGAIRSPG AANPSD
RN187	VLTGYSLFQKEKMVLKDGANTQVVAKPADAVSTQSAKNPPGATVPSGTASTKGAIGSP GAANPSD
RN189	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSP GAANPSD
RN190	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSP GAANPSD
RN193	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSP GAANPSD
K447	VLTGYSLFQKEKMVLKDGANTQVVAKPAGAVSTQSAKNPPGATVPSGTASTKGAIRSP GAANPSD
HM153 228@	N*KL*KMQY*QVIVYFKRKKWY*MKEQILKLLQSLQML*VLKVLKILQVLQYLQVLQVL KVL*DLQS
CHU5	N*KL*KMQY*QVIVYFKRKKWY*MKEQILKLLQSLQVCCKYSKC*KSSRCYSTFRYCKY*R CYRISRCCKSF
MG092 933@	*RMEQILKLLQSLQVL*VLKVLKILQVLQYLQVLQVLKVL*DLQVLQILQM
CHU15	*RMEQILKLLQSLQVL*VLKVLKILQVLQYLQVLQVLKVL*DLQVLQILQM

Figure 4.10 Amino acid sequence alignment of *msp1* block 2 of R033 types of field isolates and references from GenBank @. Asterisk (*) is site targeted amino acid recombination predictor (continued).

Phylogenetic tree of *msp1* block 2 family types in Thailand

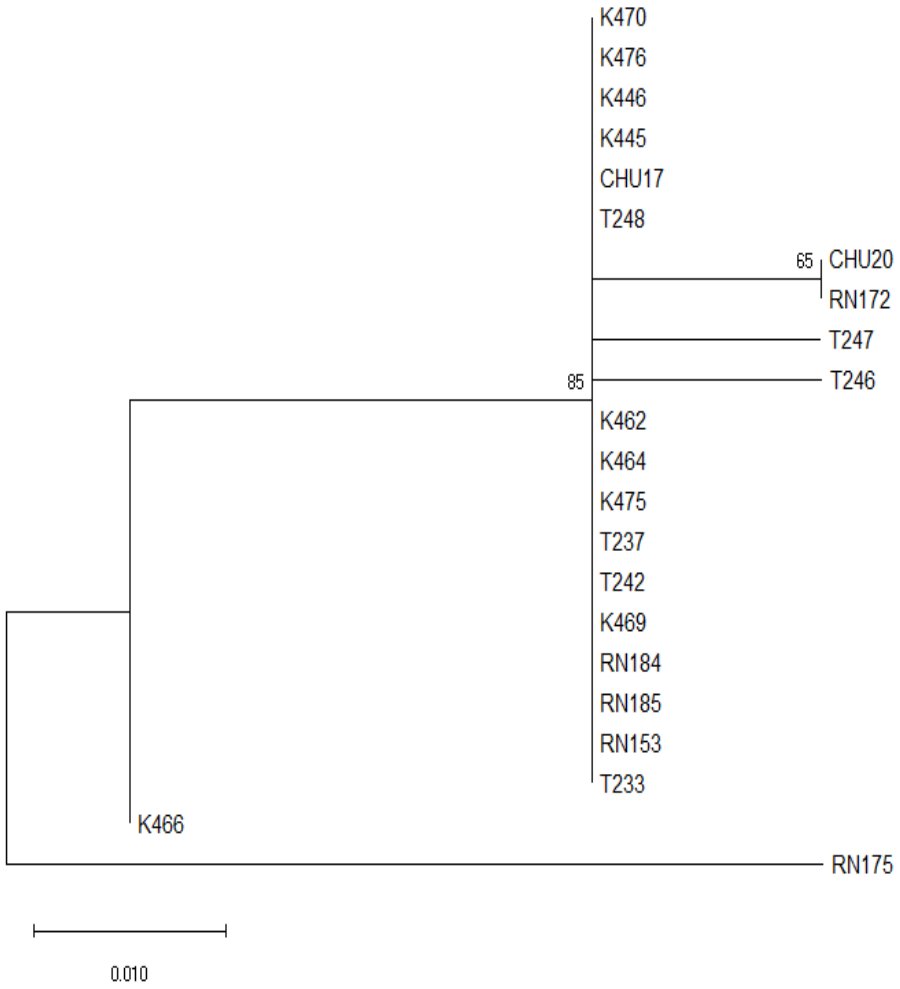


Figure 4.11 Maximum Likelihood tree of K1 allelic type. T, K, CHU, and RN stand for Tak, Kanchanaburi, Chumphon, and Ranong province, respectively. Bootstrap values are indicated at nodes.

