HETERODUPLEX TRACKING ASSAY FOR THE ANALYSIS OF *PLASMODIUM FALCIPARUM* GENETIC DIVERSITY: TECHNIQUE DEVELOPMENT, VALIDATION AND APPLICATIONS

WARUNEE NGRENNGARMLERT

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
(MEDICAL TECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005**

ISBN 974-04-6224-3

Thesis
Entitled

HETERODUPLEX TRACKING ASSAY FOR THE ANALYSIS OF *PLASMODIUM FALCIPARUM* GENETIC DIVERSITY: TECHNIQUE DEVELOPMENT, VALIDATION AND APPLICATIONS

……
Miss. Warunee Ngrenngarmlert
Candidate

……
Assoc. Prof. Dr. Wanida Ittarat,
Ph.D.
Major-Advisor

……
Dr. Chansuda Wongsrichanalai,
Ph.D.
Co-Advisor

……
Prof. Steven R. Meshnick,
Ph.D.
Co-Advisor

……
Assoc. Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

……
Assoc. Prof. Virapong Prachayasittikul ,
Ph.D.
Chair
Doctor of Philosophy Programme
in Medical Technology
Faculty of Medical Technology

Thesis

Entitled

HETERODUPLEX TRACKING ASSAY FOR THE ANALYSIS OF *PLASMODIUM FALCIPARUM* GENETIC DIVERSITY: TECHNIQUE DEVELOPMENT, VALIDATION AND APPLICATIONS

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Doctor of Philosophy (Medical Technology)

on
May 20, 2005

…….
Miss. Warunee Ngrenngarmlert
Candidate

…….....
Assoc. Prof. Wanida Ittarat,
Ph.D.
Chair

…….
Dr. Chansuda Wongsrichanalai,
Ph.D.
Member

…….. ……
Dr. Pongchai Harnyuttanakorn
Ph.D.
Member

Asst. Prof. Thareerat Kalumbaheti,
Ph.D.
Member

…….. ……
Assoc. Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Assoc. Prof. Chatchai Sornchai,
M.D.
Dean
Faculty of Medical Technology
Mahidol University

ACKNOWLEDGEMENTS

I deeply thank my advisor, Assoc. Prof. Dr. Wanida Ittarat, Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand, for her extensive support and valuable advice.

I wish to express my sincere gratitude to my co-advisor, Prof. Dr. Steven R. Meshnick, Department of Epidemiology and Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, for authorizing me to work and use facilities in Meshnick's laboratory at Mary Ellen Jones Building, University of North Carolina at Chapel Hill, and for his valuable guidance in this research.

I would like to express my deepest gratitude to my co-advisor, Dr. Chansuda Wongsrichanalai, Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, for her assistance and providing DNA samples used in this study.

I wish to express my gratefulness to Assist. Prof. Thareerat Kalambaheti, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and Dr. Pongchai Harnyuttanakorn, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, for being committee and giving valuable suggestions.

My sincere gratitude is also given to Prof. Ronald Swanstrom and Kimberly Ritola, Center for AIDS Research, University of North Carolina at Chapel Hill, Chapel Hill North Carolina, for their encouraging guidance, criticism, kindness and valuable suggestion in Heteroduplex tracking assay.

And at last, I would like to express my deepest appreciation to my family and my friends for their encouragement and understanding throughout my study.

Warunee Ngrenngarmlert

HETERODUPLEX TRACKING ASSAY FOR THE ANALYSIS OF *PLASMODIUM FALCIPARUM* GENETIC DIVERSITY: TECHNIQUE DEVELOPMENT, VALIDATION AND APPLICATIONS

WARUNEE NGRENNGARMLERT 4437275 MTMT/D

Ph.D. (MEDICAL TECHNOLOGY)

THESIS ADVISORS: WANIDA ITTARAT, Ph.D., STEVEN R. MESHNICK, Ph.D., CHANSUDA WONGSRICHANALAI, Ph.D.

ABSTRACT

Plasmodium falciparum infections are frequently composed of a mixture of different strains that exhibit significant different genotypes. Three highly polymorphic genes of the merozoite surface protein 1 and 2 (*MSP1* and *MSP2*) and the glutamine-rich protein (*GLURP*) have always been used to genotype *P. falciparum* by nested polymerase chain reaction (nested PCR). Three main disadvantages of nested PCR are an inability to detect nucleotide sequence polymorphisms, a diminished sensitivity at low parasite densities, and poor resolution at high parasitemia. In this study, Heteroduplex Tracking Assay (HTA) was developed to genotype the *MSP1* gene of *P. falciparum.* Previously determined for their allelic type by nested PCR, four DNA samples were selected for generation of four recombinant clones. The AF63, AF22, AF42 and MHP 1452 clones, which are the K1-, MAD20-, RO33-, and K1-type of the block 2 *MSP1* sequences, were used as either probes and known *MSP1* sequence controls in HTA. PCR with minor modifications was used to amplify the block 2 *MSP1* gene. The PCR products were re-annealed with the radio labeled probes and the prominent heteroduplex bands were defined as different genotypes of *P. falciparum* infection. The number of these distinct bands, which is known as the multiplicity of infection (MOI), reflects the number of genotypes in each isolate. Mean of MOI determined from 39 samples was 1.92 $\pm$ 0.13. Thirteen samples (33.3%) had one genotype, 18 samples (46.2%) had two genotypes, 6 samples (15.4%) had three genotypes, and 2 samples (5.1%) had four genotypes. In addition to identification of genetic diversity, HTA was also applied to distinguish recrudescence from re-infection in 17 paired admission and recrudescent samples. It was found that 10 out of 17 pairs (59%) showed true recrudescence since recrudescent samples contained identical heteroduplex bands or a subset of heteroduplex bands in the admission samples. Seven cases (41%) might have resulted from both recrudescence and re-infection because the appearances of new heteroduplex bands imply either new infection or the undetectable genotype at admission. This study is the first to apply HTA to identify genetic diversity of *P. falciparum* infections and also to identify recrudescence and re-infection.

KEYWORDS: HETERODUPLEX TRACKING ASSAY/ *P. FALCIPARUM*/ GENETIC DIVERSITY/ RECRUDESCENCE

110 P. ISBN 974-04-6224-3

การประยุกต์ใช้เทคนิคHeteroduplex Tracking Assay เพื่อวิเคราะห์ความหลากหลายทางพันธุกรรมของเชื้อฟัลซิปารัม มาลาเรีย (HETERODUPLEX TRACKING ASSAY FOR THE ANALYSIS OF *P. FALCIPARUM* GENETIC DIVERSITY: TECHNIQUE DEVELOPMENT, VALIDATION, AND APPLICATIONS)

วารุณี เงินงามเลิศ 4437275 MTMT/D

ปร.ด. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์: วนิดา อิฐรัตน์ ปร.ด., Steven R. Meshnick Ph.D., จันทน์สุดา วงศ์ศรีชนาลัย ปร.ด.

บทคัดย่อ

การติดเชื้อฟัลซิปารัม มาลาเรีย มักประกอบด้วยหลายสายพันธุ์ซึ่งมีความแตกต่างทางพันธุกรรมอย่างชัดเจน โดยทั่วไปความแตกต่างนี้นิยมตรวจจากยีน*MSP1 MSP2* และ *GLURP* โดยวิธีNested PCR ซึ่งมีข้อจำกัดที่สำคัญคือ1)วิธีนี้ไม่สามารถตรวจพบความหลากหลายของลำดับนิวคลีโอไทด์ 2)ความไวของวิธีนี้ลดลงถ้าตัวอย่างมีความหนาแน่นของเชื้อน้อย และ3)เมื่อตัวอย่างมีความหนาแน่นของเชื้อมากวิธีนี้จะมีความละเอียดลดลง ในการศึกษาครั้งนี้ ได้ทำการพัฒนาวิธีHTAเพื่อใช้ตรวจยีน *MSP1* ในเชื้อฟัลซิปารัม มาลาเรีย และทำการสร้างProbeที่มียีน*MSP1*ที่แตกต่างกันทั้ง 3 ชนิด วิธี HTAเริ่มจากการเพิ่มจำนวนส่วนของยีน*MSP1*จากตัวอย่างผู้ป่วยและนำผลผลิตนั้นมาคัดแยกโดยใช้Probe ที่ติดฉลากด้วยสารรังสี พบว่าHeteroduplexที่แตกต่างกัน แสดงถึงความแตกต่างของยีน *MSP1*โดยจำนวนของHeteroduplexบ่งบอกถึงจำนวนสายพันธุ์ของเชื้อฟัลซิปารัม มาลาเรีย ในแต่ละตัวอย่าง ผลการศึกษาครั้งนี้พบว่าค่าเฉลี่ยของจำนวนดังกล่าวที่ตรวจจาก 39ตัวอย่าง เท่ากับ1.92 ± 0.13 ซึ่ง13จาก39รายมีเชื้อฟัลซิปารัม มาลาเรีย1สายพันธุ์ 18รายมี2สายพันธ์ 6รายมี3สายพันธุ์ และ2รายมี4สายพันธุ์ นอกจากนี้ยังประยุกต์ใช้วิธีHTAในการวินิจฉัยแยกระหว่างการติดเชื้อซ้ำแบบRecrudescence และแบบRe-infection พบว่าผู้ป่วย 10จาก39ราย(59 %) เป็นการติดเชื้อซ้ำแบบRecrudescence เนื่องจากพบHeteroduplexที่เหมือนกันแต่ผู้ป่วย7ราย(41%)เป็นการติดเชื้อซ้ำจากทั้งRecrudescenceและRe-infectionเนื่องจากพบHeteroduplexที่แตกต่างกันซึ่งหมายถึง การติดเชื้อซ้ำของสายพันธุ์ใหม่หรือการติดเชื้อซ้ำของสายพันธุ์เดิมที่ตรวจไม่พบในระยะแรก การศึกษานี้จัดเป็นครั้งแรกที่ประยุกต์ใช้วิธีHTAเพื่อวินิจฉัยความหลากหลายทางพันธุกรรมของเชื้อฟัลซิปารัม มาลาเรีย และวิธีนี้ยังช่วยในการวินิจฉัยแยกระหว่างการติดเชื้อซ้ำแบบRecrudescenceและแบบRe-infection

110 หน้า ISBN 974-04-6224-3

CONTENTS

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATIONS

Abbreviation or Symbol	Term
cm	centimeter
^{o}C	degree Celsius
dNTP	deoxynucleoside 5’ triphosphate
dGTP	deoxyguanosine 5’ triphosphate
DNA	deoxyribonucleic acid
hr	hour
L	liter
M	molar
mm	millimeter
min	minute
mA	milli ampare
mg	milligram
ml	milliliter
mM	millimolar
mmol	millimole
mV	millivolt
$MgCl_2$	magnesium chloride
ng	nanogram
nM	nanomolar
SDS	sodium dodecyl sulfate
Sec	second
U	unit
μCi	microcuri
μg	microgram
μl	microliter
μM	micromolar

CHAPTER 1

INTRODUCTION

Plasmodium falciparum, the most virulent of the four parasite species causing human malaria, exhibits significant genetic diversity (1). The ability to generate genetic diversity is widely assumed to be a mechanism of immune evasion for the parasites to escape host immune responses (2), (3), (4). Three highly polymorphic genes of *P. falciparum* that are the merozoite surface protein-1 (*MSP1*) and the merozoite surface protein-2 (*MSP2*) genes, and the glutamine rich protein (*GLURP*) gene have been used as genetic markers to identify the genotype of *P. falciparum* (5-8).

Polymerase chain reaction (PCR) is valuable technique for the analysis of genetic diversity because this technique can provide a large amount of specific DNA fragments (9). Molecular genotyping methods based on PCR including nested PCR (5), restriction fragment length polymorphism (RFLP) (10), single strand conformation polymorphism (SSCP) (11), and direct DNA sequencing are used most often to analyze genetic diversity although some limitations still occur (12, 13). For example, by nested PCR to detect size polymorphisms (14), different DNA sequences with the same size will co-migrate as one band in a gel so it is misinterpreted as one sequence.

Heteroduplex tracking assay (HTA), which involves the amplication of variable region from the polymorphic genes, followed by the analysis of different genotypes in native polyacrylamide gel electrophoresis, has been used to analyze genetic variations in various pathogenic microorganisms such as in Human Immunodeficiency Virus (HIV) (15), (16), (17), Epstein-barr virus (EBV) (18), and *Mycrobacterium tuberculosis* (19). This technique relies on both size and sequence polymorphisms, thus the potential of HTA to detect sequence differences in heteroduplexes with a

labeled DNA probe may be useful not only for determination of DNA polymorphisms in *P. falciparum* population but also to distinguish recrudescence from re-infection after treatment failure.

The present study was designed to develope PCR-based HTA for genotyping *P. falciarum* infections, and also to evaluate the technique validation and applications, especially the estimation of multiplicity of infection and the assessment of recrudescence or reinfection after antimalarial drug treatment.

CHAPTER 2

OBJECTIVES

The aims of this research are as follows:

1. To develop and validate the heteroduplex tracking assay for the analysis of *P. falciparum* genetic diversity.
2. To evaluate the applicability and reproducibility of this novel technique for screening of field specimens by
    2.1 investigation of the extent of *P. falciparum* genetic diversity
    2.2 identification of recrudescence from re-infection after antimalarial drug treatment
3. To compare nested PCR and HTA for estimation of the multitiplicity of *P. falciparum* infection.

CHAPTER 3

REVIEW OF THE LITERATURES

1. Human Malaria

Malaria is one of the most important infectious diseases in the world, particulary in many tropical countries, causing 300–500 million infections worldwide and 1-2 million deaths annually (20). Malaria threatens approximately 41% of the world's population (21), most of which are in the world's poorest countries, especially in rural areas with poor access to health services or have limited internal resources to combat the disease. Malaria is a curable disease but life-threatening severe malaria may occur if prompt diagnosis and adequate treatment are not given, or if the parasites are resistant to treatment.

Malaria parasites belong to the genus *Plasmodium*, the intraerythrocytic protozoa. This genus is classified in the phylum Apicomplexa, family Plasmodidae, class Sporozoea. It is consisting of almost 5,000 species which are different in host specificity and disease manifestation due to wide variations in genome composition and codon usage (22). The causative agents of human malaria are four species of *Plasmodium* protozoa; *P. falciparum, P. vivax, P. ovale* and *P. malariae*. The geographical distribution of malaria mainly follows the distribution of Anopheles, disease vector. Anopheles can survive and reproduce well in tropical climates (23).

Vivax malaria is the predominant malarial parasite in most part of the world (23). It is found almost everywhere of malaria endemic area. *P. vivax* is the only one *Plasmodium* that has a range extending into the temperate regions such as Eastern Europe, the Middle East, and the lower Korean peninsula. The distribution of ovale malaria is more limited; it seems to be rather widely distributed in tropical Africa, almost entirely of the Western Africa coast (23). It has also been found in South America and Asia. *P. malariae* occurs primarily in subtropical and temperate areas,

particularly in Africa. It has a broad distribution, but is less frequently found than other species of malaria (23). Finally, falciparum malaria is widespread. It is prevalent in most areas where malaria is endemic (23).

Of the four species of malaria, *P. falciparum* is the most virulent and is responsible for the high mortality rate, about 95% of malaria deaths worldwide. The virulent of this parasite is thought to result from many factors including intrinsic genetic and biological components (24). For example, in a patient infected with falciparum malaria the parasite burden can reach as high as 10^{11} organisms. Red blood cells, more over, infected with falciparum malaria can adhere to the endothelium of small blood vessels of the brain, and cause cerebral malaria; the most severe form of malaria infection. Additionally, drug resistant falciparum malaria increases the severity and widespread of malaria infection.

1.1 The Plasmodium life cycle

The *Plasmodium* protozoan requires two hosts, human and female mosquito genus Anopheles as shown in Figure 1 (23). The *Plasmodium* life cycle in human begins with the bite of an infected female Anopheles. The sporozoites in the mosquito's salivary gland are injected into the blood circulation of the human host. The introduced sporozoites then invade liver cells, beginning the exo-erythrocytic part of the cycle. Subsequent development of *P. falciparum* and *P. malariae* differs from that seen in *P. vivax* and *P. ovale*. In all species, an asexual multiplication takes place within the liver cells, but in *P. vivax* and *P. ovale* a proportion of the infecting sporozoites are believed to enter a resting stage before undergoing asexual multiplication, while others undergo this multiplication without delay. The resting stage of the parasite in the liver cells is known as a hypnozoite. After a period of weeks or months, reactivation of the hypnozoite initiates asexual division. Asexual multiplication in the liver cells results in the production of thousands of tiny merozoites in each infected cell. Rupture of these cells releases merozoites into the blood circulation. In the blood stream, an asexual cycle, known as schizogony, takes place within the red cells. The merozoites invade the erythrocytes to begin the erythrocytic cycle and develop into ring stage, trophozoites, and schizont;

respectively. Merozoites bud from mature schizonts, and eventually the infected erythrocyte ruptures, releasing merozoites into the bloodstream. The merozoites invade the new erythrocytes and new erythrocytic cycle start again. By unknown mechanism, merozoites, instead of continuing the asexual replication cycle, enter a sexual replication cycle and differentiate into male and female gametocytes.

The infection in a mosquito host (the sporogonic cycle) occurs when a female Anopheles takes up the gametocytes during a blood meal. Inside the mosquito, the gametocytes are transformed into gametes and fertilization takes place. The zygote develops into an ookinete, which transverses the midgut epithelium of the mosquito. At this stage, the ookinete develops into an oocyst. The oocyst undergoes asexual replication (sporogony) to produce thousands of sporozoites. After sporozoite maturation the oocyst bursts and the sporozoites release into the body cavity of the mosquito. The motile sporozoites enter the mosquito's salivary gland and travel to the lumen of the gland. If the infected anopheline mosquito takes a blood meal from a new human host, these sporozoites will continue the *Plasmodium* life cycle in human.