Maximum likelihood tree is for probability of nucleotide substitutions of sequences In the phylogenetic tree of K1 type, 22 nucleotide sequences of field samples; Tak 6 samples, Kanchanaburi 9 samples, Chumphon 2 samples and Ranong 5 samples were analyzed. *P. falciparum* isolates from Tak, Kanchanaburi, Chumphon and Ranong had the relation mostly with monophyletic clade. Ranong isolates had the unique sequence (Figure 4.11).

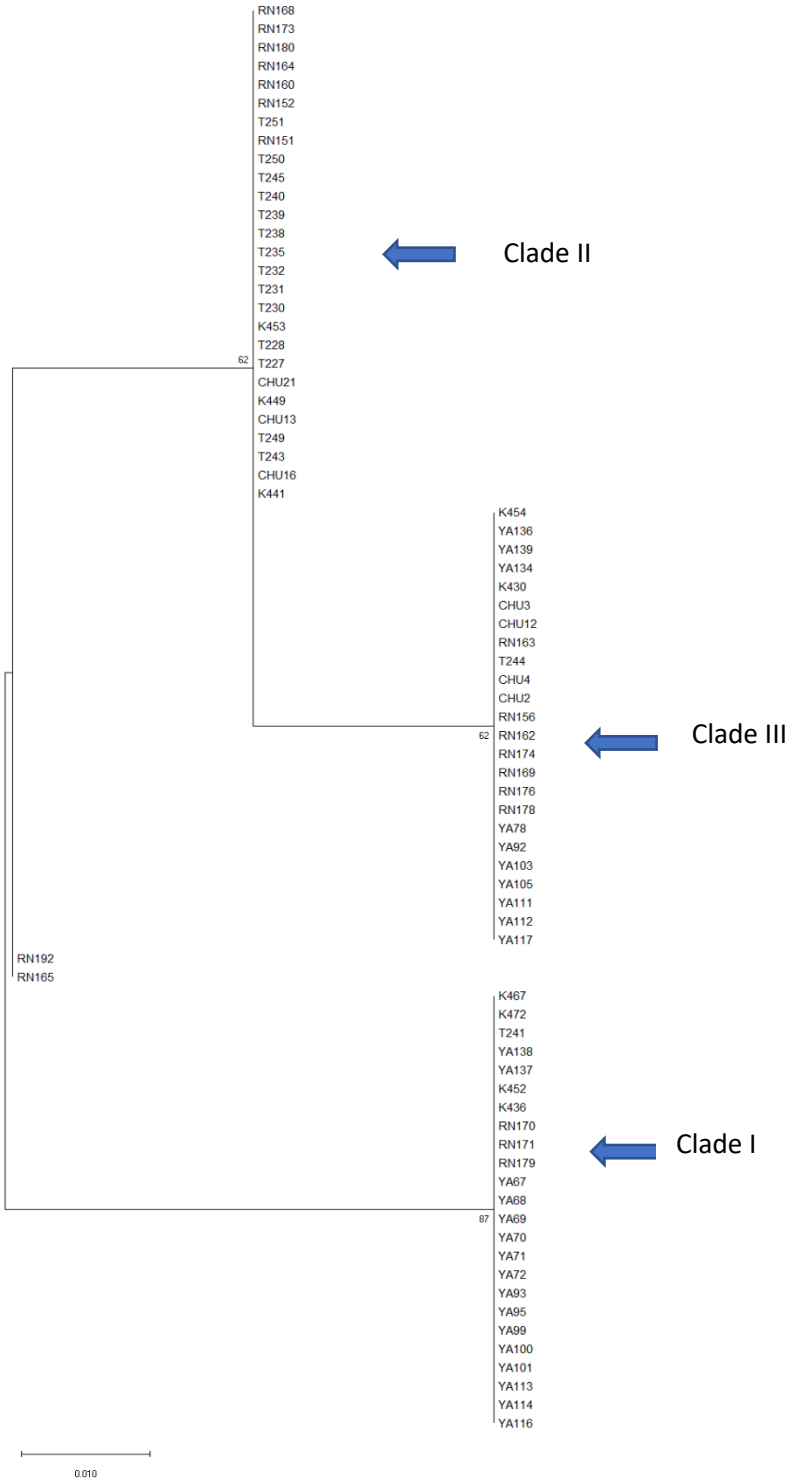


Figure 4.12 Maximum likelihood tree of MAD20 allelic type. T, K, CHU, RN, and YA stand for Tak, Kanchanaburi, Chumphon, Ranong and Yala province. Bootstrap values are indicated at nodes.

In the phylogenetic tree of MAD20 type, 77 nucleotide sequences of field samples; Tak 16 samples, Kanchanaburi 9 samples, Chumphon 7 samples, Ranong 19 samples and Yala 26 samples. The first clade had the close relation of Tak, Kanchanaburi, Ranong and Yala. The second clade had the relationship of isolate from Tak, Kanchanaburi, Chumphon and Ranong. At the far end of the tree, isolate sequences from five provinces were related respectively (Figure 4.12).

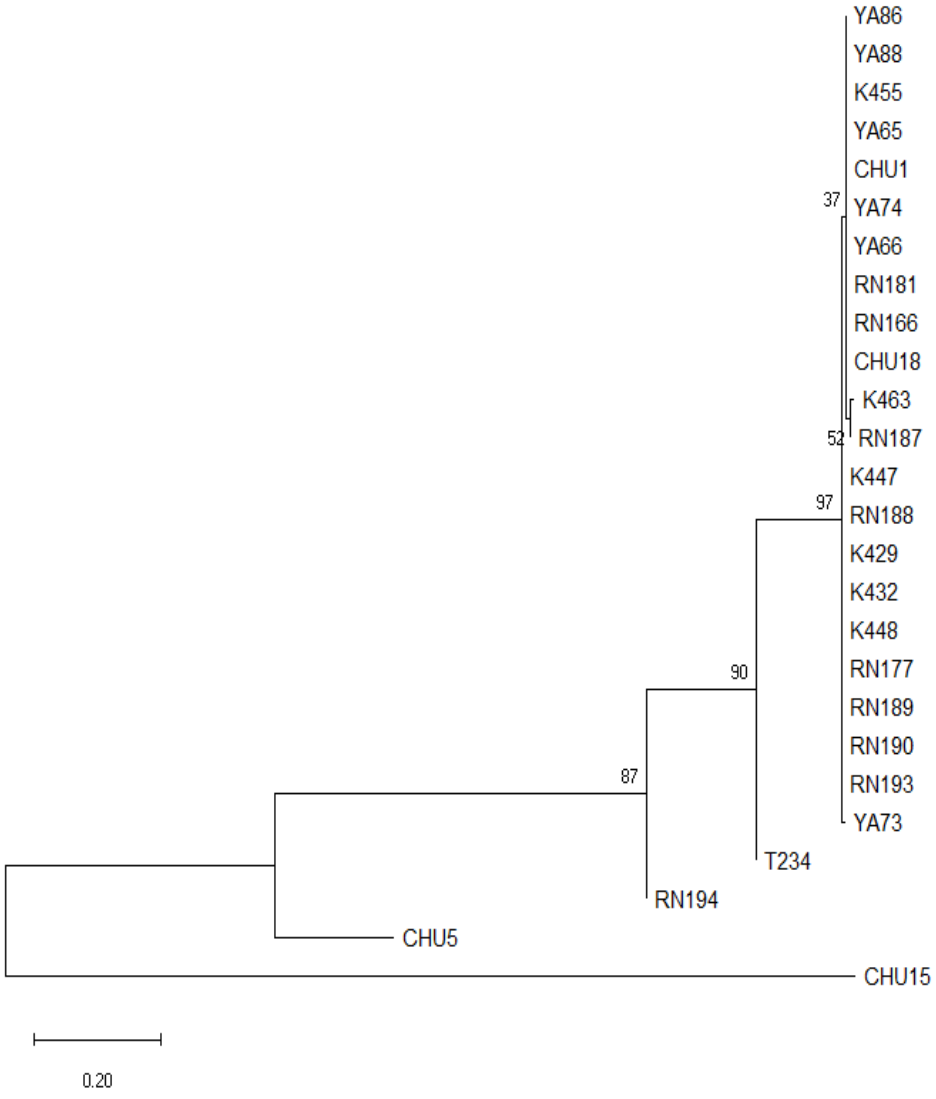


Figure 4.13 Maximum Likelihood tree of R033 allelic type.

T, K, CHU, RN, and YA stand for Tak, Kanchanaburi, Chumphon, Ranong and Yala province respectively. Bootstrap values are indicated at nodes.

In the tree of R033 type. 26 nucleotide sequences of field samples; Tak 1 sample, Kanchanaburi 6 samples, Chumphon 4 samples, Ranong 9 samples and Yala 6 samples were analyzed. Most of the sequences from Kanchanaburi, Chumphon, Ranong and Yala were grouped as the same clade. They had closely related each other. Tak isolate was different and related with different clade. Furthermore, two *Plasmodium falciparum* isolates from Chumphon (CHU 5 and CHU 15) are unique (Figure 4.13).

CHAPTER V

Discussion and Conclusion

Discussion

Most people know about the malaria disease. However, it has still the challenge to eliminate the malaria all over the world. Because of the complicated life cycle of malaria parasite, mosquito vector, human host, many *Plasmodium* species and their gene variation. Now five *Plasmodium* species are transmitted to human by *Anopheles* mosquitoes. Among them, cerebral malaria, *Plasmodium falciparum* is the most virulent species. Long time ago, people used many drugs for treatment and prevention of malaria. However, Africa still has the high death rate of maternal and children by malaria. Therefore, it is found that malaria vaccine is also essential like hepatitis B virus vaccine. For that, the expected frequencies and observed frequencies of potential vaccine gene of *P. falciparum* is needed. In this study, the diversity of block 2 region, *msp1* gene of *P. falciparum* from high endemic regions of Thailand was investigated. Moreover, the relatedness of *P. falciparum* populations in these five regions of Thailand was analyzed with phylogenetic tree.