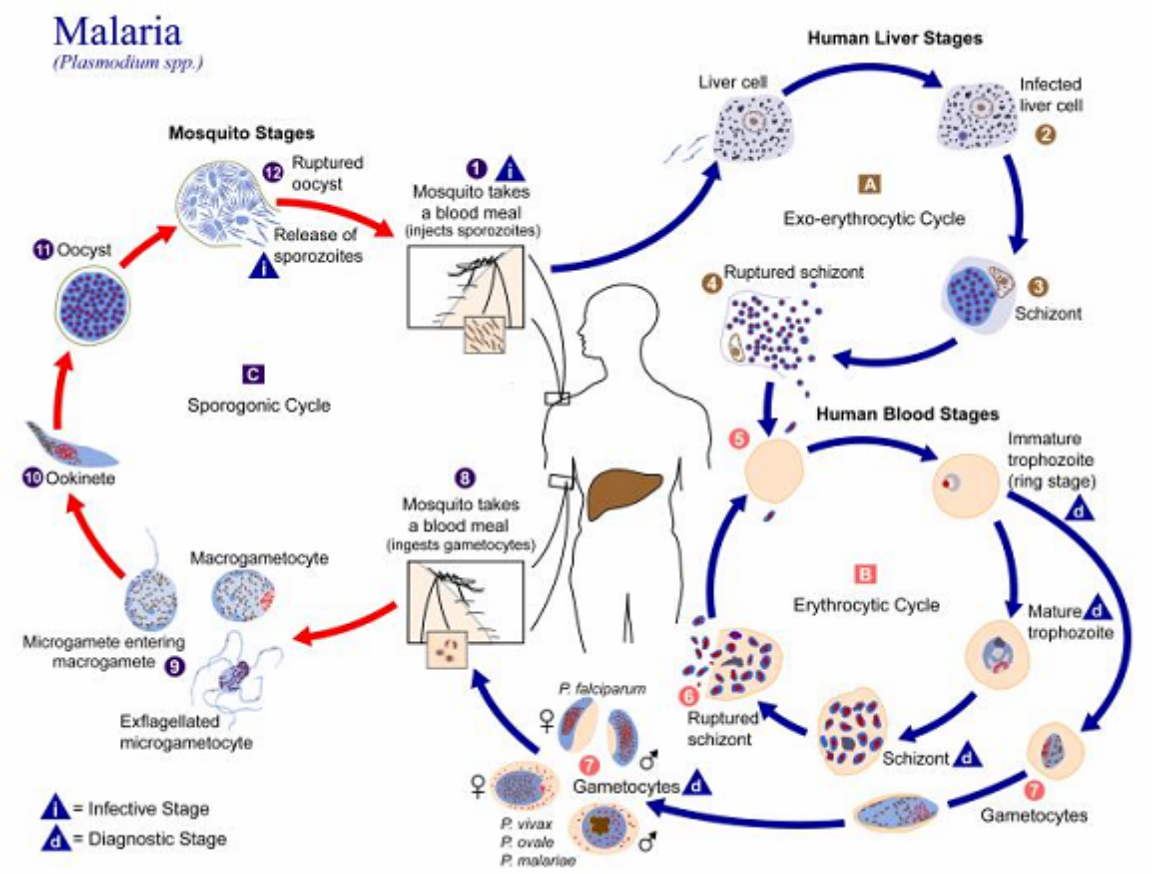


Figure 1. Life cycle of the malaria parasite. A, Exo-erythrocytic cycle. B, Erythrocytic cycle. C, Sporogonic cycle.

(http://www.cdc.gov/malaria/ biology/life_cycle.htm)

Source: CDC/Laboratory for Identification of Parasites of Public Health Concern. By Alexander J. da Silva, PhD and Melanie Moser.

1.2 Epidemiology of malaria infection

Malaria is particularly prevalent in tropical regions and occurs in over 100 countries in Africa, Central and South America, Indian subcontinent, and South-east Asia (Figure 2). All of the malaria parasites are transmitted by female Anopheline mosquitoes. Transmission of malaria is affected by climate and geography. When prevalence is relatively steady from year to year and season to season, transmission is year round and malaria is said to be stable, but when there are wide differences in incidence from year to year or season to season, transmission is seasonal or sporadic and malaria is unstable.

In areas of highly endemic or stable malaria including much of sub-Saharan Africa, tropical areas of Asia, and South America, the parasite and mosquito development can occur year-round. Thus, transmission also occurs year-round at moderate to very high levels, and the incidence is fairly constant. In these areas the vast majority of deaths occur among young children (20). Due to constant exposure and infection over their lifetimes, adults have a constant low-level of immunity to malaria infection which can prevent serious diseases. However, pregnant women suffer higher morbidity and mortality rates than the general adult population. Due to maternal antibodies, infants generally have a high level immunity.

In areas of seasonal or unstable transmission, such as parts of Southeast Asia, South America, and the highlands of Africa, the climate does not necessarily allow rapid or year-round development of the parasite and mosquito. Children and adults in these areas have similar malaria morbidity and mortality, as there is either no immunity or very low level of immunity. These areas have potential for malaria epidemics, and incidence may vary widely year to year.

In Southeast Asia, there are approximately 27 million cases of malaria yearly, including 30,000 deaths (25). Over the last decade there has been no detectable decline in malaria rates in the countries of this region (25). Epidemics often occur because of cross-border migration patterns, bringing non-immune workers into areas that carry malaria risk.

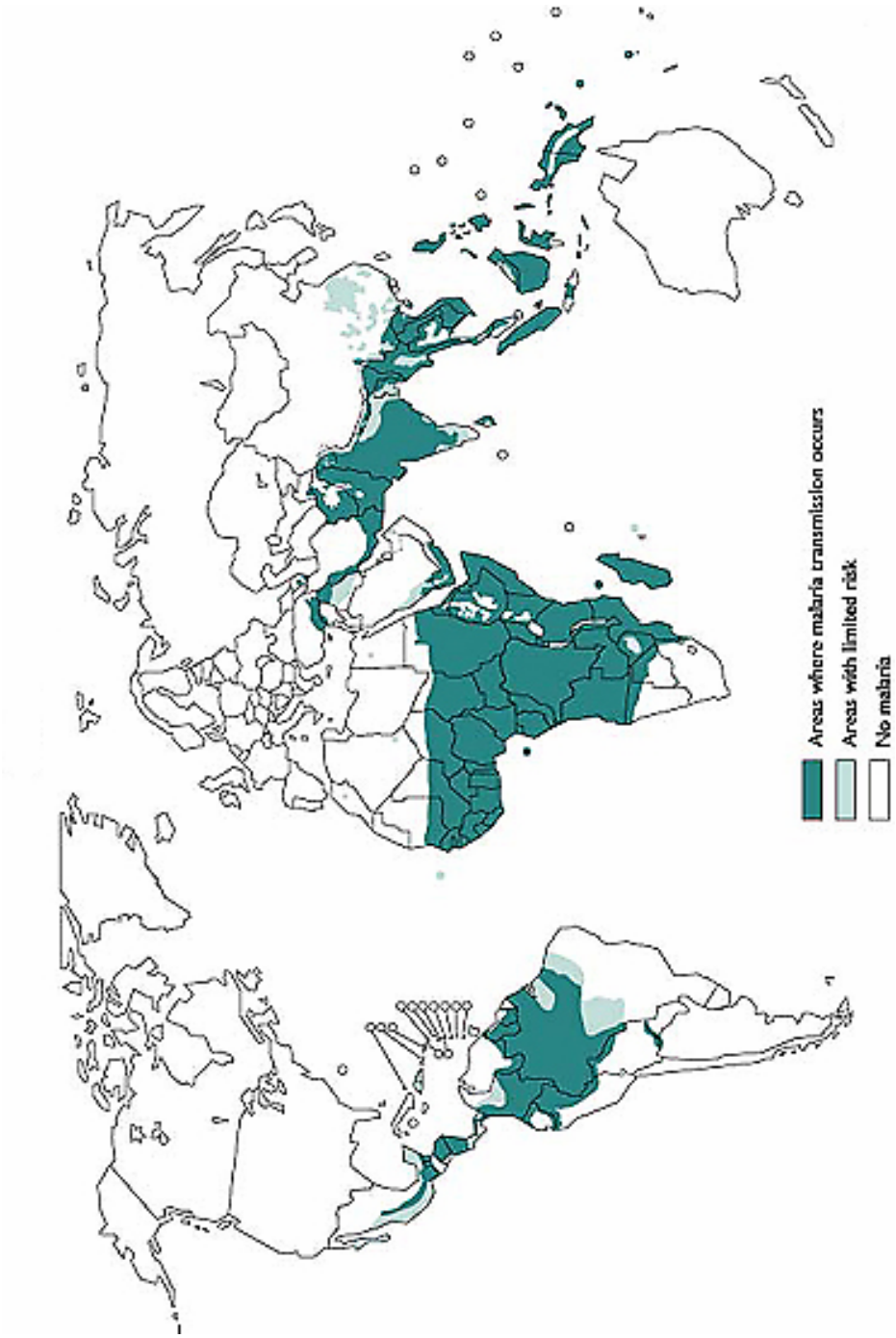


Figure 2. World map of malaria-endemic geographies
(http://www.who.int/ith/fr/ chapter05_m08_malaria.html)
Source: World Health Organization. *International Travel and Health*. Chapter 5: "Infectious Diseases".

1.3 Symptoms of malaria infection

Symptoms of malaria typically consist of definite paroxysms of chill, fever, and sweating, which occur at regular intervals depending on the time of erythrocytic burst. However the chills and fever are not as significant as the periodicity. Some of the features of the four different malarias are compared in Table 1 (23).

Table 1. Clinical Comparison of the types of Malaria (23)

Feature	*P. vivax*	*P. ovale*	*P. malariae*	*P. falciparum*
Incubation period	10-17 days		18-40 days	8-11 days
	Sometimes prolonged for months to years			
Prodromal symptoms	May be influenza-like in all four types			
Severity	++	+	++	+
Initial fever pattern	Irregular to quotidian		Usually regular every 72 hours	Continuous, remittent, or quotidian
Periodicity	44-48 hours	48-50 hours	72 hours	36-48 hours
Initial paroxysym Usual severity Average duration	Modurate to severe 10 hours	Mild 10 hours	Modurate to severe 11 hours	Severe 16-36 hours
Duration of untreated primary attack	3-8+ weeks	2-3 weeks	3-24 weeks	2-3 weeks
Duration of untreated infection	5-8 years[a]	12-20 months[a]	20-50+ years[b]	6-17 months 20-50+ years
Anemia	++	+	++	++++
CNS involvement	±	±	±	++++
Nephrotic syndrome	±	-	+++	+

[a]Including relapses, [b]Including recrudescences

1.4 Malaria treatment

Currently, the three main catagories of antimalarial drugs (26) are:

(1) Quinoline-containing drugs include type-1 drugs (the 4-aminoquinolines e.g. chloroquine) and type-2 drugs (the aryl-amino alcohols e.g. quinine, mefloquine, halofantrine), which act on hemoglobin degradation and parasite food vacuoles. The two groups differ in that type-1 drugs are weak bases, diprotonated and hydrophilic at neutral pH, whereas type-2 drugs are weaker bases and lipid soluble at neutral pH. In addition, the two groups appear to interact differently with their putative target (27) and show an inverse relationship with respect to parasite sensitivities.

(2) Folate antagonists inhibit enzymes of the protozoan folic acid biosynthesis pathway. The antifolate drugs are dihydrofolate reductase (DHFR) inhibitors (e.g. pyrimetamine, cycloquanil, and chlorcycloquanil), and dihydropteroate synthase (DHPS) inhibitors (e.g. sulfonamides and sulfones), mimic *p*-aminobenzoic acid (PABA). The effect of the DHFR inhibitors on malaria parasites is to reduce the biosynthesis of folate resulting in decreased synthesis of pyrimidines and blockage of DNA replication. Also, decreased conversion of glycine to serine and decreased methionine synthesis can be found (24). The DHPS inhibitors compete for the active site of DHPS that is a bifunctional enzyme with 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase, the enzyme that mediates the previous step in the biosynthetic (24).

(3) Endoperoxides of artemisinin-type compounds are either the natural extract artemisinin or the semi-synthetic derivatives (dihydroartemisinin, artesunate, artemether, arteether). These compounds contain stable endoperoxide bridges. Artemisininis believed to act via a two-step mechanism (28). It is first activated by intraparasitic heme-iron which catalyses the cleavage of this endoperoxide. A resulting free radical intermediate may then kill the parasite by alkylating and poisoning one or more essential malaria proteins. No clinically relevant artemisinin-resistant human malaria has yet been reported.

2. Emergence of Malaria Drug Resistance

Although malaria is a fatal disease, illness and death from malaria are largely preventable. Management of malaria is based on three principles: chemotherapy, transmission blocking and vaccination. In areas where malaria transmission is intense, chemotherapy is the most practical approach for control. Transmission blockers, which include insecticides and bed nets, can be effective, particularly where transmission is less intense (29). Considerable effort has been focused on vaccine development, but no effective vaccine has been deployed yet (30).

Resistance is the ability of parasites to survive doses of drugs that would normally kill parasites of the same species and stage. It is inherited and selected for because the survivors of drug treatments pass genes of resistance on to their offsprings. The global malaria crises include emergence of widespread resistance to available, affordable, and previously highly effective antimalarial drugs (31). Inadequate health systems and resources are also carrying widespread malaria resistance. Antimalarial drug resistance is now widespread throughout most malaria endemic areas. The worldwide distribution of drug-resistant malaria is shown in Figure 3. The problem of drug resistance is a major challenge for replacement with new effective drugs; however, resistance to antimalarials is emerging faster than new drugs are being developed. In particular, *P. falciparum*, the species that causes the most severe form of the disease, is resistant to quinolines and antifolates. The consequences of resistance are an increase in mortality and morbidity. It has been estimated that mortality to falciparum malaria increases up to fivefold in areas where resistance to the antimalarial chloroquine is established (32). The majority of multidrug resistant to *P. falciparum* cases were reported from Thai-Myanmar and Thai-Cambodian border.

Two aims for monitoring of resistance are to detect resistance after it has emerges and to detect resistance when it is at low frequency (33). In order to maintain drug susceptibility, very sensitive surveillance methods are needed in the future of malaria control, particularly for guiding therapeutic decisions. Effective drugs can be identified and retained for use while other drugs that are no longer effective can be removed.

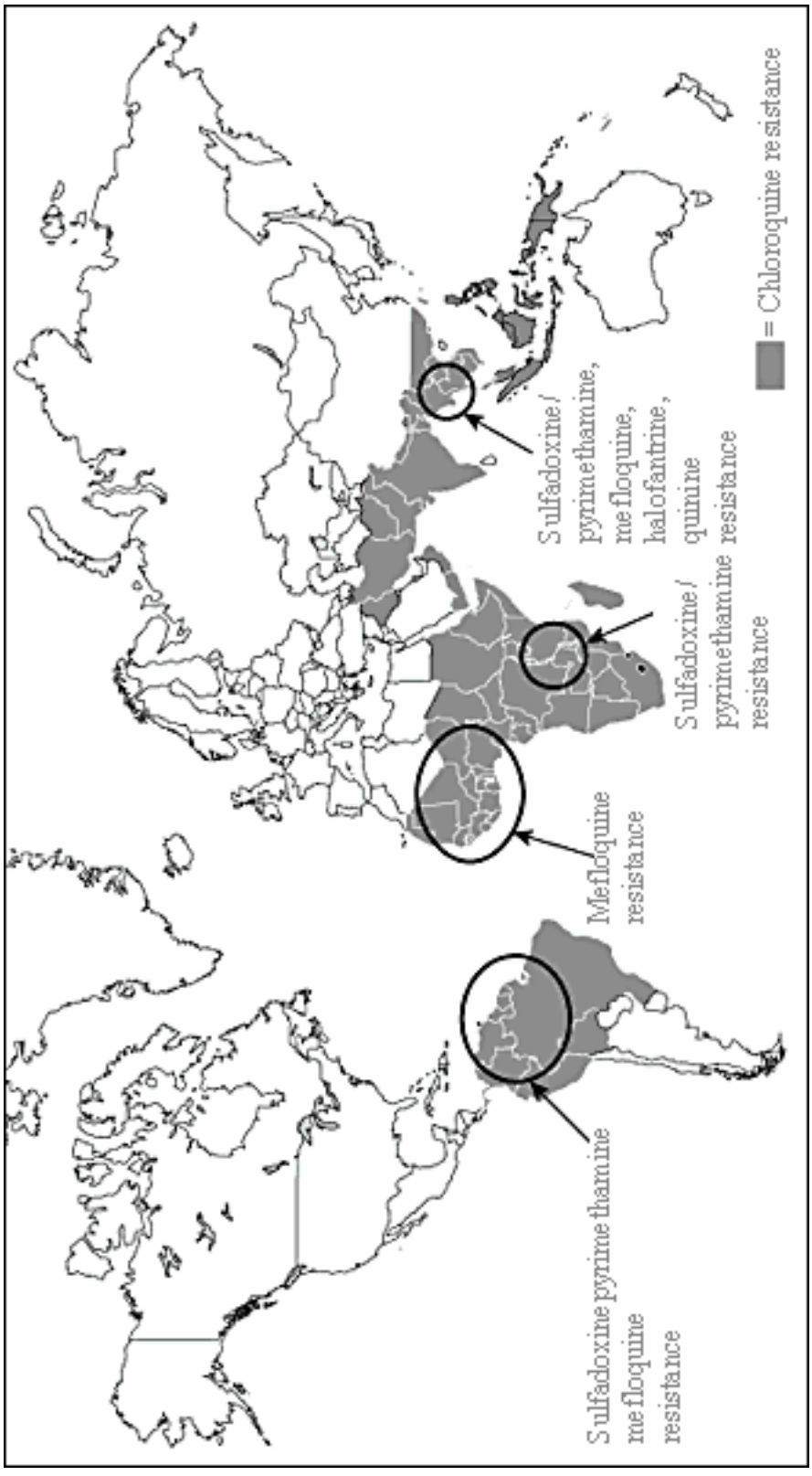


Figure 3. Distribution of drug resistant malaria
(http://www.cdc.gov/ncidod/emergplan/box23.htm)

2.1 Assessment of antimalarial susceptibility

Classical methods for drug efficacy measurement are the clinical response of patients to a standard drug treatment (*in vivo*), and the sensitivity of parasites to drugs (*in vitro*). An *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. The results of *in vivo* tests do not necessarily reflect the true level of pure antimalarial drug resistance. However, this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions.

In vivo response to drugs was originally defined by the World Health Organization (WHO) in terms of parasite clearance (sensitivity [S] and three degrees of resistance [RI, RII, RIII]. This classification remains valid for areas with low or no malaria transmission, but it is difficult to apply in intensive transmission areas, where new infections may be mistaken for recrudescences. Therefore, WHO introduced in 1996 a modified protocol based on clinical outcome (adequate clinical response, early treatment failure and late treatment failure) targeted at a practical assessment of therapeutic responses in areas with intense transmission, where parasitaemia in the absence of clinical signs and symptoms is common (31). Classifications of *in vivo* antimalarial drug sensitivity test outcomes according to the original WHO protocol and the modification were shown in Table 2.

Table 2. Classifications of *in vivo* antimalarial drug sensitivity test outcomes according to the original WHO protocol (34) and the modification (1996) for areas with substantial malaria transmission

Original classification	Definition
S (Sensitive)	Reduction to <25% of initial parasitemia on Day 2 with negative malaria smears from Day 7 till the end of the follow-up period (28 days, or longer for drugs with a long half-life, e.g. mefloquine).
RI response	Initial clearance of parasitemia, a negative smear on Day 7, followed by recrudescence 8 or more days after treatment.
RII response	Initial clearance or substantial reduction of parasitemia (<25% of the initial count on Day 2) but with persistence or recrudescence of parasitemia during Days 4-7.
RIII response	Lack of significant reduction of parasitemia.

Modified classification	Definition
Early treatment failure (ETF)	Aggravation or persistence of clinical symptoms in the presence of parasitaemia during the first 3 days of follow-up.
Late treatment failure (LTF)	Reappearance of symptoms in the presence of parasitemia during Days 4 –14 of follow-up
Adequate clinical response (ACR)	Absence of parasitaemia on day 14 irrespective of fever, or absence of clinical symptoms irrespective of parasitemia, in patients not meeting ETF or LTF criteria.

In the most frequently used procedure of the *in vitro* tests, the micro-technique, parasites obtained from a blood sample are exposed in microplates to precisely known quantities of drug and observed for inhibition of maturation into schizonts (35). This test more accurately reflects “pure” antimalarial drug resistance. Multiple tests can be performed on isolates; several drugs can be assessed simultaneously.

However, these methods both *in vivo* and *in vitro* tests are time consuming require highly trained personnel and are difficult to standardize (20). Recently, the use of molecular markers has been proposed as an additional tool for early detection of drug resistance (10),(13),(5),(36),(37),(7). Molecular techniques are mainly based on polymerase chain reaction (PCR). PCR is useful because the reaction conditions can be designed to be highly specific, easily automated and capable of amplifying small amounts of sample. These molecular data obtained from this technique are potentially powerful public health tools for surveillance of drug resistance. However, it is relatively complex how to relate the molecular data on parasite genotypes to clinical outcomes and this requires detailed knowledge of resistance mechanisms at the molecular level. Genetic markers are not available for all drug resistant phenotype and it is important to find the gene(s) that involve drug susceptibility when there are the mutations.

2.2 Determinants of antimalarial resistance

Factors contributing to the development and spread of resistant malaria parasites include the interaction of drug treatment patterns, drug characteristics, human host factors, parasite characteristics, vector and environmental factors (31). In nature, resistant malaria parasites appear to occur through gene mutations that confer reduced sensitivity to a given drug or group of drugs. Single or multiple point mutations in the *Plasmodium* genome may confer resistance in the face of chemotherapy.

Characteristics of the drug are important determinants of resistance. First, drugs with a long half-life may exert substantial residual selection on new infections if the drug persists at subtherapeutic concentrations in the plasma, especially in areas with intense malaria transmission. Second, the maintenance of adequate drug concentrations over a long time is important for clearing the entire population of

parasites within a given individual. Subtherapeutic drug concentrations eliminate the most susceptible parasites and leave the resistant parasites to recover and reproduce. As a result, the therapeutic dose may increase the maximum tolerated, and the manifestation of drug resistance will emerge. Third, widespread use of drugs at high intensity increases drug pressure and selection for resistant parasite populations.

The efficacy of chemotherapy could be increased by host immunity. Partially drug resistant parasites might be cure in a semi-immune pateint. Finally, vector and environmental factors may influence the proliferation of resistant parasites. For example, chloroquine resistant parasites may be able to reproduce better in certain anopheline mosquitoes than in non-resistant strains (38).

3. Genetic Diversity of Malaria Parasites

P. falciparum population in endemic area is extremely diverse. The parasite genotypes are unique for each strain. Several mechanisms including genetic recombination, gene conversion, and duplication generate new strains in *P. falciparum* populations (39, 40). Different parasite strains with different genotypes result in different phenotypes. Analysis of the genetic diversity could indicate the structure of parasite populations. The number of genotypes of *P. falciparum* per infected person is defined as the multiplicity of infection (MOI). The MOI that play an important role for the monitoring and control of malaria disease (41) has been used to study the development of protective immunity and to understand the dynamics of malaria disease (42),(41).

P. falciparum displays extensive genetic variation in many antigens. Marked diversity has been found in genes encoding the merozoite surface protein 1 (MSP1), the merozoite surface protein-2 (MSP2), and the glutamine rich protein (GLURP). These highly polymorphic genes have been frequently used as genetic markers for genotyping of *P. falciparum* (5-7).

3.1 The merozoite surface protein-1 (*MSP1*) gene

The MSP1 is the primary surface antigen of *P. falciparum* merozoite. This glycoprotein of about 200 kDa appears to play an important role in the receptor-ligand interactions at the initial attachment of the merozoite to erythrocyte. A large number of *MSP1* alleles, a gene coding for a major malaria vaccine candidate, have been sequenced. Size and allelic polymorphisms in *MSP1* gene are generated by ether intragenic recombination (43) or gene conversion (40). The various *MSP1* alleles can all be grouped into two major allelic families (43), (40). The gene has been divided into 17 blocks that are conserved, semi-conserved or variable (Figure 4) (43).

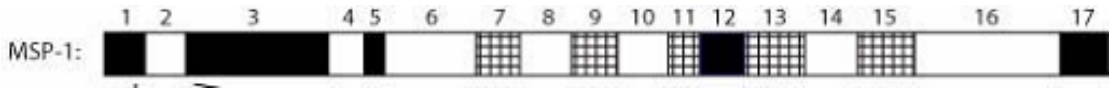


Figure 4. Schematic diagram of the *MSP1* gene illustrating the blocks 1-17 (43). Conserved, semiconserved, and variable blocks of the gene are shown as closed, hatched, and open boxes, respectively

The block 2 region of *MSP1* is particularly polymorphic and occurs as one of three distinct allelic families: K1, MAD20, and RO33. The two major families; K1 and MAD20 have been characterized by different repeated sequence units of the dimorphic variable block 2 of *MSP1* whereas the RO33; a part of the MAD20 family, has a unique sequence without tripeptide repeats. The most polymorphic block 2 of *MSP1* is widely used as a genetic marker for analysis of genetic diversity (44),(45),(46). Analysis of genetic diversity in the block 2 at the 5' end of the *MSP1* gene revealed an association between *MSP1* specific antibodies and malaria protection (3, 4, 47). But the actual consequence of the *MSP1* polymorphism on the human immune responses is still unclear.

3.2 The merozoite surface protein 2 (*MSP2*) gene

A second family of merozoite surface antigens is a 43 - 50 kDa glycoprotein and has been localized on the plasma membrane of intracellular and free merozoites. This molecule anchored in the merozoite membrane by a glycosylphosphatidylinositol moiety. Immune responses to this protein (MSP2) can inhibit parasite multiplication (48), (49).

The *MSP2* of *P. falciparum* displays extreme size and allelic polymorphism. Sequence polymorphism of *MSP2* has been characterized at the DNA level in laboratory-established isolates (48), (49) and field isolates (50),(51), (52). These data reveal highly conserved 5' and 3' sequence that flank a central variable region. This central region is composed of repeats flanked by nonrepetitive sequences. The nonrepetitive sequences can be divided into two allele families of *MSP2,* which are defined as FC27 (53) and 3D7 (54). The central repeats are more variable and define the individual alleles of *MSP2*.

The *MSP2* is organized into five blocks of sequence. The N-terminal and C-terminal translated sequence, block 1 and block 5; respectively. Figure 5 shows common *MSP2* of all isolates studied to date. The block 3 of *MSP2* is a polymorphic central region flanked by two nonrepetitive regions (block 2 and block 4).

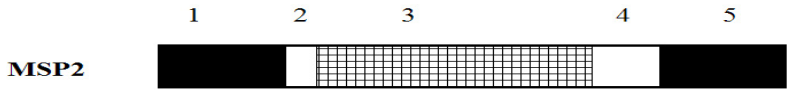


Figure 5. Schematic diagram of the *MSP2* gene illustrating the blocks 1-5. Conserved, family specific non-repetitive, and allele-specific repetitive regions are shown as closed, open, and hatched boxes, respectively.

3.3 The glutamine rich protein (*GLURP*) gene

The Glutamine Rich Protein is a 220-kDa exoantigen found in the parasitophorous vacuole. The single full-length *GLURP* sequence available to date (Laboratory strain F32) shows two amino acid repeat regions (R1 and R2) with degenerate repeat motifs found in both (Figure 6). Diversity in *GLURP* has been indicated by different sized PCR products from the R2 region of various laboratory-adapted (55) and field isolates (56).

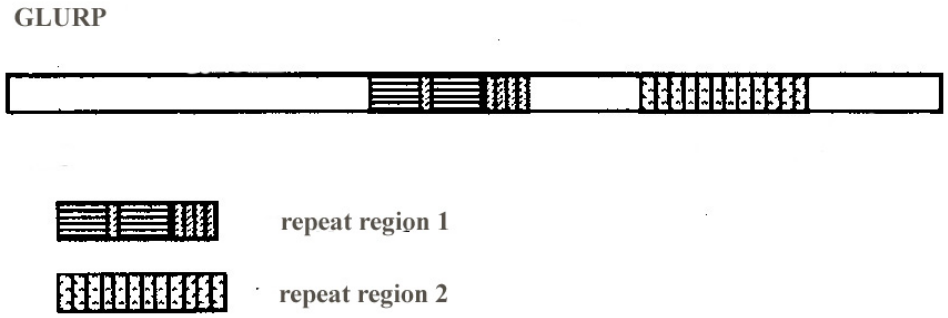


Figure 6. Schematic diagram of the *GLURP* gene highlighting patterns of diversity (6).

4. Genotyping of *Plasmodium falciparum*

The most frequently used techniques with interesting potential for identifying genetic variations of *P. falciparum* are based on the PCR (9). PCR is a major breakthrough in molecular research because its sensitivity allows the analysis of genes from small amount of parasite materials. The process of genetic analysis is formally divided into two steps: 1) the identification of genetic variations according to the physical or enzymatic principle used to reflect the change in the DNA primary structure and 2) the visualization of the detectable products, which involves ways of making this change visible.

4.1 Nested PCR

Nested PCR uses two sets of amplification primers (57, 58). The target DNA sequence of one set of primers (termed "inner" primers) is located within the target sequence of the second set of primers (termed "outer" primers). In practice, a standard PCR reaction is first run with the patient sample using the "outer primers". Then a second PCR reaction is run with the "inner primers" using the product of the first reaction as the amplification target. This procedure increases the sensitivity of the assay by reamplifying the product of the first reaction in a second reaction. The specificity of the assay is increased because the inner primers amplify only if the first PCR reaction yielded a specific product. However, nested PCR could not identify different DNA sequences with the same size.

4.2 Single Strand Conformation Polymorphism (SSCP)

The SSCP analysis involves the denaturing of PCR-amplified fragments with the subsequent formation of sequence-specific secondary and tertiary structures of the single strands during non-denaturing gel electrophoresis. The electrophoretic mobility of a single-stranded DNA molecule is dependent on its structure (conformation) and size. The conformations as a result of base pairing between nucleotides within each strand are dependent on the length of the strand, and the location and number of regions of base pairing. Specific DNA regions are labeled, denatured, and then analyzed in non-denaturing polyacrylamide gels. In non-denaturing conditions, the

single-stranded molecules with different bases can be separated because of changes in their mobility as a consequence of differing conformations. Due to its simplicity and relatively high sensitivity, this method is extensively used in malaria research (11). However, SSCP is most effective only for DNA fragments with size less than 150 bps (12).

4.3 Restriction Fragment Length Polymorphism (RFLP)

RFLP is the production of DNA fragments of different lengths by a given restriction enzyme. Due to inherited differences in a restriction site, a particular sequence of nucleotide bases in DNA allows a specific restriction enzyme to cleave the DNA molecule at that site. RFLP involves the PCR amplification of an interested fragment and the subsequent digestion with a restriction enzyme (10). The innocuous single nucleotide changes in a restriction site occur so that a restriction enzyme can no longer cut DNA at that site, thus, producing a longer DNA fragment. Because of its simplicity and specificity, the method has been widely used, although it confers certain limitations. Only a subset of polymorphisms that happen to reside in an endonuclease restriction site can be studied with this method.

4.4 DNA Sequencing

In the late 1970s, methods for determining the base sequences in DNA were developed (59, 60). These methods are relatively simple and rapid, so that even long sections of DNA can be sequenced. Genetic information is thus directly accessible and does not require prior knowledge of the gene product. Two principal methods of DNA sequencing are available: Maxam-Gilbert chemical degradation (60) and Sanger dideoxy termination (59) methods. The principle of DNA Sequencing by chemical method is based on base-specific cleavage of DNA. The DNA segment to be sequenced is analyzed for each of the four bases in reaction mixtures, where it is cleaved into fragments by chemical modification. The sizes of the fragment depend on the positions of the respective nucleotide bases. By exact comparison of all the fragments formed in the four reaction mixtures, the positions of the individual nucleotide bases can be inferred and their sequence determined. The principle of Sanger DNA sequencing method based on the interrupting the synthesis of the 5’ to 3’

strand at specific bases of the DNA segment to be sequenced. As a consequence, DNA fragments of different sizes are formed. The sizes of the fragments reflect the positions of the respective bases. In this way, the base sequence of a given DNA segment can be determined.

DNA sequencing has been claimed to be the “gold standard” used to establish the identity of both known and unknown sequence specific nucleotide variations. However, these methods have limited sensitivity and do not easily analyze multiple different DNA sequences in a clinical sample.

4.5 Heteroduplex analysis

Heteroduplex analysis is based on the principle that a DNA duplex with mismatches and/or size differences (defined as heteroduplex) will migrate through a native polyacrylamide gel electrophoresis (PAGE), more slowly than a DNA duplex with completely complementary strands (defined as homoduplex) Heteroduplex is a malformations (Figure 7A). The principle of the universal heteroduplex generator is that mismatches in heteroduplex do not result in significant mobility changes if they do not cluster or contain less than 1 to 2 % of mismatches (Figure 7B). Heteroduplex Mobility Assay (HMA) and Heteroduplex Tracking Assay (HTA), which are the modification of heteroduplex analysis, can be used as more rapid and more sensitive methods for the detection of genotypic polymorphisms within a population.

Heteroduplex mobility assay (HMA) is a technique that has been used for either rapid characterization of heterogeneity between two segments of DNA. Usually PCR amplicons (61, 62) or rapid molecular characterization of a wide range of microorganisms is identified by HMA (62). The technique comprises mixing together a reference DNA of known sequence with a test sample, heating to denature and cool slowly. During re-annealing, single strands reform the two original homoduplexes plus heteroduplexes composed of one strand from each amplicon present in the mixture. Homoduplex and heteroduplex bands can be characterised by polyacrylamide gel electrophoresis: heteroduplexes with mismatched bases migrate more slowly than the homoduplex.

Heteroduplex tracking assay (HTA), a screening method able to determine whether multiple different DNA sequences exist in a sample, is used to detect

uncharacterized sequence variations on double-stranded DNA. Only heteroduplexes that contain a labeled strand (probe) are visualized, in contrast to HMA, in which all heteroduplexs are visualized by staining with ethidium bromide. If the sequence mixtures contain more than two different sequences, HMA will contain complex banding patterns that are difficult to interpret the results.

HTA has been widely used in the field of viral genetic, particularly HIV type 1, for the study of genetic diversity (15, 17). The potential of HTA to detect genetic diversity of *P. falciparum* was developed in the present study. The technique was also applied to identify recrudescence from re-infection after treatment failure.

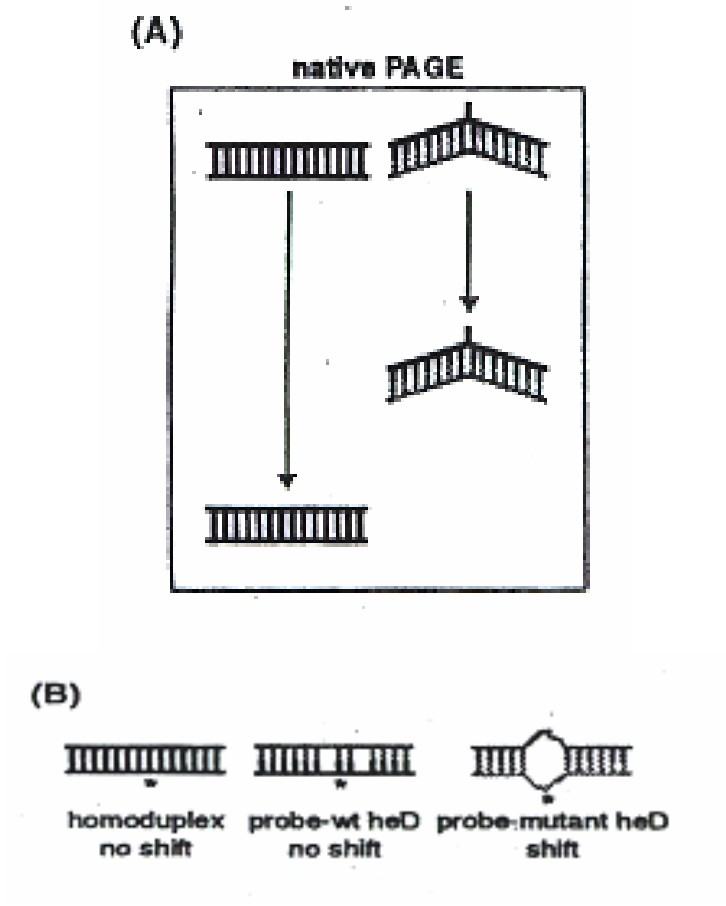


Figure 7. (A) DNA heteroduplexes migrate slower in native PAGE than a DNA homoduplex of equal size. (B) Principle of the universal heteroduplex generator (UHG). Mismatches in DNA heteroduplexes do not result in significant mobility changes if they do not cluster.

Source: Heteroduplex tracking assay – General protocol and notes on designing single mismatch sensitive probes. By Wolfgang Resch, August 16, 2001.

CHAPTER 4

MATERIALS AND METHODS

1. Background

This international study was conducted in collaboration with investigators from Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand; from Departments of Epidemiology, Microbiology and Immunology, University of North Carolina (UNC) at Chapel Hill, Chapel Hill, North Carolina, USA; and from Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand. Dr. Chansuda Wongsrichanalai of AFRIMS conducted the majority of the fieldwork, did the tracking and storing of biological samples, and performed DNA extractions. Prof. Steve R Meshnick of UNC supported for nested PCR genotyping and heteroduplex tracking assay (HTA). Nested PCR was previously performed by Deborah D. Kamwendo, Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan. Assoc. Prof. Wanida Ittarat and Warunee Ngrenngarmlert of Mahidol University were responsible for developing HTA for analysis of *P. falciparum* genetic diversity. The study setting was summarized in Figure 8

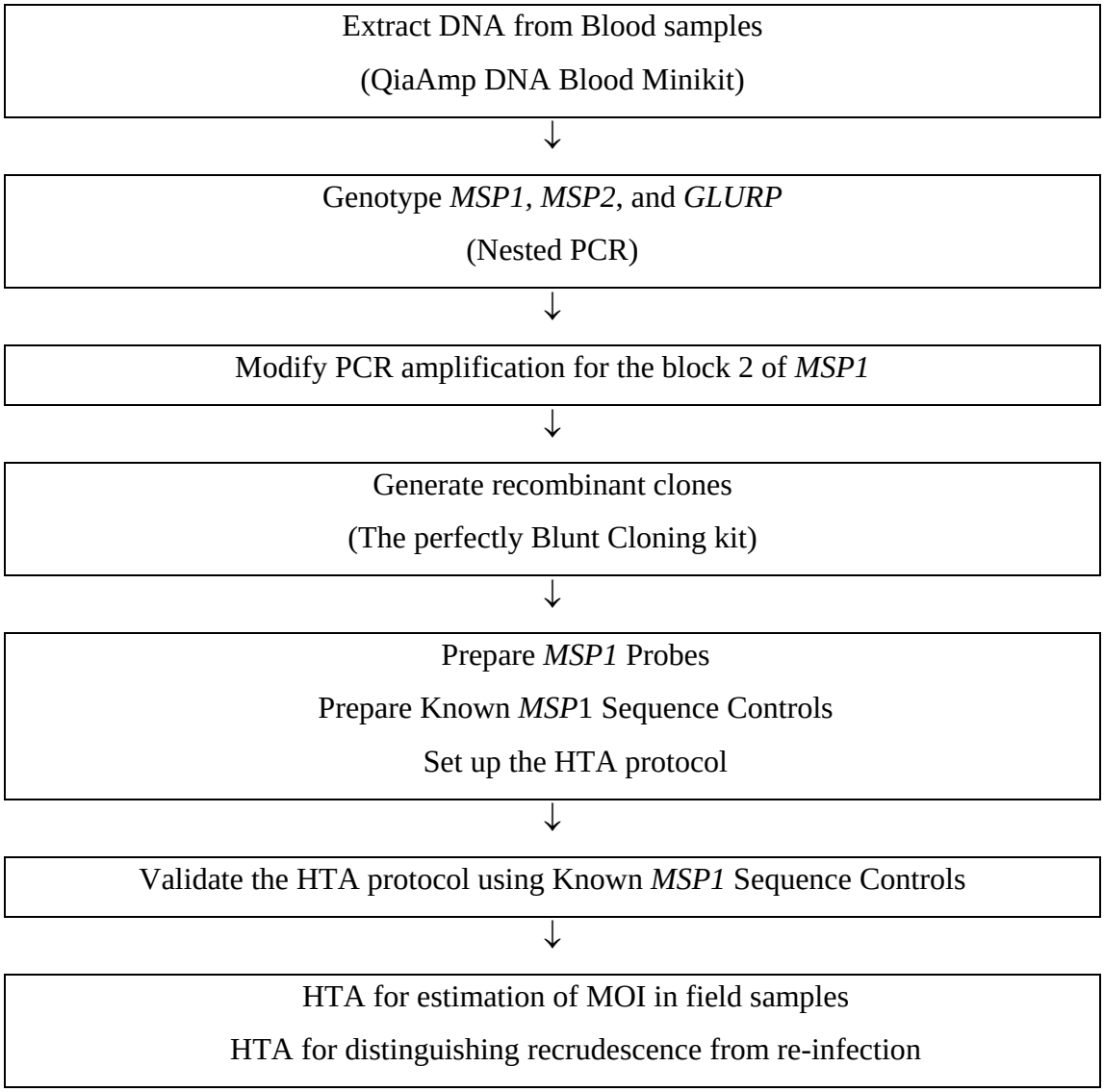


Figure 8. A summary diagram of the whole process to develop HTA for *P. falciparum*

2. DNA Samples

All samples used in this study were drawn from two previous projects; AF study, and GR study. The studies had ethical approval from the Thai Ministry of Public Health and the University of North Carolina IRB. In the AF study, a cross-sectional study in South-East Asia (63), cryopreserved parasite isolates from various sites in Southeast Asia (Figure 9) were collected from patients with falciparum malaria. These samples were transported to the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok for *in vitro* cultivation (64). Table 3 showed the list of parasite isolates used in this study. The GR study was a cohort study in Kanchanaburi Province on the western border of Thailand. This region is considered an area of low malaria transmission. Patients with falciparum malaria were treated with mefloquine, and those found with recurrent *P. falciparum* infection within 42 days of enrolment were retreated with artesunate-mefloquine. Paired admission and recurrent infection samples were obtained and the parasitemia per microliter was quantitated by light microscopy of Giemsa stained thick blood smears (Table 4).