To distinguish polymorphism in allele-specific families in this study, all DNA products from each allele-specific family were detected by nested PCR. After analyzing the base pair of PCR products, the isolates carrying MAD20 allelic type were found to be the most prevalent of total samples in Thailand (135/236, 57.2%). The isolates with RO33 and K1 allelic types of block 2 were (74/236, 31.3 %) and (37/236, 15.7 %) respectively. Goh, et. al also expressed that MAD20 was the highest type (74, 42.8%) and followed by R033 (52, 30%) and K1 (18, 10.4%) of the total samples from southern part of Thailand in 2008-2014 (66). The prevalence of polymorphic strains was found in Kanchanaburi (9/45, 20%), Chumphon (1/21, 4.8%), Ranong (5/44, 11.4%) and Yala (2/98, 2.0%).

About 7.2% (17/236) of *P. falciparum* infections in Thailand in this study were multiple clonal infections of the three allele types studied. MOI in Thailand was low as not exceed 1.2. The overall multiplicity of infection (MOI) was 2.25 by Muzamil Mahdi Abdel Hamid et al. in Sudan, 2012 (94) and 2.0 in Ethiopia, 2017 by Abdulhakim Abamecha et al. (95) and 1.94 in Myanmar, 2009-2010 by Than Naing Soe et al. (96). Many reports showed that MOI would reflect that infected individual has immunity

against malaria. Multiplicity of infection (MOI), also termed complexity of infection (COI) is defined as the number of different parasite strains co-infecting a single host. MOI can be a useful indicator of immune status and transmission level. Traditionally, MOI was assessed by PCR genotyping of antigen protein gene (*msp1)* and microsatellite markers, which were regarded as the gold standard because of their high polymorphism. However, these methods were unable to distinguish sequence variation among parasite strains and detect minority clones within a host. By using Sanger sequencing, the minority clone could be detected as low as 0.5% within-host infection frequency.