Genomic DNA samples were kindly provided by Dr. Chansuda Wongsrichanalai of AFRIMS in Bangkok using QiaAmp DNA Blood Minikit Blood and Body Fluid (Qiagen, USA). Briefly, cultured samples (the AF samples) and blood samples (the GR samples) are enzymatically lysed with protease in optimized buffers that stabilize nucleic acids, and enhance selective DNA adsorption to the silica-gel membrane. DNA binds specifically to the membrane while contaminants pass through. Proteins are completely removed in two efficient wash steps with alcohol, leaving pure DNA to be eluted with either water or a low-salt buffer provided with the kit. For long-term storage of DNA, eluting in a buffer and storing at –20ºC is recommended since DNA stored in water is subject to acid hydrolysis. DNA samples were shipped to the University of North Calorina for analysis of *P. falciparum* genetic diversity using nested PCR and HTA.

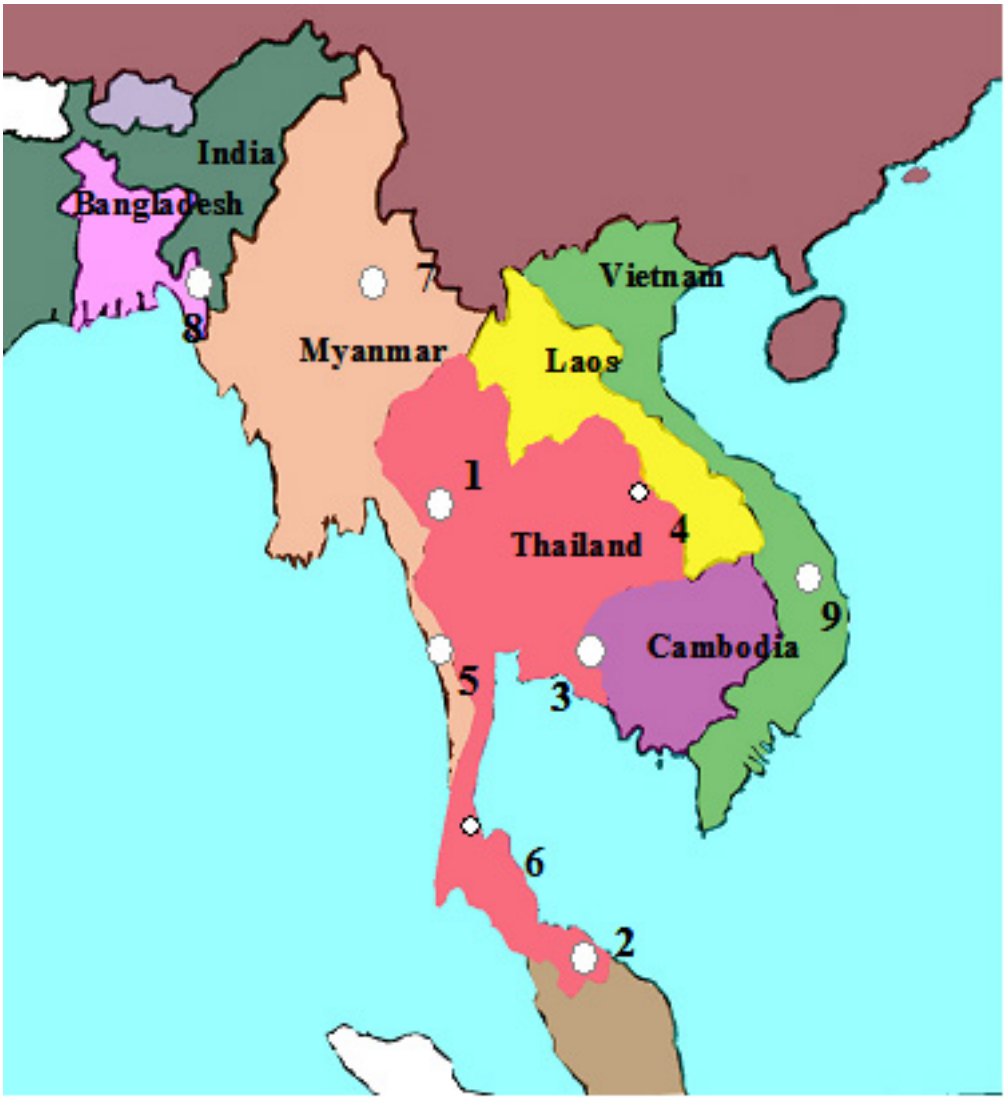


Figure 9. Various sites in Southeast Asia where blood samples were collected. 1: Tak; 2: Yala; 3: Trad; 4: Ubon; 5: Kanchanaburi; 6: Ranong; 7: Myanmar; 8: Bangladesh; 9: Vietnam

Table 3. Parasite isolates which were collected from various sites in Southeast Asia. The parasite isolate number is as used in the text.

Sites	Number of Samples	Parasite isolate number
Thailand	23	
Kanchanaburi	3	AF3, AF27, AF29
Tak (Maesod)	9	AF7, AF9, AF12, AF15, AF16, AF17, AF18, AF19, AF22
Trad	3	AF23, AF25, AF64
Yala	3	AF51, AF54, AF56
Ranong	2	AF58, AF59
Ubon	3	AF71, AF73, AF74
Bangladesh	6	AF6, AF8, AF31, AF32, AF69, AF70
Vietnam	3	AF10, AF34, AF37
Myanmar	7	AF38, AF42, AF43, AF44, AF60, AF62, AF63,
Total	39	

Table 4. Participant characteristics. The patient number is as used in the text. Also shown are parasite density, sex, and age of patients.

Patient number		Parasite Density (Parasites / μl of Blood)	Sex	Age (Years)
GR001	day 0 day 29	134,000 1,040	M	22
GR004	day 0 day 29	496 432	M	36
GR008	day 0 day 28	9,000 272	M	20
GR010	day 0 day 42	27,000 12,000	F	56
GR012	day 0 day 21	72,000 48	F	28
GR014	day 0 day 35	11,000 80	M	25
GR022	day 0 day 35	65,000 144	M	45
GR027	day 0 day 8	14,000 3,728	M	24
GR030	day 0 day 35	36,000 3,008	M	23
GR031	day 0 day 28	110,000 1,760	M	28
GR032	day 0 day 42	13,000 48	F	22
GR034	day 0 day 44	67,000 64	M	23
GR037	day 0 day 20	126,000 9,000	M	28
GR041	day 0 day 7	59,000 528	M	20
GR045	day 0 day 28	45,000 80	F	60
GR046	day 0 day 8	137,000 432	M	19
GR050	day 0 day 7	625,000 416	M	40

3. Assessment of *P. falciparum* Polymorphisms in MSP1, MSP2, and GLURP by Nested PCR

Nested PCR was previously performed by Deborah D. Kamwendo, Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, Michigan for genotyping *P. falciparum* parasites (57, 65). DNA amplification of *MSP1*, *MSP2* and *GLURP* involved two rounds of PCR, using the nested primers in the second round. Oligonucleotide primers, which hybridize conserved sequences flanking the repeat polymorphic regions of the genes, are used in the first amplification reaction (Nest 1). The product of the first reaction is then used as the DNA template for separate second amplification reactions (Nest 2). In Nest *2,* the oligonucleotide primers used recognize sequences contained within the DNA fragment amplified in the first reaction (Figure 10). For *MSP1*, three separate Nest 2 reactions were performed to complete the genotyping of parasites using *MSP1* marker (Figure 10): one specific for the K1 family (M1-KF and M1-KR), one specific for the MAD20 family (M1-KF and M1-MR), and one specific for the RO33 family (M1-RF and M1-RR). For *MSP2,* two separate Nest 2 reactions were performed to complete the genotyping of parasites using *MSP-2* marker (Figure 10): one specific for the FC27 family (M2-FCF and M2-FCR), and the last specific for the 3D7/IC family (M2-ICF and M2-ICR). For *GLURP*, a Nested 2 reaction was carried out to complete the genotyping of parasites with the antisense primer G-OR used in Nest 1 and G-NF, which is internal but overlaps with G-OF (Figure 10).

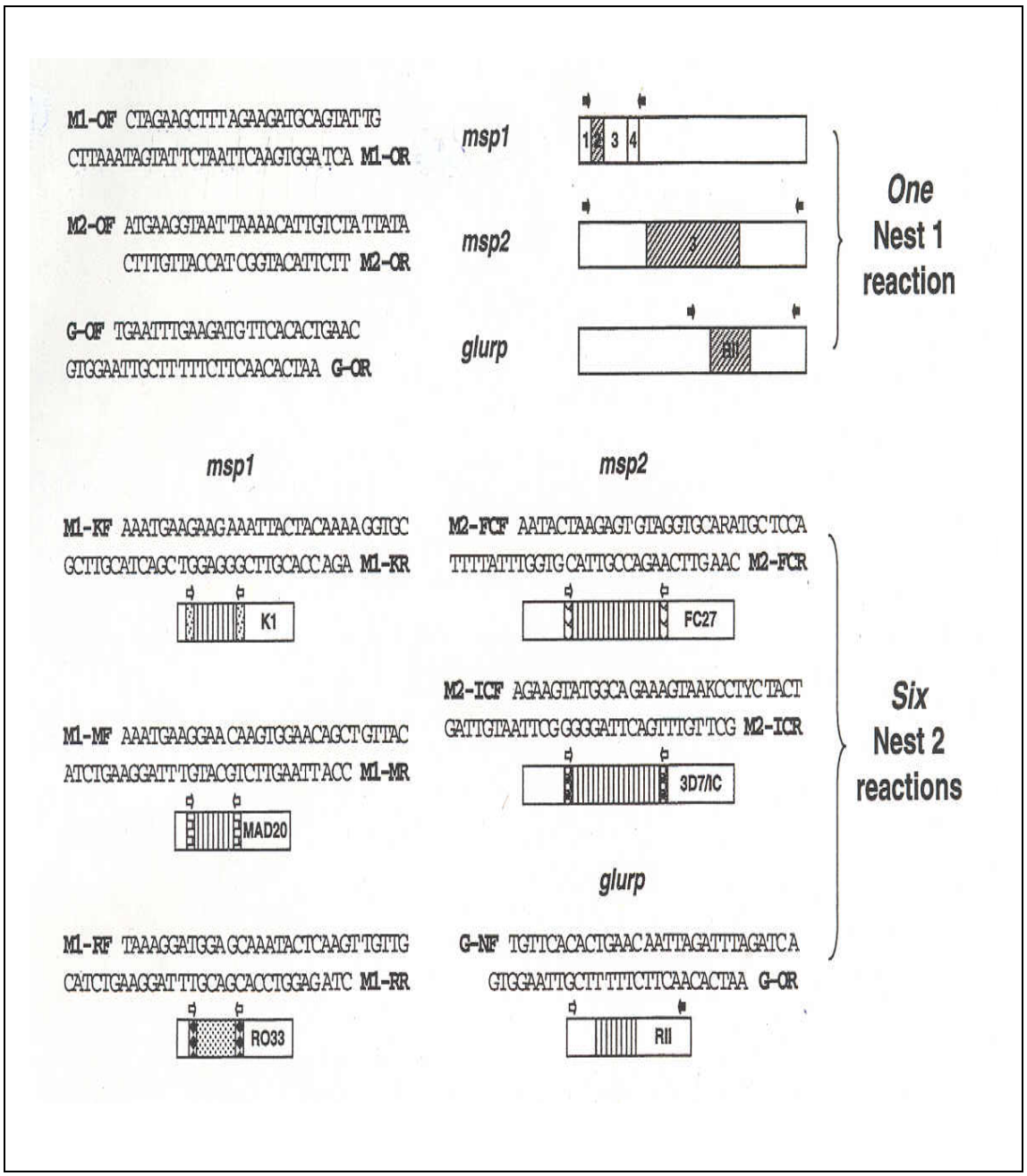


Figure 10. Schematic representation of *MSP1*, *MSP2*, and *GLURP* genes with the sequences of the different oligonucleotide primer pairs and their approximate positions and that of their products (57). The name of each oligonucleotide primer is in bold, and placed before the sequence (presented 5' to 3') for "sense" primers, and after the sequence for "antisense" primers.

The amplification reactions started with initial denaturation at 95°C for 5 min, followed by PCR cycle conditions as summarized in Table 4, and ended with extension at 72°C for 5 min. These conditions have been performed in a PTC-200 Thermalcycler (MJ Research). A total of 20 µl of the reaction mixture contained (1x) PCR buffer with 1.0 mM $MgCl_2$ (Invitrogen, USA), 125 µM of dNTP (Invitrogen, USA) containing each of dATP, dCTP, dGTP, and dTTP, 0.4 unit of *Taq* DNA Polymerase (Invitrogen, USA), oligonucleotide primers, and 1 µl of DNA template. All the oligonucleotide primers for the Nest 1 and Nest 2 reactions are used at a final concentration of 250 nM, except for the *GLURP*, Nest 1 primers, which are used at a 125 nM. In all secondary PCRs, two negative controls were used: the amplicon of the negative control for the first PCR and a triple distilled water control.

A 10 µl of the reaction mixture with 2 µl of (6x) loading buffer (Appendix) was analyzed by 1.5% agarose gel electrophoresis in (1x) TBE buffer (Appendix) at 120 mV for 25 min. Following ethidium bromide staining (Appendix), DNA bands were visualized under ultraviolet light by a UV transluminator. The PCR product lengths, in terms of number of base pairs (bp), were calculated according to their mobility relative to the molecular size standard (100 bp weight marker) (Promega, USA). The approximate size range of the PCR products differs for the different markers: 125-250 bp for the *MSP1* sequences, 250-450 bp for the FC27 family of *MSP2*, 450-700 bp for the 3D7/IC family of *MSP2*, and 600-1200 bp for the *GLURP* sequences.

Table 5. Primers and cycle conditions of nested PCR for amplification of merozoite surface protein 1 block-2 (*MSP1*), merozoite surface protein 2 block-3 (*MSP2*) and glutamine rich protein (*GLURP*)

Locus	Primers	Cycle conditions
MSP-1 (Block-2)	Nest 1: M1-OF, M1-OR	94ºC/1 min: 58ºC/2 min: 72ºC/1 min: 25 cycles
	Nest 2: M1-KF, M1-KR (K1 family) M1-MF, M1-MR (MAD20 family) M1-RF, M1-RR (RO33 family)	94ºC/1 min: 61ºC/2 min: 72ºC/1 min: 30 cycles
MSP-2 (Block-3)	Nest 1: M2-OF, M2-OR	94ºC/1 min: 58ºC/2 min: 72ºC/1 min: 25 cycles
	Nest 2: M2-FCF, M2-FCR (FC27 family) M2-ICF, M2-ICR (3D7/IC family)	94ºC/1 min: 61ºC/2 min: 72ºC/1 min: 30 cycles
GLURP	Nest 1: G-OF, G-OR	94ºC/1 min: 58ºC/2 min: 72ºC/1 min: 25 cycles
	Nest 2: G-NF, G-OR	94ºC/1 min: 58ºC/2 min: 72ºC/1 min: 30 cycles

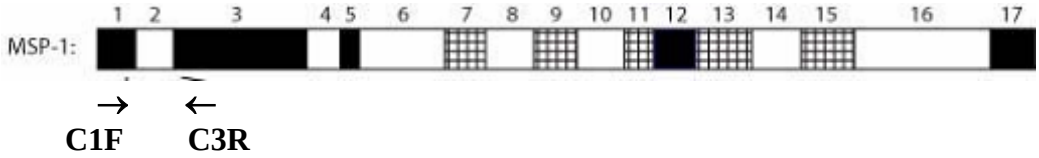
4. PCR Amplification for the Block 2 of *MSP1* Sequence

4.1 Primers

Oligonucleotide primers (Qiagen, USA) used in this study were synthesized corresponding to highly conserved DNA sequences flanking the block 2 of *MSP1*. The forward primer is, C1F: 5'–GAAGATGCAGTATTGACAGG-3' and the reverse primer is, C3R: 5'–TGATTGGTTAAATCAAAGAG- 3'. The final concentration of the primers in the DNA amplification reaction was 250 nM.

4.2 Reaction conditions

PCR amplifications with minor modifications (66) were performed in a final volume of 50 µl. With 2 µl of parasite DNA, the reactions were carried out in (1x) PCR buffer with 1.5 mM $MgCl_2$ (Qiagen, USA), 125 uM of dNTP (Invitrogen, USA) containing each of dATP, dCTP, dGTP, and dTTP, 1 unit of *HotStarTaq* DNA Polymerase (Qiagen, USA) and 250 nM of each primer. The final volume was adjusted to 50 µl with triple distilled water. The amplification was performed in a PTC-200 thermalcycler (MJ Research). The reaction condition consisted of a preheat to 95^{o}C for 5 min followed by 25 cycles of 94^{o}C for 1 min, annealing at 50^{o}C for 2 min, and extension at 72^{o}C for 2 min, and final extension at 72^{o}C for 5 min (Figure 11). All PCR reactions were done in duplicate to ensure reproducible sampling and included genomic *P. falciparum* strain 3D7 DNA (Appendix) as a positive control and triple distilled water as negative control.