When comparing the three allelic types in each province separately, this study found that the MAD20 allelic type was high in Tak 67.8 % (19/28), Ranong 63.6 % (28/44), and Yala 58.1 % (57/98). K1 allelic type was the most frequent in Kanchanaburi 48.8 % (22/45) and R033 was the dominant in Chumphon 52.4 % (11/21). We found that Yala province had the parasite isolates carrying two family types (MAD20 and R033) without K1 family type. By comparing with the previous paper of Congpuong K et al., K1 type was the highest in Kanchanaburi province (74.5%) while MAD20 type was highest in Tak (59.6%) (69). The fragment size of K1 was 100-160 bp, MAD20 (110-220 bp) and RO33 (100-170 bp).

In 1997, Diana Gomez et al. expressed that Colombia isolates carried the highest MAD20 type and followed by R033 and K1 (61). In 2000, Agnès Aubouy et al. in Gabon, in 2005, Kevin K. A et al. in Northern Zambia and Praveen K Bharti et al. in Central India, and then 2015, Brice Pembet Singana et al. in Congo showed that the K1 type was the most frequent family (83, 85, 97, 98). India isolates changed the allelic pattern in block 2 as the highest R033 type in 2009 (85). In 2007, Lili Yuan et al. in China-Myanmar and in 2013-17 in our study in Thailand had the MAD20 type as a dominant one (99).

The information of the different allele types in malaria endemic regions are useful for monitoring of drug efficacy, the conditions about the parasite transmission and the human immunity to the parasites (100). Regarding to the number of alleles, we observed 6 alleles for K1 type, 12 alleles for MAD20 type and 8 alleles for RO33. The number of alleles observed in this study was different from the reports in 1991-1992, the alleles of K1 types were 4, MAD20 types were 5 and R033 type was 1 (64), in 1992-

1995, K1 family types were 20 alleles, MAD20 family types were 15 alleles among 362 samples without R033 type (101) and in 2008-2014, the alleles of K1 were 6, MAD20 were 13 and R033 were 2 (66). This fluctuation of dominant allelic family and its allele frequency showed the dynamic of the parasite population in the different period. By comparing these data, block 2 of *msp1* carried MAD20 and R033 types more and more in the later.

In the previous studies of Myanmar, Lao, northeastern india and Brazil parasite isolates, the different numbers of tripeptide repeat were also observed and human immune response for inhibition of parasite growth (58, 87, 102, 103). Antibodies specific to R033 type was not significant although there had the association between antibodies to K1 and MAD20 types of block 2 against malaria (6, 104). The present study provides a detailed assessment of genetic diversity and multiplicity of infection of *P. falciparum* parasites from five provinces of Thailand. The association between IgG (especially IgG3) responses to block 2 and a significantly reduced risk of clinical disease is important evidence that the block 2 region of *msp1* is a significant target of protective immune responses to *P. falciparum*. Antibodies to most full-length antigens from the two common block 2 types, namely the K1 and MAD20-like types, were significantly associated with protection (105). Moreover, Kevin K. A. Tetteh et al., expressed that allele-specific antibodies have the reaction with K1 repeated sequence (98). In contrast, K1 and MAD20 repeated sequences and flanking region had the strong correlation with high antibodies (106). The diversity of parasites implied that transmission, treatment and vaccine strategies. The parasite population with high diversity causes the high transmission and treatment failure. Moreover, it causes the various epitopes and still have the problems to success the vaccine development. The combination of different factors such as the genetics and immune response of the host, differential adaptation of the parasite to the vector, preferential distribution of certain vectors, and the genetic diversity of the parasite itself, among other aspects, plays a decisive role in the development of malaria (107).

Based on sequence data analysis and phylogenetic tree of each K1, MAD20 and R033 allelic type sequences in Thailand showed that the most allelic types are not unique and related with each other and circulating in the whole country. MAD20 haplotypes were related with many monophyletic clades due to high sequence diversity.