Cycle condition: 95°C for 5 min
94°C for 1 min
50°C for 2 min (25 cycles)
72°C for 2 min
72°C for 5 min
hold @ 4°C

Figure 11 PCR cycle condition for amplification of the block 2 of *MSP1*

4.3 Agarose gel electrophoresis and ethidium bromide staining

The PCR products were analysed by 1.2% agarose gel electrophoresis (67). Briefly, weighted DNA grade agarose was heated to dissolve in (1x) TBE buffer (Appendix) and cooled to 50°C before pouring into the gel tray (Bio-Rad Laboratory). After insert the appropriate gel comb into the gel, the gel was allowed to set at 25°C for 20-30 min. Ten microliters from each reaction was mixed with a one-fifth volume of the (6x) DNA loading buffer (Appendix) before loading into the wells. Electrophoresis was carried out at 120 mV for 25 min**.** in (1x) TBE buffer for 30 min or until the dye front reached two-third of the gel. The gel was then removed and stained with 0.5 µg/ml ethidium bromide for 20 min. The DNA bands were visualized under ultraviolet light by a UV transluminator. Successful amplification resulted in products of approximately 300 bps after sized against a 100-bp molecular weight marker (Promega, USA)

5. Construction of Recombinant Clones

5.1 Blunt End Cloning and Screening of Recombinants

Three samples (AF63, AF22, and AF42) from the AF study and one sample (MHP1452), which was collected from the malaria positive, pregnant woman in the Malaria and HIV in Pregnancy (MHP) study (68), contain K1-, MAD20-, RO33-, and K1-types of *MSP1*, respectively. These DNA samples were amplified for the block 2 of *MSP1* as described in this chapter, section 3. The purified PCR products were blunt cloned into the pT7Blue vector using the perfectly blunt cloning kit (Novagen, USA) according to the manufacturer protocol (Figure 12). Briefly, the insert DNA is first converted to a blunt, phosphorylated form in a single reaction. Following a heat inactivation step, the blunt phosphorylated insert is combined with blunt dephosphorylated pT7Blue vector and then ligate. Subsequent transformation into the competent cells generates recombinant colonies that are visualized easily by blue/white screening.

The map of the pT7Blue vector that provide for blue/white screening of recombinants was shown in Figure 13. The plasmid encodes a functional *lacZ* α-peptide that complements the *lacZ* ω-fragment expressed by the host strain. The resulting active β-galactosidase can cleave the chromogenic substrate, which is 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), to yield a blue colony phenotype. Inserts are cloned into a dephosphorylated *EcoR* V site in pT7Blue. This site is located within the α-peptide open reading frame (ORF). Inserts disrupt this ORF, thereby preventing the production of functional α-peptide. This prevention results in the white colony phenotype when plated on LB agar plates containing 100 μg/ml of carbenicilin (Appendix), 50 μg/ml of tetracycline (Appendix), 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG) (Appendix) and 40 μg/ml of X-Gal (Appendix).

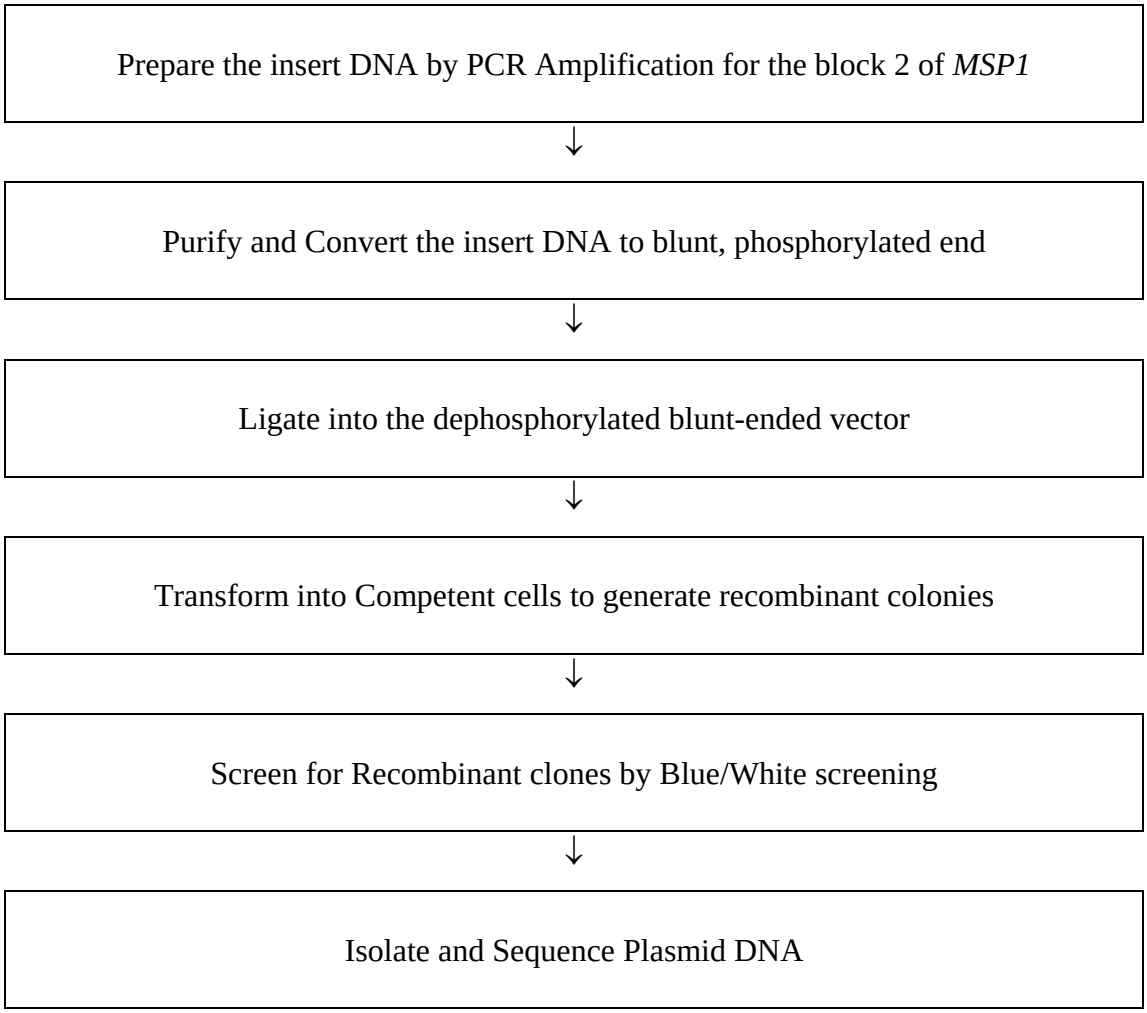
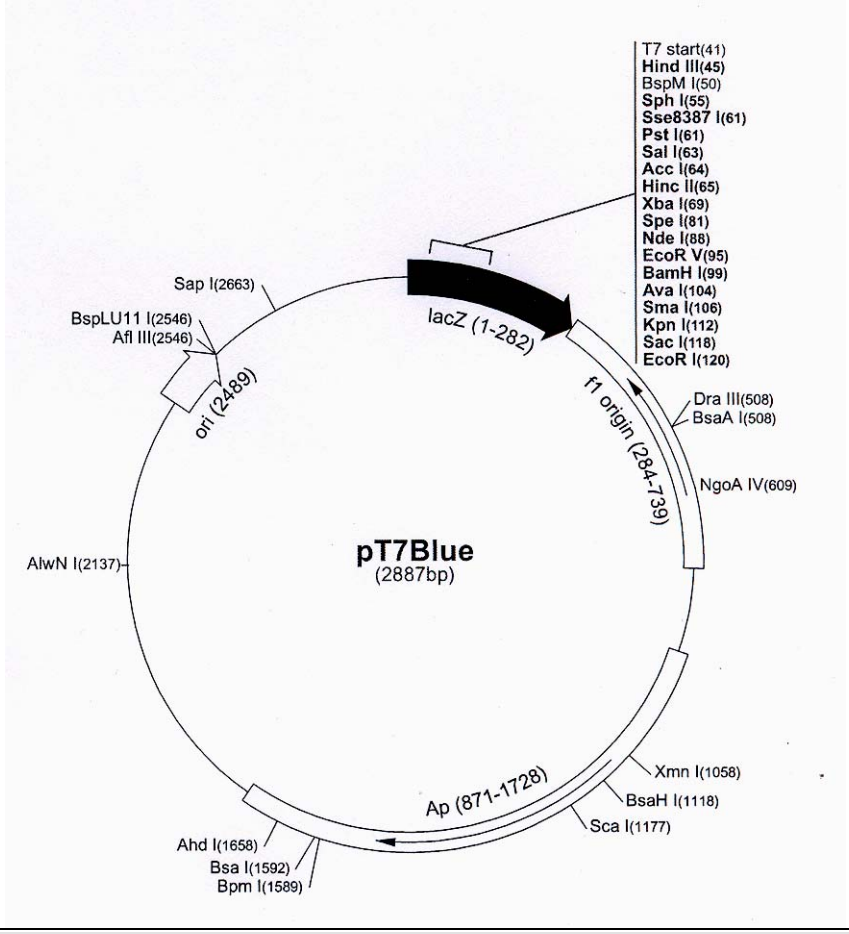
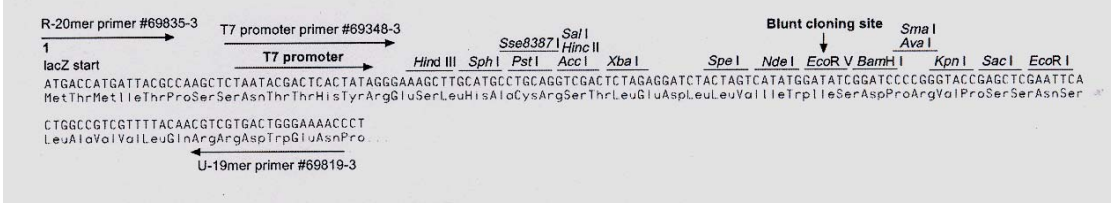


Figure 12. The process of cloning with the Novagen Perfectly Blunt Vectors.

pT7Blue sequence landmarks

*lacZ*start codon	1
*lacZ*α-peptide	1-282
T7 promoter	24-40
T7 transcription start	41
Muliple cloning region (Hind III – EcoR I)	45-125
fl origin	284-739
bla coding sequence	871-1728
pUC origin	2489





pT7Blue cloning/expression regions

Figure 13. The map of the pT7Blue vector

(http://www.merckbiosciences.com/docs/docs/PROT/TB017.pdf)

5.2 Plasmid Preparation and Clone Storage

Prior to growing colonies for plasmid isolation, the presence of the appropriate insert can be determined using PCR screening method with the original insert-specific primers (the C1F and C3R primers). The white colonies (lacking *lac Z*) are grown overnight in LB with carbenicilin and tetracycline (Appendix) at 37^{o}C. All plasmid DNA from the overnight culture are isolated by using the Wizard DNA minipreparation kit (Promega, USA) according to the manufacturer protocol. Briefly, Bacteria are lysed by treatment with sodium hydroxide/sodium dodecyl sulfate (NaOH/SDS). Chromosomal DNA and plasmid DNA are denatured by NaOH and SDS is a detergent that denatures bacterial proteins. The mixture is neutralized by the addition of an acidic potassium acetate solution, which allows the small covalently closed plasmid DNA to renature. The denatured chromosomal DNA and proteins are removed by centrifugation, while the plasmid DNA, RNA, and some proteins remain in solution. The soluble proteins are removed by phenol/chloroform extraction. Plasmid DNA and RNA are precipitated out of solution twice with alcohol. Ribonuclease A solution is used to degrade the RNA.

Once the clone has been confirmed for the correct DNA insertion, clone storage is done by making a stock of the pure culture (67). The pure bacterial culture of *E. coli* is grown in LB broth containing 100 μg/ml of carbenicilin, 50 μg/ml of tetracycline (Appendix). A 920 μl of culture is transfer into a polypropylene tube and 80 μl sterile glycerol is added to make an 8% glycerol freezing solution. The mixture is vortexed to evenly mix the glycerol throughout the culture. The stock of the pure culture is kept at -80^{o}C until used.

5.3. DNA-Sequencing and Sequence Analysis

The plasmid DNA was sequenced at the University of North Carolina–Chapel Hill Automated DNA Sequencing Facility on a 3100 Genetic Analyzer (Applied Biosystems) using the ABI PRISM ^{TM} BigDye^{TM}Terminator Cycle Sequencing Ready Reaction Kit FS with AmpliTaq^{R} DNA Polymerase (Applied Biosystems). Sequencing was performed from both directions using the C1F and C3R primers. DNA sequences were aligned with Clustal-X (69) (http://www2.ebi.ac.uk/clustalw/)

and displayed using GeneDoc (70) (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed with Clustal-X and displayed with NJPlot (71) (http://pbil.univ-lyon1.fr/software/njplot.html). The MAD20, K1, and RO33 sequences used in the construction of the phylogenetic tree were obtained from PubMed.

6. Heteroduplex Tracking Assay (HTA)

6.1 Probe labeling

Plasmid DNAs from four recombinant clones, which are the AF22, AF42, AF63, and the MHP1452, were isolated and purified by using Wizard Plus SV miniprep DNA Purification System (Promega, USA) as previously described in section 4.2. An aliquot of 10 µg of plasmid DNA in the *BamHI* reaction buffer (Fermentas, USA) was digested with *BamHI* (Fermentas, USA) and *PstI* (Fermentas, USA) at 37°C for 1 hr to release inserted *MSP1* fragments from the vector. The insert (probe) was purified by QIA quick gel extraction kit (Qiagen, USA) and eluted in a final volume of 70 µl. After adding 10 mM DTT (Appendix), 50 µM dGTP (Appendix), 50 µCi of [α-^{35}S]dATP (1,250 Ci/mmol) (ICN, USA), and 10 U of Klenow fragment (3'$\rightarrow$5' exo$^-$) of DNA polymerase I (Fermentas, USA), the fragment (probe) in the *EcoPol* reaction buffer (Fermentas, USA) was labeled by filling in the *BamHI* overhangs in 120 µl at 25°C for 15 min. The DNA polymerase was inactivated by the addition of 10 mM EDTA (Appendix) followed by 75°C incubation for 15 min. Unincorporated nucleotides were removed by column purification over a Centri spin column (Princeton, USA). The labeled probe was brought to a final volume of 150 µl using TE buffer (Appendix). All probes were used in a MSP1-HTA for estimation of *P. falciparum MSP1* genotypes per infected person (MOI).

6.2 Preparation of *MSP1* known sequence controls

Plasmid DNAs from four recombinant clones, which are AF63,AF22, AF42, and MHP1452, were used as DNA templates in PCR amplification for the block 2 of *MSP1* as described in this chapter, section 3, to provide K1-, MAD20-, RO33-, and

K1-known sequence controls, respectively. These *MSP1* known sequences were used as known controls for the validation of HTA.

6.3 HTA protocol

The schematic diagram of HTA protocol was shown in Figure 14. A 25-ml of polyacrylamide gel solution (6%T) was prepared by adding of 5.0 ml of 30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide Stock solution (national diagnostics, USA), 2.5 ml of (10x) TBE buffer (Appendix), 17.5 ml of distilled water, 125 µl of freshly prepared 25% ammonium persulphate solution and 19 µl of TEMED. The gel solution was mixed and poured into 20 cm ×20 cm × 0.8-mm glass plates. A 24-well comb was inserted and the gel was allowed to polymerize for approximately 45 min at 25^{o}C.

Heteroduplex annealing reaction mixtures consisted of 5 µl of PCR product containing the block-2 of *MSP1* or 5 µl gel purified amplicons from the plasmid DNA controls, 1 µl of (10x) annealing buffer (Appendix), 0.1 µM C1F primers, and 1 µl of labeled probe in a total volume of 10 µl. Extra C1F primers were added to the annealing reaction mixture to bind unannealed probe and make its migration more uniform. The mixtures of the PCR product and the radiolabeled *MSP1* probe were denatured at 95^{o}C for 2 min and allowed to reanneal at 25^{o}C for 5 min. The product containing heteroduplex and homoduplex was mixed with (6x) DNA loading buffer (Promega, USA), and then separated by electrophoresis in a 6% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) in 1x TBE buffer (Appendix) by using a model FB-VE20-1 vertical gel apparatus (Fisher) run at 17 mA per gel for 4 hr 30 min or until the second dye front just run off. The gel was dried onto filter paper (Whatman, UK)) using a gel dryer. The dried gel was exposed to BioMax MR X-ray film (Kodak, Japan) for approximately 18 hr at 25ºC. Alternatively, for quantitative data, the dried gels were exposed to a phorphorimager screen for 2 days. The relative abundance of each band within a sample and the band intensities were quantitated by using FragmeNT analysis software (Molecular Dynamics).

HTA was determined by observing distinct migration pattern of heteroduplexes. Only the prominent bands with approximately 10% or greater of the total signal in an individual lane were considered true heteroduplexes. Bands with less than 10% of the total signal, or common bands to all lanes were not counted since they could represent

either true minority genotypes or PCR artifacts. The exclusion of faint bands could potentially bias this study towards an underestimation of MOI. In each lane should contain at least three bands; single strand probe, probe-target heteroduplex, and probe homoduplex. For interpretation, individual bands were identified as *MSP1* genotypes by the following criteria. Genotypes that had length differences were well separated and easy to distinguish. For genotypes with only a few of base paired mismatches, the bands were much closer together and thus more difficult to distinguish. Close bands were characterized as discrete when they were visibly separated by one bandwidth.

Figure 15 outlines the potential probe-target interactions observed in HTA experiments; the probe sequence is in bold type and the target, or unknown sequence, is in regular type. Single-stranded probes, as shown in Figure 15A, are unordered and flexible and therefore migrate more slowly than probes annealed to their complement (homoduplexes) (Figure 15B). If the probe and the target are not complementary, one of three structural distortions occurs after annealing: 1) clustered base pair mismatches cause a bubble between the probe and the target (Figure 15C), 2) deletions in the target cause the probe to loop out (Figure 15D), or 3) insertions in the target are looped out (Figure 15E). Any one of the latter 3 complexes, either alone or in combination, retards the migration of the probe-target complexes compared to the homoduplex.

Genomic DNA Extraction

↓

PCR for the block 2 of *MSP1*

↓ + Probe labeled with ^{35}S

Annealing Reaction
Denatured at 95°C, 2 min
Re-annealed at RT, 5 min

↓

6% non denaturing PAGE,
17mA/gel, for 4hr 30min

↓

Exposure to Kodak BioMax MR film,
at 25°C, for overnight

Figure 14. Schematic diagram of HTA protocol

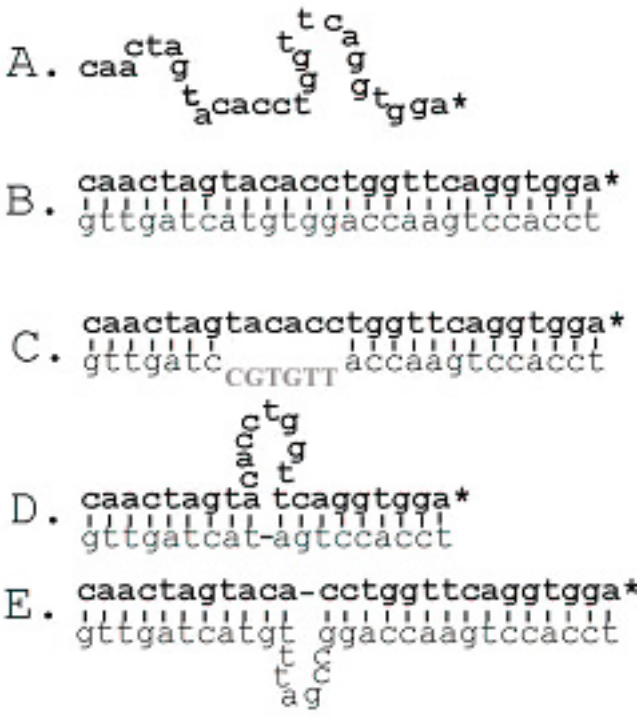


Figure 15. The principle of Heteroduplex tracking assay. Single stranded probes (bold) are radiolabeled and annealed to *MSP1* amplicons from patient. Radiolabeled probes migrate on the gel alone as in A) single stranded probe, or in complexes with the complementary DNA, such as B) homoduplexes, C) heteroduplexes with clustered base-pair mismatches, D) heteroduplexes with sample deletion(s), and E) heteroduplexes with sample insertion(s).

7. Data Analysis

7.1 The estimation for the multiplicity of infection

7.1.1 Using nested PCR

Generally, the multiple bands in the PCR product of nested PCR reflect the presence of many different strains in an isolate (57). The MOI is estimated from the number of bands observed in an agarose gel. For *MSP1*, the numbers of K1, MAD20, and RO33 bands are added, and for *MSP2*, the numbers of FC27 and 3D7 bands are added. In similar way to *MSP1* and *MSP2*, the number of *GLURP* bands is counted. Comparing these numbers from all three genes, the highest number at any of three genes; *MSP1*, *MSP2*, *GLURP*, is assumed to be the MOI of that isolate.

7.1.2 Using HTA

The heteroduplex bands reflect the presence of many different *P. falciparum* strains in an isolate. The MOI is estimated from the number of prominent bands observed in a non denaturing poloacrylamide gel. Bands with less than 10% of the total signal, or common bands to all lanes were not counted.

7.2 Comparison of the MOI estimated by HTA and nested PCR methods

Kappa statistics were used to assess the agreement of the number of MOI estimated by HTA and nested PCR methods. The kappa coefficient (*k*) (72) was calculated by the SPSS statistical package. In 1977, Landis and Koch interpreted *k* values between 0 and 1 (73); the definitions of *k* value are the possible following guidelines with slight modification;

Value of *k*	Strength of agreement
<0.20	Poor
0.21-0.40	Fair
0.41-0.60	Good
0.61-0.80	Very good
0.81-1.00	Excellent

7.3 The identity of the heteroduplex mobility patterns between paired admission and recurrent infection samples

The identity of heteroduplex mobility patterns between admission and recurrent infection samples is based on the number and mobility rate of heteroduplex bands observed in 6% non denaturing polyacrylamide gel electrophoresis. The identity is defined using a modification of the coefficient of similarity, *F* value (74). In this modification, $F = 2Nxy/(Nx + Ny)$; where *Nx* is the total number of heteroduplex bands in the recurrent infection sample, and *Ny* is the total number of heteroduplex bands in the admission sample and *Nxy* is the number of fragments shared in paired samples. Therefore an *F* value of 1.0 indicates that the heteroduplex mobility pattern of the recurrent infection sample is identical to, or contained within that of the admission sample. Value less than 1.0 indicates non-identity.

CHAPTER 5

RESULTS

1. Polymorphisms of *MSP1, MSP2,* and *GLURP* of *P.falciparum*

The polymorphisms of *P.falciparum* in each sample were assessed by determining the number and the size variation (alleles) of the followings genes: *MSP1, MSP2* and *GLURP* as described previously in Chapter 4, section 2. DNA amplification of *MSP1, MSP2* and *GLURP* from 39 AF samples involved two rounds of polymerase chain reaction (PCR), using the nested primers in the second round. Primer sequences are shown in Figure 10. All PCR reactions started with incubation at 95°C for 1 min and ended with elongation at 72°C for 5 min. Following electrophoresis and ethidium bromide staining, the PCR product lengths, in terms of number of base pairs (bp), were calculated according to their mobility relative to the 100 bp weight marker (Promega, USA). The positive or negative results for given allelic families were consistent since a random 10% of samples done as a formal "random rerun" both allelic family and band size were identical. After the PCR products of these samples were separated by 1.2% agarose gel electrophoresis, the number of bands with different sizes was defined as the number of genotypes.

1.1 Allelic types of *MSP1, MSP2,* and *GLURP*

Thirty-nine of AF samples used in this study were successfully amplified for *MSP1, MSP2,* and *GLURP*. The number of genotypes in each sample (the clone number) analyzed by nested PCR was shown in Table 6. From these observations, it appears that the number of genotypes of *MSP1* and *MSP2* ranged from 1 to 3 but it was 1 to 2 in *GLURP.*

Table 6: The number of genotypes analyzed by nested PCR

Sample No	MSP-1	Allelic Family of MSP-1			MSP-2	Allelic Family of MSP-2		GLURP	Total
		MAD20	K1	RO33		IC	FC27		
AF003	1	1	0	0	1	1	0	1	1
AF006	2	1	1	0	1	1	0	1	2
AF007	1	1	0	0	2	1	1	2	2
AF008	3	1	1	1	2	1	1	2	3
AF009	1	1	0	0	2	1	1	1	2
AF010	2	1	0	1	2	1	1	1	2
AF012	1	0	1	0	1	1	0	1	1
AF015	2	1	1	0	1	1	0	1	2
AF016	1	0	1	0	1	0	1	1	1
AF017	1	0	1	0	1	1	0	1	1
AF018	1	1	0	0	1	1	0	1	1
AF019	1	1	0	0	1	0	1	1	1
AF022	2	1	1	0	3	1	2	1	3
AF023	2	1	1	0	2	1	1	1	2
AF025	2	1	1	0	2	1	1	1	2
AF027	2	1	0	1	2	1	1	1	2
AF029	1	1	0	0	2	1	1	1	2
AF031	1	1	0	0	1	0	1	1	1
AF032	3	2	1	0	2	1	1	2	3
AF034	2	1	1	0	1	1	0	1	2

Table 6: The number of genotypes analyzed by nested PCR (Continued)

Sample No	*MSP-1*	Allelic Family of *MSP-1*			*MSP-2*	Allelic Family of *MSP-2*		*GLURP*	Total
		MAD20	K1	RO33		IC	FC27		
AF037	1	1	0	0	1	1	0	1	1
AF038	2	1	1	0	2	1	1	1	2
AF042	1	0	0	1	1	1	0	1	1
AF043	3	1	1	1	1	1	0	1	3
AF044	1	1	0	0	1	0	1	1	1
AF051	1	1	0	0	1	1	0	1	1
AF054	1	0	1	0	2	1	1	1	2
AF056	2	1	1	0	2	1	1	1	2
AF058	2	1	1	0	2	1	1	1	2
AF059	2	1	1	0	1	1	0	1	2
AF060	2	1	1	0	2	1	1	1	2
AF062	1	1	0	0	3	1	2	1	3
AF063	1	0	1	0	1	1	0	1	1
AF064	1	1	0	0	2	1	1	1	2
AF069	3	1	1	1	1	1	0	1	3
AF070	3	1	1	1	2	1	1	1	3
AF071	3	1	1	1	1	1	0	1	3
AF073	2	1	1	0	2	1	1	1	2
AF074	1	1	0	0	1	0	1	1	1

The sample number is as used in the text. Some case numbers highlighted in bold are selected to clone, and the recombinant clones are used for verification of the HTA. The result of PCR where (+) illustrates a DNA product and (-) no product obtained from PCR.

The predominant allele of *MSP1* was MAD20 as 32 out of 39 samples (82.1%) harboured MAD20-type, 23 out of 39 samples (59.0%) were K1-type, and 8 samples out of 39 samples (20.5%) were typed as RO33. At the *MSP2* loci, 34/39 (87.2%) samples contained 3D7/IC-type and 22/39 (56.4%) samples contained FC27-type (Table 7).

A total of 12 different *MSP1* genotypes (6 MAD20-type alleles, 5 K1-type alleles and only one RO33-type alleles), 10 different *MSP2* genotypes (5 3D7/IC-type alleles and 5 FC27-type alleles), and 7 different *GLURP* genotypes were identified based on the size of DNA fragments (Table 7).

1.2 The Multiplicity of Infection (MOI)

The MOI or the clone number was the highest number of alleles at any of the three loci. For example, the AF22 contained two alleles of *MSP1*, three alleles of *MSP2*, and one allele of *GLURP*. Therefore, the clone number or the MOI was taken to be 3 (Table 7). At least 13 of the 39 samples (33.3%) were monoclonal, 46.2% of samples (18/39) contained at least two clones, and 20.5% of samples (8/39) carried at least three clones. Allelic typing of *MSP1* and *MSP2* were detected 51.3% and 48.7% of the multiclonal infections, respectively, while only 7.7% of the multiclonal infections were detected by typing of *GLURP*. The overall mean MOI was 1.87 $\pm$ 0.12 (the total number of clones divided by the total number of isolates. All of these observations were summarized in Table 7.

Table 7. Polymorphism in the merozoite surface protein 1 (*MSP1*), the merozoite surface protein 2 (*MSP2*) and glutamine rich protein (*GLURP*) of *P. falciparum* isolates from Thailand

Gene	*MSP-1*	Allelic Family of *MSP1*			*MSP-2*	Allelic Family of *MSP2*		*GLURP*	Total
		MAD20	K1	RO33		3D7/IC	FC27		
Number (%) of samples containing alleles of the corresponding family	39 (100)	33 (84.6)	23 (59.0)	8 (20.5)	39 (100)	34 (87.2)	24 (61.5)	39 (100)	39 (100)
Number of different bands (Observed on agarose gel)	12	6	5	1	10	5	5	7	29
MOI* (± SE)	1.67 (± 0.12)	0.87 (± 0.07)	0.59 (± 0.08)	0.21 (± 0.07)	1.53 (± 0.10)	0.87 (± 0.05)	0.67 (± 0.09)	1.08 (± 0.04)	1.87 (± 0.12)
Number (%) of samples contained one genotype	19 (48.7)	32 (82.1)	23 (59.0)	8 (20.5)	20 (51.3)	34 (87.2)	22 (56.4)	36 (92.3)	13 (33.3)
Number (%) of samples contained two genotypes	14 (35.9)	1 (2.6)	0	0	17 (43.6)	0	2 (5.1)	3 (7.7)	18 (46.2)
Number (%) of samples contained three genotypes	6 (15.4)	0	0	0	2 (5.1)	0	0	0	8 (20.5)

*Multiplicity of Infection

2. The Optimal Conditions for PCR Amplification of the Block 2 of *MSP1* Sequence

2.1 Choice of Enzyme DNA Polymerase

Three DNA Polymerases; *Taq* Polymerase (Invitrogen, USA), *Vent* Polymerase (Invitrogen, USA), and *HotstarTaq* Polymerase (Qiagen, USA), were used in the *MSP1* amplification reaction. It was shown that a single specific band at 398 bp was observed in the *MSP1* amplification reaction using *HotstarTaq* Polymerase (Figure 16A). The non-specific bands were seen in the *MSP1* amplification reaction using *Taq* Polymerase (Figure 16B) whereas *Vent* Polymerase was negative for a specific band at 398 bp (Figure 16C). Therefore, the *HotstarTaq* Polymerase was chosen to be used throughout the study for amplification of the block 2 of *MSP1*.

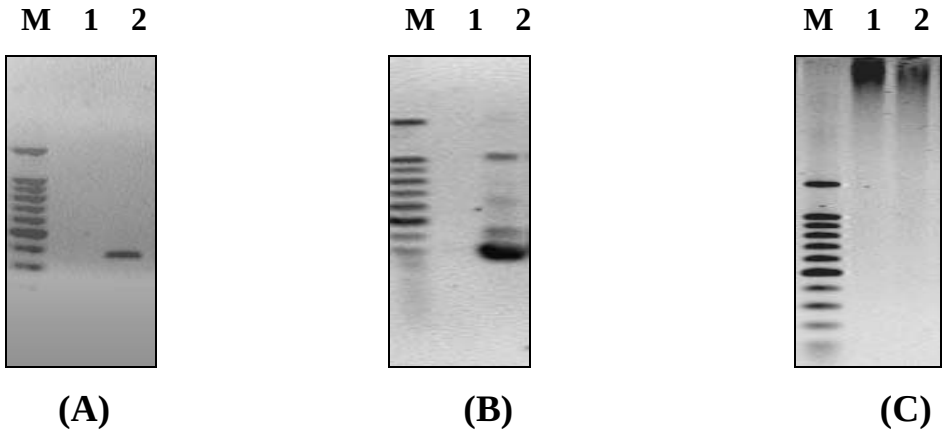


Figure 16. PCR-amplified fragments from *P. falciparum* strain 3D7 using three types of DNA polymerase. (A) *HotStartaq polymerase* (B) *Taq polymerase* (C) *Vent polymerase*. Primers were prepared to the sequences of the conserved blocks 1 and 3 of the *MSP1* gene: C1F, 5'–GAAGATGCAGTATTGACAGG-3'; and C3R: 5'–TGATTGGTTAAATCAAAGAG- 3'. Template DNA was isolated from the cultured 3D7 (lane 3). Lane M, contained DNA molecular size markers of 1000, 900, 800, 700, 600, 500, 400, 300, and 200 bps, respectively. Lane 2, No template DNA.

2.2 Annealing temperature

The effect of the annealing temperature on the *MSP1* amplification of *P. falciparum* strain 3D7 was determined by gradient PCR, varying the annealing temperatures at 40°C, 45.1°C, 48.2°C, and 50°C. It was found that the single band at 398 bp were seen at all four annealing temperatures with similar ethidium bromide staining intensity (Figure 17). Therefore, the annealing temperature at 50°C was selected throughout the study to prevent non-specific DNA amplification at lower temperature.

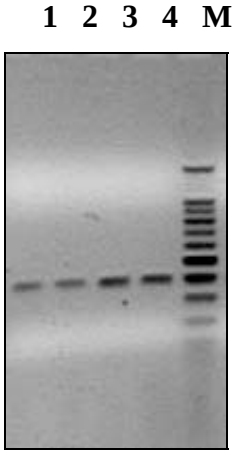


Figure 17. PCR-amplified fragments from *P. falciparum* strain 3D7 using primers to *MSP1* and varying the annealing temperatures at 40°C (lane 1), 45.1°C (lane 2), 48.2°C (lane 3), and 50°C (lane 4). Primers were prepared to the sequences of the conserved blocks 1 and 3 of the *MSP1* gene: C1F, 5'–GAAGATGCAGTATTGACAGG-3'; and C3R: 5'–TGATTGGTTAAATCAAAGAG- 3'. Lane M, contained DNA molecular size markers of 1000, 900, 800, 700, 600, 500, 400, 300, and 200 bps, respectively.

2.3 Analytical specificity

In many areas where malaria is endemic, several *Plasmodium* species are in a single circulation. The specificity of the *MSP1* amplification was examined whether the primers and PCR conditions chosen were specific for *P. falciparum*. DNAs from *P. falciparum, P. malariae*, and *P. vivax* were amplified with the primers C1F and C3R. The result is shown in Figure 18. For all reactions, amplification was restricted to only *P. falciparum* DNA, whereas the other two species of *Plasmodium* were negative. This indicated that the reactions with *P. falciparum*-derived primers were species specific.

Figure 18. PCR-amplified fragments from *P. falciparum* strain 3D7 and patient blood using primers to *MSP1*. Primers were prepared to the sequences of the conserved blocks 1 and 3 of the *MSP1* gene: C1F, 5'–GAAGATGCAGTATTGACAGG-3'; and C3R: 5'–TGATTGGTTAAATCAAAGAG- 3'. Template DNAs were isolated from the cultured *P. falciparum* strain 3D7 (lane 1), and the blood patients infected with *P. malaraie* (lane 2), *P. vivax* (lane 3), and. *P. falciparum* (lane 4). Lane M, contained DNA molecular size markers of 1000, 900, 800, 700, 600, 500, 400, 300, and 200 base pairs, respectively. Lane N, No template DNA.

3. Comparison of the Block 2 of *MSP1* Sequences in Recombinant Clones

3.1 Size and Allelic Family

Plasmid DNAs of four recombinant clones constructed from four samples, AF22, AF42, AF63, and MHP1452 have been used as templates for PCR amplification of the block2 of *MSP1* as described in chapter 4, section 3. The PCR amplicons contained the *MSP1* inserts were directly sequenced and resulted in products of 344, 317, 335, and 371 base pairs (AF22, AF42, AF63, and MHP-1452, respectively) as shown in the multiple alignment (Figure 19). The *MSP1* sequences of the AF22, AF42, AF63 and MHP1452 clone were compared to the *MSP1* known type K1, MAD20 and RO33 (75) and their relationship was shown in a phylogenetic tree (Figure 20). This suggested that the recombinant clone of AF63 and MHP1452 are allelic type K1, the AF22 clone is MAD20, and the AF42 clone is type RO33. Since the repetitive nature of *MSP1* makes estimates of genetic variation between *MSP1* alleles difficult to quantify (75, 76), the *MSP1* family assignments should be interpreted with caution.

Additionally, the *MSP1* sequences of each clone were aligned with the allelic specific sequences for the block 2 of *MSP1* gene. Their alignments revealed that the *MSP1* sequence of AF63 and MHP1452 clones were complementary with the K1 specific sequence whereas the *MSP1* sequences of AF22 and AF42 clones were matched with type MAD20 and RO33, respectively. According to this information, it is more likely that the recombinant clones of AF63 and MHP1452 were allelic type K1. In contrast, the AF22 clone was type MAD20, and the AF42 clone was type RO33. The characteristics of recombinant clones used in this study were shown in Table 8.