R033 types were grouped together and there had the unique pattern from Yala province. Relationship of block 2 allelic family type in endemic areas of Thailand indicated that the transmission was circulated in the whole countries. Here we have shown that variation in the number, sequence, and arrangement of repeat units within block 2 of *msp1* alleles. Tandem repeat sequences showed the drug pressure and may be due to the high recombination (108). Block 2 alleles have the specific antibody response determined by Conway et al. (56). The new variants in block 2 regions during mitosis have the potential to produce immune response (109). A total of 125 different alleles of *msp1* were used for sequence analysis. Sixteen variant subtypes for K1 type, 46 variant subtypes for MAD20, and 11 variant subtypes for RO33 were detected.

All K1-type alleles started with EITTKGA at the 5′-end and the central variable region always started with SAQ and terminated with SGT containing the difference number of tripeptide repetition, i.e., SAQ, SGP, and SGT. All MAD20-type alleles started with NEGTSGTAVTTSTP and at the 5′-end with SGNSRRT and the central variable region with different repetition of SVT, SGG and SVA. The parasite population in Myanmar and Thailand have same amino acid repetitions except SKG is not found in parasite isolates of Thailand (59). In Chumphon, Ranong, Kanchanaburi and Yala, it was found that haplotypes for R033 type were similar. However, Chumphon had the isolate carrying the different pattern for R033 type.

From the phylogenetic tree analysis, most of R033 type of block 2, *msp1* isolates from four provinces had the close relation. The isolates from Tak, Chumphon and Ranong had the different variation and related with different nodes. It was found that the isolates carrying with R033 type were migrated in all provinces except in Yala. In the tree with K1 type, most of the isolates from all five provinces had closely related. Chumphon, Ranong and Tak had the isolates carrying K1 type with different variation. For MAD20 type, the neighbouring provinces; Tak and Kanchanaburi, Kanchanaburi and Chumphon, Chumphon and Ranong, Ranong and Yala are related in monophyletic clade. Moreover, the isolate from Tak (northern part of Thailand) and Yala (southern part of Thailand) are related in monophyletic clade. The parasite isolates from each province have the relation and not found for isolate with unique pattern. All provinces have the gene flow and parasite migration.

Therefore, genetic diversity plays a crucial role in the natural acquisition of immunity in malaria disease and it is a major problem in the pathway of control strategies to prevent the distribution of the infection. Combating drug resistance in malaria parasites or producing potential vaccines require comprehension of population structure of the parasite. For this purpose, a lot of studies have been conducted by researchers to access genomic markers and polymorphism of the parasite. Isolating the genes that involve in producing cerebral malaria provides a valuable opportunity for prevention of deleterious complications of falciparum malaria in endemic areas. Phylogenetic tree revealed that there is an extensive diversity range between isolates so that some of the isolates achieved in the present study. There is high diversity in the gene in the specific population of the parasite. It is, therefore, important to characterize the level of parasite genetic variation in diverse geographical locations to identify the prevailing parasite strains. This provides a comprehensive description of *P. falciparum* diversity for immunogenic segment of *msp1* gene in disparate malaria affected regions of Thailand. Point mutation and repeat instability due to recombination are the major factors responsible for variability of K1 and MAD20. Several factors, such as transmission intensity, parasite recombination rate, increase in antimalarial drug resistance and effective malaria control measures can contribute the evolution of genetically diverse parasites.

Conclusion

In Thailand, *Plasmodium falciparum* population have the high genetic diversity for block 2 of *msp1* gene. It indicates the polymorphic nature and evolutionary aspect of the vaccine candidate antigens in the *P. falciparum* population.They also have the recombination site in their sequences. The parasite can exist with new gene and can also cause the high transmission with new isolates in the community. Therefore, continuous molecular epidemiological surveillance to monitor the genetic variation and gene flow of the parasite in Thailand would be necessary. Further studies to examine the malaria transmission intensity based on entomological inoculation rates, and the immune status of *P. falciparum*-infected individuals, may contribute to the guidance of malaria interventions.

Recommendation

The allelic variants of block 2 region of *Plasmodium falciparum* are different. These results are valuable for future epidemiological study of malaria transmission and human immune response to the parasites.

Appendix

Appendix 1

Reagents;

To get 10% of Chelex-100 reagent, weigh 10 grams of chelex resin and add 90 ml sterile water and mix with magnetic stirrer. After that autoclaved and keep as stock solution.