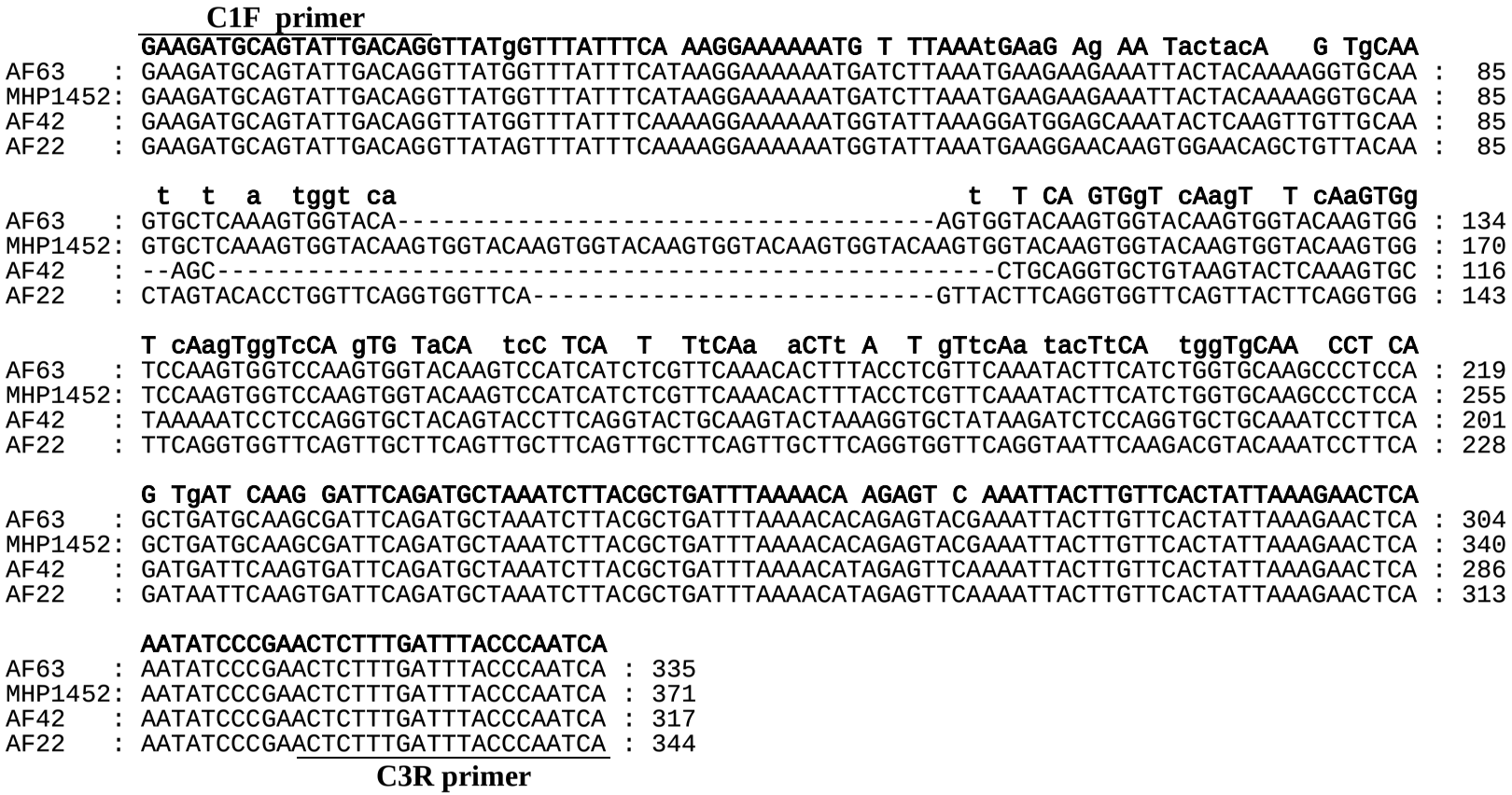


Figure 19. DNA sequence alignment for the recombinant clones, AF22, AF42, AF63, and MHP1452. The sequences have been aligned to maximize the matched residues. Dashs indicate the deletion in AF22, AF42, and AF63 relative to MHP1452. The capital letters indicate identical nucleotides among four sequences. The positions of the PCR primers are overscored.

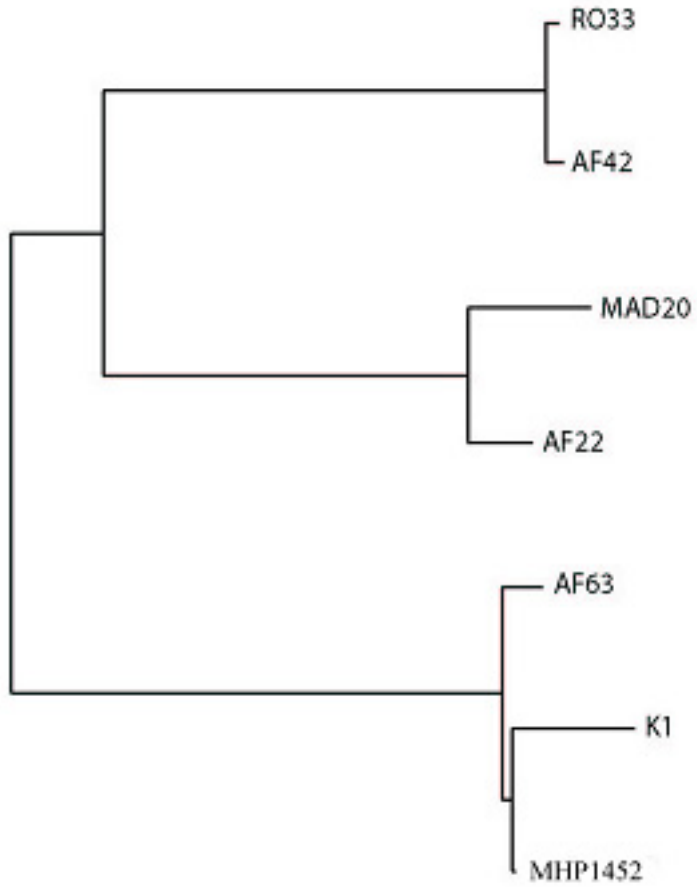


Figure 20. A phylogenic tree showing the relationships between the block2 of *MSP1* sequences. From recombinant clones of AF22, AF42, AF63, and MHP1452, the block 2 of *MSP1* sequences were compared to the known *MSP1* sequences

Table 8. Characteristics of recombinant clones used in this study.

Recombinant Clones	Size	Allelic family
AF 63	335 bp	K1
MHP 1452	371 bp	K1
AF 22	344 bp	MAD20
AF 42	317 bp	RO33

3.2 Sequence Differences determined by Pairwise Alignment

The *MSP1* sequence differences between recombinant clone AF63 and other clones were determined by pairwise alignment. It was found that MHP1452 comparing to AF63 had 1 insertion of 36 nucleotides and no base-pair mismatches, which were equal to 11% difference (Figure 21). The alignment between AF63 and AF42 revealed 1 insertion of 18 bp and 68 mismatches (26% difference) (Figure 22). Sequence differences between AF63 and AF22 were 1 deletion of 9 nucleotides and 75 base-pair mismatches (25% difference) as shown in Figure 23.

```
               C1F  primer
                       *        20         *        40         *
          GAAGATGCAGTATTGACAGGTTATGGTTTATTTCATAAGGAAAAAATGAT
AF63    : GAAGATGCAGTATTGACAGGTTATGGTTTATTTCATAAGGAAAAAATGAT :  50
MHP1452: GAAGATGCAGTATTGACAGGTTATGGTTTATTTCATAAGGAAAAAATGAT :  50

                 60         *        80         *       100
          CTTAAATGAAGAAGAAATTACTACAAAAGGTGCAAGTGCTCAAAGTGGTA
AF63    : CTTAAATGAAGAAGAAATTACTACAAAAGGTGCAAGTGCTCAAAGTGGTA : 100
MHP1452: CTTAAATGAAGAAGAAATTACTACAAAAGGTGCAAGTGCTCAAAGTGGTA : 100

                   *       120         *       140         *
          CAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGT
AF63    : CAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGT--------------- : 135
MHP1452: CAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGTACAAGT : 150

                 160         *       180         *       200
                               CCAAGTGGTCCAAGTGGTACAAGTCCATC
AF63    : ---------------------CCAAGTGGTCCAAGTGGTACAAGTCCATC : 164
MHP1452: GGTACAAGTGGTACAAGTGGTCCAAGTGGTCCAAGTGGTACAAGTCCATC : 200

                   *       220         *       240         *
          ATCTCGTTCAAACACTTTACCTCGTTCAAATACTTCATCTGGTGCAAGCC
AF63    : ATCTCGTTCAAACACTTTACCTCGTTCAAATACTTCATCTGGTGCAAGCC : 214
MHP1452: ATCTCGTTCAAACACTTTACCTCGTTCAAATACTTCATCTGGTGCAAGCC : 250

                 260         *       280         *       300
          CTCCAGCTGATGCAAGCGATTCAGATGCTAAATCTTACGCTGATTTAAAA
AF63    : CTCCAGCTGATGCAAGCGATTCAGATGCTAAATCTTACGCTGATTTAAAA : 264
MHP1452: CTCCAGCTGATGCAAGCGATTCAGATGCTAAATCTTACGCTGATTTAAAA : 300

                   *       320         *       340         *
          CACAGAGTACGAAATTACTTGTTCACTATTAAAGAACTCAAATATCCCGA
AF63    : CACAGAGTACGAAATTACTTGTTCACTATTAAAGAACTCAAATATCCCGA : 314
MHP1452: CACAGAGTACGAAATTACTTGTTCACTATTAAAGAACTCAAATATCCCGA : 350

                  360         *
          ACTCTTTGATTTACCCAATCA
AF63    : ACTCTTTGATTTACCCAATCA : 335
MHP1452: ACTCTTTGATTTACCCAATCA : 371

                 C3R primer
```

Figure 21. Pairwise alignment between the block2 of *MSP1* sequences from AF63 and MHP1452 clones.

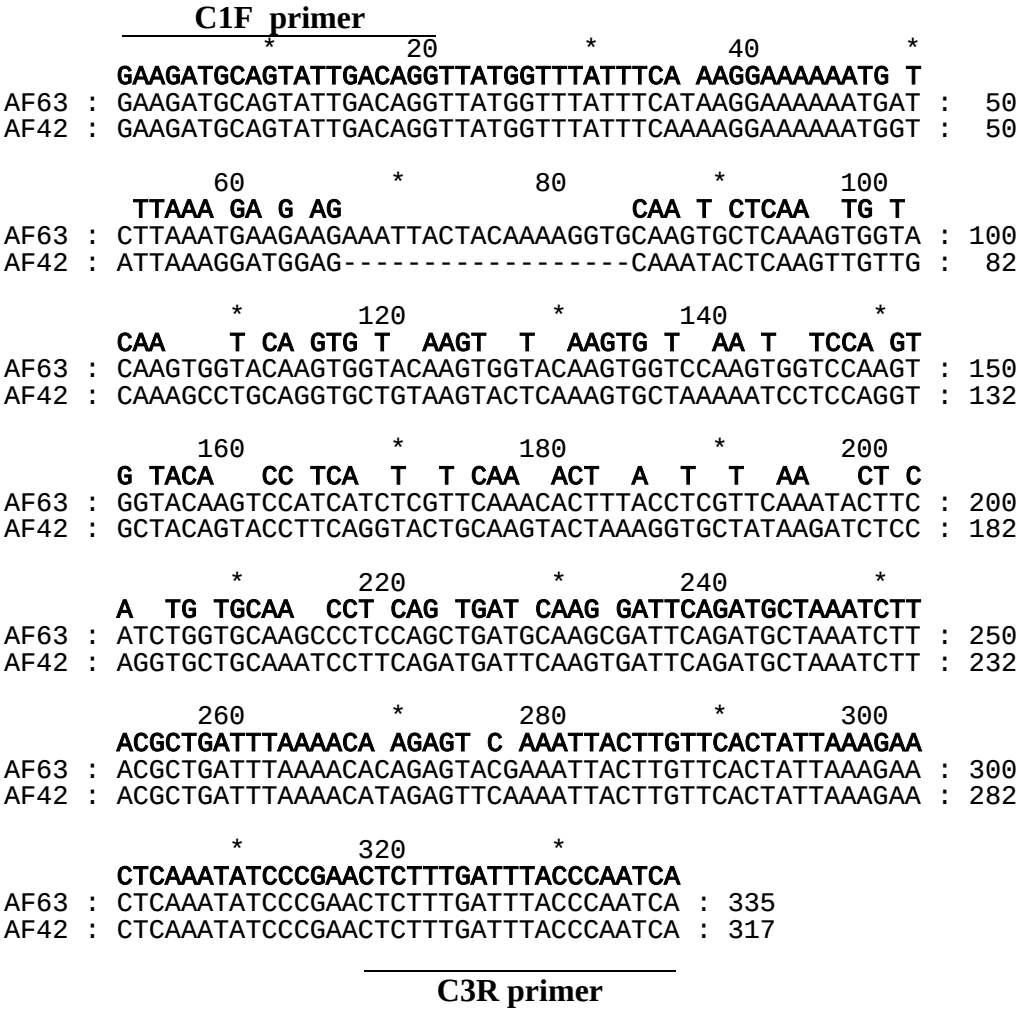


Figure 22. Pairwise alignment between the block2 of *MSP1* sequences from AF63 and AF42 clones.

```
              C1F  primer
                ____________________
                 *        20         *        40         *
       GAAGATGCAGTATTGACAGGTTAT GTTTATTTCA AAGGAAAAAATG T
AF63 : GAAGATGCAGTATTGACAGGTTATGGTTTATTTCATAAGGAAAAAATGAT :  50
AF22 : GAAGATGCAGTATTGACAGGTTATAGTTTATTTCAAAAGGAAAAAATGGT :  50

               60         *        80         *       100
        TTAAATGAAG A  AA          T  TACAA   GT CA  TG T
AF63 : CTTAAATGAAGAAGAAA---------TTACTACAAAAGGTGCAAGTGCTC :  91
AF22 : ATTAAATGAAGGAACAAGTGGAACAGCTGTTACAACTAGTACACCTGGTT : 100

                 *       120         *       140         *
        A GTGGT CA  T  T CA GTGGT CA  T  T CA GTGGT CA GT
AF63 : AAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGTACAAGTGGTCCAAGT : 141
AF22 : CAGGTGGTTCAGTTACTTCAGGTGGTTCAGTTACTTCAGGTGGTTCAGGT : 150

              160         *       180         *       200
       GGT CA  TG T CA  T C TCA  T  TTCA    CTT A  T GTTC
AF63 : GGTCCAAGTGGTACAAGTCCATCATCTCGTTCAAACACTTTACCTCGTTC : 191
AF22 : GGTTCAGTTGCTTCAGTTGCTTCAGTTGCTTCAGTTGCTTCAGGTGGTTC : 200

                 *       220         *       240         *
       A  TA TTCA    GT CAA  CCT CAG T AT CAAG GATTCAGATG
AF63 : AAATACTTCATCTGGTGCAAGCCCTCCAGCTGATGCAAGCGATTCAGATG : 241
AF22 : AGGTAATTCAAGACGTACAAATCCTTCAGATAATTCAAGTGATTCAGATG : 250

              260         *       280         *       300
       CTAAATCTTACGCTGATTTAAAACA AGAGT C AAATTACTTGTTCACT
AF63 : CTAAATCTTACGCTGATTTAAAACACAGAGTACGAAATTACTTGTTCACT : 291
AF22 : CTAAATCTTACGCTGATTTAAAACATAGAGTTCAAAATTACTTGTTCACT : 300

                 *       320         *       340
       ATTAAAGAACTCAAATATCCCGAACTCTTTGATTTACCCAATCA
AF63 : ATTAAAGAACTCAAATATCCCGAACTCTTTGATTTACCCAATCA : 335
AF22 : ATTAAAGAACTCAAATATCCCGAACTCTTTGATTTACCCAATCA : 344
                                ____________________
                                     C3R primer
```

Figure 23. Pairwise alignment between the block2 of *MSP1* sequences from AF63 and AF22 clones.

4. Development and Validation of *MSP1*- HTA

The heteroduplex tracking assay has been developed to detect as many different *MSP1* sequences as possible. The HTA probes were generated from four molecular clones, AF63, AF22, AF42, and MHP1452. These recombinant clones were also used as four *MSP1* known sequence controls. All four probes were tested in the *MSP1*-HTA with the sequence controls. These experiments showed that if the target sequences were typically different with the probe used, all of the *MSP1* heteroduplexes between the probe and PCR products exhibited reduced migration during electrophoresis.

As shown in Figure 24, all of the clones analyzed matched with one of the bands in heteroduplex mobility pattern and accurately displayed the same location in the gel. The K1 (AF63) probe annealed equally well to both MAD20 (AF22) and RO33 (AF42) families (Figure 24, lanes 2-3). In contrast, the diffuse signal in lane 4 suggests that the AF63-MHP1452 heteroduplex is an unstable, dynamic complex. Quantitation of the area of the heteroduplex bands determined that the signal of the diffuse MHP1452 complex is not equivalent to the signal of the tighter AF22 or AF42. The results supported that the AF63 probe does not anneal tightly to the MHP1452 sequence.

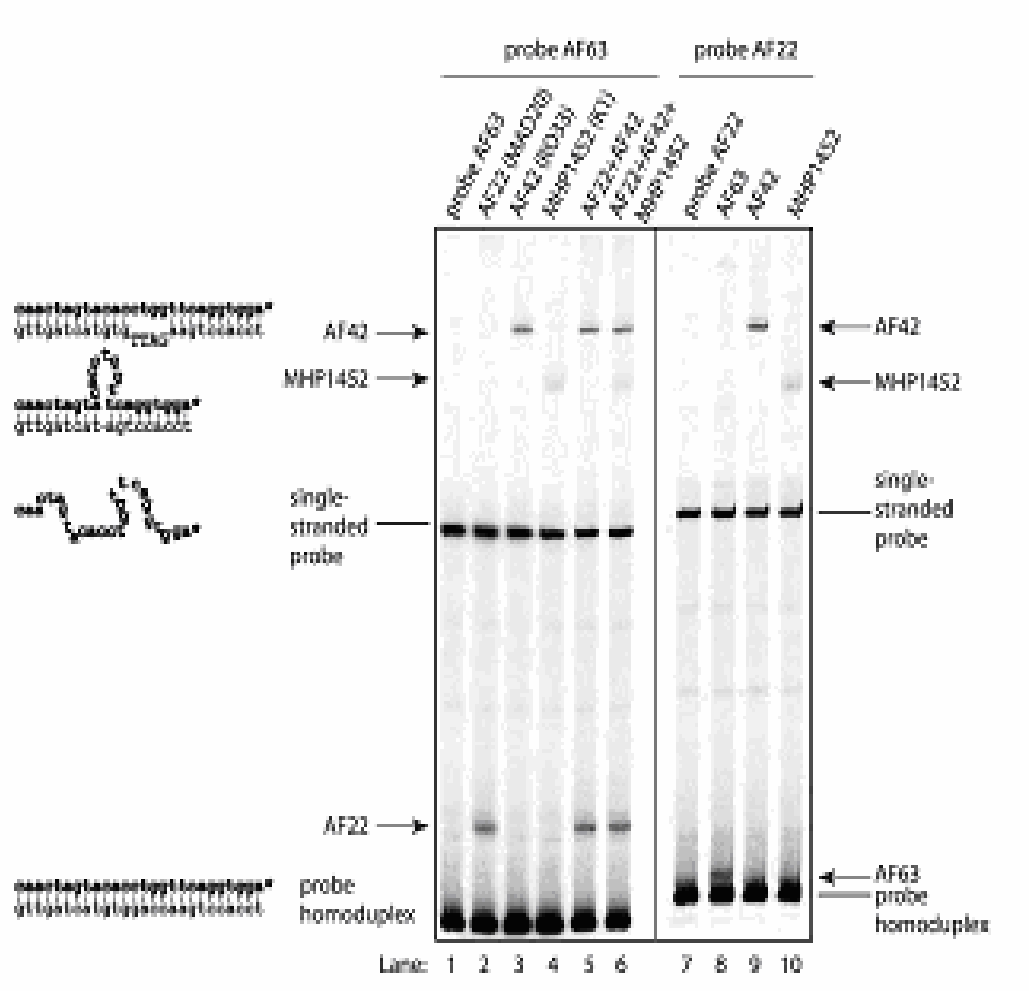


Figure 24. Autoradiogram of *MSP1*- HTA analysis using the AF63 probe or the AF22 probe. Probes were annealed with equal amounts of *MSP1* known sequence controls amplified from AF22, AF42, AF63, and MHP1452 clones. Lane 1-6 show samples annealed to probe AF63

In addition, the results with the AF63 and AF22 probes were then compared in order to determine the ability of these probes to bind to different *MSP1* families. Annealing between the plasmid-derived amplicons to the radiolabelled AF22 probe resulted in migration patterns which were similar to the AF63 probe (Figure 24, lanes 7-10). These data supported that the HTA probes developed within this study are capable of binding to sequence variants from all of the MSP1 allelic families (K1, MAD20 and RO33).