To get 0.5% Saponin, weigh 0.05 grams of saponin and add 100 ml of sterile water and adjust pH 7.2-7.4.

To get TBE (10x), weight 109 g Tris base, 55 g Boric acid dissolve in 800 ml of ddH2O, add 40ml of 0.5 m EDTA pH 8.0, then adjust volume to 1000 ml, pH should be 8.2-8.3, if not adjust with HCL. For 1x buffer, dilute 1 ml of 10x TBE buffer with 9 ml of sterile water.

To get 2.5% agarose gel, weigh 2.5 g of agarose gel and dissolve in 100 ml of TBE buffer.

To get 70% ethanol, dilute 70 ml of absolute ethanol with 30 ml of distilled water.

Appendix 2

In PCR sequencing reaction, 2 µl of Big Dye ™ Terminator 3.1 Ready Reaction Mix, 1 µl of Big Dye ™ Terminator v3.1 5X Sequencing Buffer, 0.32 µl for each primer (3.2 µM) Forward/Reverse primer, 5.68 µl of deionized water and 1 µl of template PCR product are used.

Appendix 3

Purification the sequencing reaction with ethanol/EDTA preparation

1. Prepare a 125 mM EDTA solution from 0.5 M EDTA, pH 8.0.
2. Make 70% fresh ethanol using absolute ethanol.
3. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.
4. Remove the MicroAmpTM Clear Adhesive Film from the plate.
5. Add the following in order:

Component	**Volume**
Sequencing reaction (starting volume)	10 ul
125 mM EDTA solution	2.5 ul
Absolute ethanol	30 ul
Total volume	42.5 ul

6. Cover the plate with Micro Amp™ Clear Adhesive Film. then centrifuge at 1,870 x g (4'C) for 15 minutes.
7. Slowly take out the Micro Amp™ Clear Adhesive Film to prevent disruption of the pellet. Use 4 layers of tissue paper to absorb the left ethanol and place it into the centrifuge bucket and carefully invert the plate onto that paper. Centrifuge with slow speed at 185 x g for 1 minute to remove the reagent. Don't tip out liquid first. Don't tap plate to help with liquid removal.
8. Allow the plate to air dry, face up and protected from light, for 5 to 10 minutes at room temperature.
9. Cover the plate with Micro Amp™ Clear Adhesive Film.
10. Do vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 x g.

11. Leave the plate at room temperature for exactly 15 minutes.
12. Centrifuge the plate in a swinging bucket centrifuge at 1,870xg (4'C) for 20 minutes.
13. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge and carefully invert the plate onto the paper without dislodging the pellet. Centrifuge with slow speed at 185 x g for 1 minute to remove the reagent. Don't tip out liquid first. Don't tap plate to help with liquid removal.
14. Put 30 ul of 70% ethanol to each well.

Appendix 4

AF 01-12

The Research Ethics Review Committee for Research Involving Human Research Participants, Group I, Chulalongkorn University

Jamjuree 1 Building, 2 Floor, Phyathai Rd., Patumwan district, Bangkok 10330, Thailand,
Tel: 0-2218-3202, 0-2218-3049 E-mail: eccu@chula.ac.th

COA No. 072/2020

Certificate of Approval
Exemption for Ethics Review

Study Title No. 049.1/63: GENETIC DIVERSITY OF MEROZOITE SURFACE PROTEIN 1 GENE (*msp* 1) OF *PLASMODIUM FALCIPARUM* IN THAILAND

Principal Investigator : MISS MAU MYAT THU

Place of Proposed Study/Institution : College of Public Health Sciences, Chulalongkorn University

This Research proposal is exempted for ethics review in compliance with the Office for Human Research Protections (OHRP Exempt Categories) 45 CFR part 46.101(b).

Certified under condition: To conduct this research project, the researcher (s) must strictly adhere to procedures appeared in the research project submitted to the committee for review.

Signature: Prida Tasanapradit

(Associate Professor Prida Tasanapradit, M.D.)
Chairman

Signature: Nuntaree Chaichanawongsaroj

(Associate Professor Nuntaree Chaichanawongsaroj, Ph.D.)
Secretary

Date of Exemption : 10 March 2020

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