Upchurch and colleges reported that when the degree of divergence between two annealed sequences exceeds 4.5%, the rate of heteroduplex migration is proportional to the heterogeneity between the annealed sequences. However, as shown in Figure 24 (lane 4), the AF63/MHP1452 heteroduplex migrates much more slowly than the AF63/AF22 heteroduplex. This is unlikely since AF63 and MHP1452 are from the same allelic family (K1) and an AF63/MHP1452 heteroduplex should migrate very closely to the AF63 homoduplex. However, it has been reported that heterogeneity near the center of the heteroduplex influences migration more than heterogeneity near the ends of duplexes. Thus, the unpaired bases retard mobility more than mismatched bases.

Based on these observations, we hypothesize that the unexpected migration of AF63/MHP1452 and the AF63/AF22 heteroduplexes is related to the location of the heterogeneity between the sequences. This hypothesis is future supported by pair-wise alignment between AF63/AF22. It was found that shows that AF 22 has a 9 base-pair insertion 75 base-pairs away from the 5’end and multiple nucleotide mismatches (Figure 23), while the AF63/MHP1452 heteroduplex has no base-pair mismatches but a large, 36 base-pair insertion near the center of the heteroduplex (Figure 21).

The ability of the HTA to probe a mixture of MSP1 sequence variants were then investigated. As expected, when the block2 *MSP1* of AF22 and AF42 are mixed together and annealed to an AF63 probe, two heteroduplex bands were observed (Figure 24, lane 5). Furthermore, a mixture of AF22, AF42, and MHP1452 annealed to the AF63 probe yields three heteroduplex bands (Figure 24, lane 6). These results show that the HTA, using a single probe, can simultaneously detect multiclonal and multifamily infections.

Finally, to test the ability of the AF63 probe to detect similar sequences, the AF63 probe was annealed to an AF63 amplicon. As a consequence of *PstI* digestion of the AF63 probe from the pT7Blue vector, the AF63 probe has 34 unpaired 5' nucleotides, which retards the migration of the AF63 amplicon/AF63 probe compared to the probe AF63 homoduplex. Digestion of the AF63 probe with *NdeI*, which cuts 7 nucleotides away from the *MSP1* insert, caused the AF63/AF63 probe to run concurrently with the probe AF63 homoduplex. These observations suggest that the AF63 probe digested with *NdeI* can detect the sequence variants that are very similar to the probe sequence.

5. HTA for Detection of *MSP1* Genotypes of Field Samples

DNA samples from 17 AF samples were chosen to initially analyze by HTA. All four probes were used in order to determine if one HTA probe is more sensitive than others in the detection of *MSP1* genotypes. These DNA samples were amplified for the block 2 of *MSP1* and the PCR products from each sample were annealed individually with all four probes. Based on the criteria that the number of *MSP1* genotypes was counted from distinct hetroduplex bands and the bands commonly observed were defined as PCR amplification artifact. Figure 25 shows the HTA patterns of four examples of patient samples (AF18, AF38, AF43, and AF62). The numbers of *MSP1* genotypes detected by HTA using four probes were summarized in Table 9. Each of the probes presented different heteroduplex mobility patterns. It was noted that in some cases the probes identified an unequal number of genotypes in each sample. However, these differences were not due to the reproducibility of the MSP1-HTA or biased genotype sampling, since all samples were PCR amplified in duplicate and also two independent PCR products were run side by side in the same gel. The heteroduplex mobility patterns of all duplicates were identical and the same numbers of heteroduplex bands were observed in all duplicates. Examples of identical heteroduplex mobility patterns of the three AF samples were present in Figure 26.

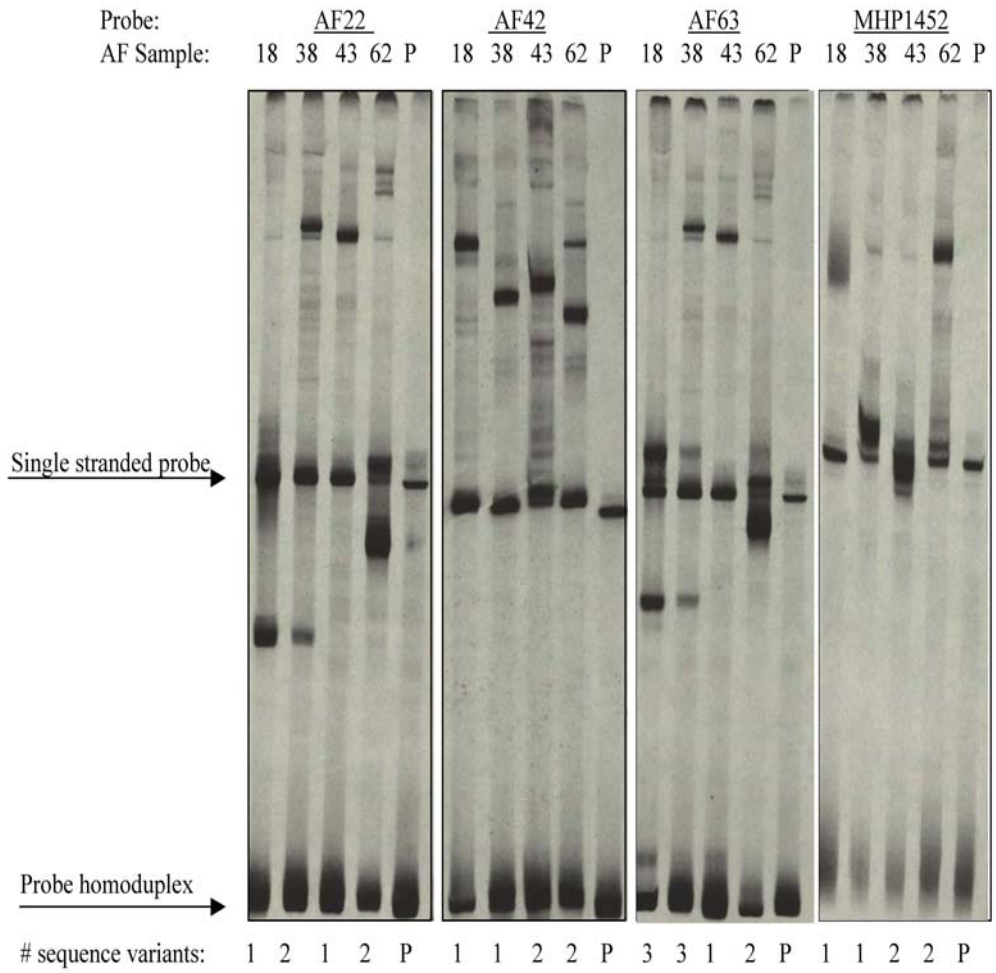


Figure 25. The HTA patterns of four representative patient samples. Autoradiogram of MSP1- HTA analysis using four probes (AF22, AF42, AF63, and MHP1452). The number of distinct heteroduplex bands as shown at the bottom of each lane was defined as the number of *MSP1* genotypes present in each sample.

Table 9: The number of *MSP1* genotypes determined by HTA using all probes

Patient No. (N=17)	HTA (ProbeAF22)	HTA (ProbeAF42)	HTA (ProbeAF63)	HTA (ProbeMHP1452)
AF3	2	1	**2**	1
AF6	2	2	**3**	1
AF7	1	1	**3**	1
AF8	2	1	**2**	3
AF12	1	1	**2**	3
AF16	1	1	**1**	1
AF17	1	1	**1**	1
AF18	1	1	**3**	1
AF29	1	1	**1**	2
AF31	2	2	**1**	2
AF32	4	2	**4**	2
AF38	2	1	**3**	1
AF43	1	2	**1**	2
AF62	2	2	**2**	2
AF69	1	2	**2**	2
AF70	2	3	**3**	2
AF71	3	1	**3**	2

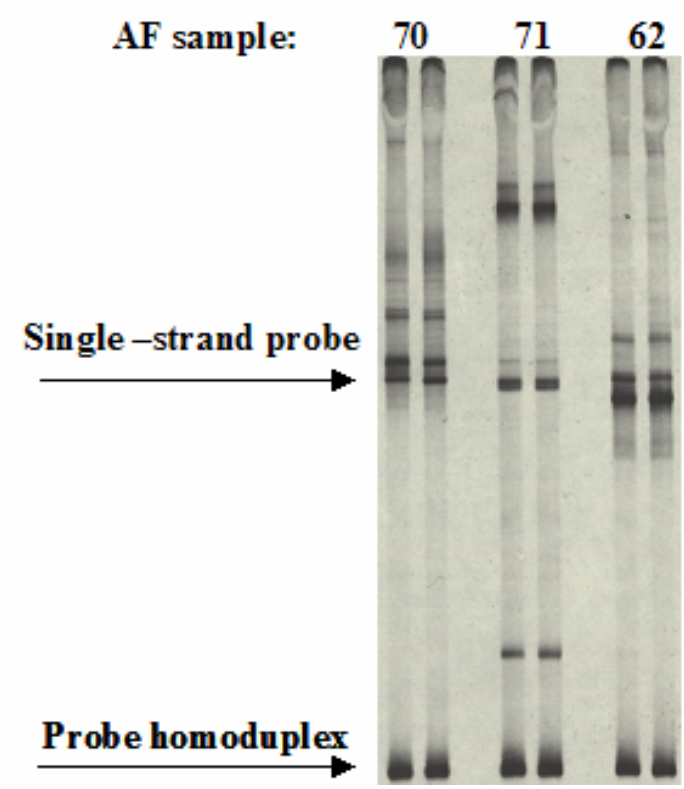


Figure 26. Autoradiogram of MSP-1 HTA analysis using the probe developed from the AF63 clone. The probe was annealed with PCR products amplified in duplicate from three samples (AF 70, AF 71, and AF62). The arrows designate the single strand probe (Top) and the probe homoduplex (bottom). All PCR duplicates displayed identical heteroduplex mobility patterns.

The sensitivity of the HTA in relation to nested PCR method was determined by tallying the number of MSP1 genotypes detected by both methods for the same 17 AF samples (Figure 27). The largest number of distinct heteroduplex bands detected by HTA using four probes can be used to estimate the clone number in each sample. It was found that the clone number detected by HTA was more than those detected by nested PCR in ten samples; 10/17 (58.8%), less than in two samples; 2/17 (11.8%) and equal to in five samples; 5/17 (29.4%). Also, the comparison of nested PCR to HTA using the AF63 probe alone resulted in the HTA detecting more genotypes in 8/17(47%) isolates, less genotypes in 3/17 (18%) isolates, and an equal number in 6/17(35%). These observations suggest that the HTA, even using a single probe, is equivalent or better than Nested PCR in determining the clone number of *P.falciparum* in a patient sample. Although no probe always detected more genotypes than the others, the AF63 probe is the most consistent probe which can detect the largest number of *MSP1* genotypes. Therefore, the AF63 probe was chosen for the subsequent analysis.

DNA samples from 39 AF samples were analyzed by HTA using the AF63 probe in order to estimate the multiplicity of *P. falciparum* infection. It was found that the MOI in parasite isolates obtained from various sites in Southeast Asia was 1.92 $\pm$ 0.13 (Table 10). Thirteen samples (33.3%) contained one genotype of *MSP1*, 18 samples (46.2%) contained two genotypes of *MSP1,* 6 samples contained three genotypes of *MSP1,* and 2 samples contained four genotypes of *MSP1.* Within DNA samples from Thailand, the MOI from each province were 2.00 $\pm$ 0.37 in Tak, 1.67 $\pm$ 0.33 in Kanchanaburi, 2.33 $\pm$ 0.33 in Ubon, 1.67 $\pm$ 0.33 in Trad, 2.00 $\pm$ 0.00 in Ranong, and 1.33 $\pm$ 0.33 in Yala (Table 11).

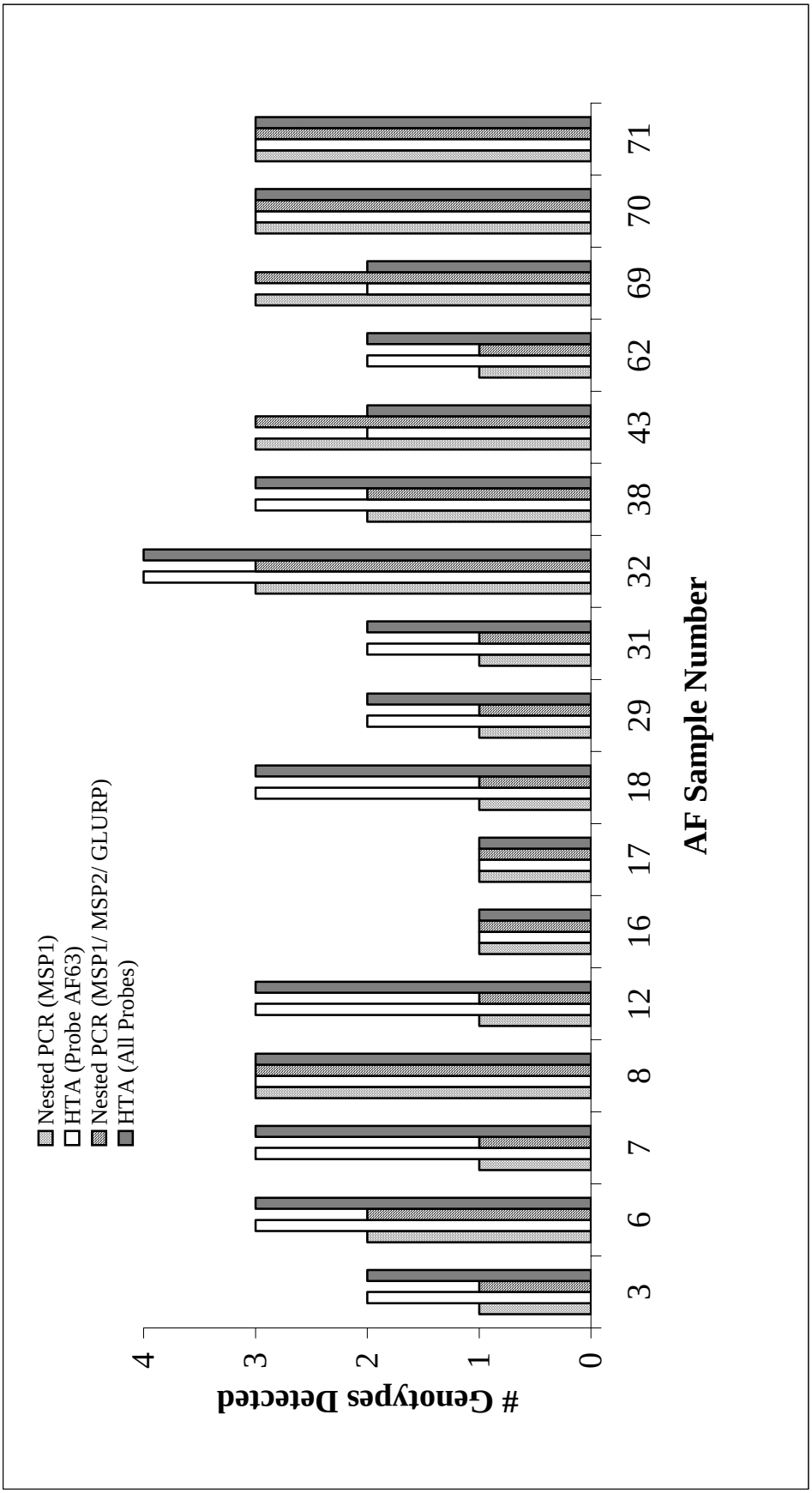


Figure 27. Comparison the numbers of genotypes determined by two methods; nested PCR and HTA.

Table 10. The multiplicity of *P. falciparum* infection in AF samples from various sites of Southeast Asia analyzed by HTA using probe AF63

Sites	Thailand	Myanmar	Bangladesh	Vietnam	Total
Number (%) of samples	23 (59.0)	7 (18)	6 (15.4)	3 (7.7)	39 (100)
MOI* (± SE)	1.87 (± 0.17)	1.57 (± 0.30)	2.50 (± 0.43)	2.00 (± 0.00)	1.92 (± 0.13)
Number (%) of samples contained one genotype	8 (20.5)	4 (10.3)	1 (2.6)	0	13 (33.3)
Number (%) of samples contained two genotypes	11 (28.2)	2 (5.1)	2 (5.1)	3 (7.7)	18 (46.2)
Number (%) of samples contained three genotypes	3 (7.7)	1 (2.6)	2 (5.1)	0	6 (15.4)
Number (%) of samples contained four genotypes	1 (2.6)	0	1 (2.6)	0	2 (5.1)

*Multiplicity of Infection

Table 11. The multiplicity of *P. falciparum* infection in AF samples from various sites of Thailand analysed by HTA using probe AF63

Sites	Province						Total
	Tak (Maesod)	Kanchanaburi	Ubon	Trad	Ranong	Yala	
Number of samples	9	3	3	3	2	3	23
MOI* (± SE)	2.00 (± 0.37)	1.67 (± 0.33)	2.33 (± 0.33)	1.67 (± 0.33)	2.00 (± 0.00)	1.33 (± 0.33)	1.87 (± 0.17)
Number of samples contained one genotype	4	1	0	1	0	2	8
Number of samples contained two genotypes	2	2	2	2	2	1	11
Number of samples contained three genotypes	2	0	1	0	0	0	3
Number of samples contained four genotypes	1	0	0	0	0	0	1

*Multiplicity of Infection

Comparison of the clone numbers determined by HTA using the AF63 probe and by Nested PCR (*MSP1/MSP2/GLURP*) in 37 field samples was shown in Table 12. Significant difference, calculated by kappa coefficient (*k*) (72) was used to correlate detection the number of clone in *P. falciparum* infections by nested PCR and HTA. The kappa coefficient beyond chance was 0.262 which indicate fair agreement beyond chance between HTA using probe AF63 and nested PCR with three polymorphic genes (*MSP1/MSP2/GLURP*).

Table 12. The multiplicity of *P.* falciparum infection in AF samples detected by Nested PCR (*MSP1/MSP2/GLURP*) and HTA

		Nested PCR (*MSP1/MSP2/GLURP*)			Total
		1	2	3	
HTA (ProbeAF63)	1	8	4	1	13
	2	4	11	4	18
	3	1	3	2	6
Total		13	17	7	37

Chi-Square Tests
P-value = 0.154

Kappa coefficient calculation

Kappa coefficient	=	0.262
Standard error of Kappa	=	0.128
P-value	=	0.031

Table 13 showed the comparison of the clone numbers determined by HTA using the AF63 probe and by Nested PCR (*MSP1*) in 37 field samples. Significant difference was calculated by kappa coefficient (*k*) (72). The *k* value was used to correlate detection the number of clone in *P. falciparum* infection by nested PCR and HTA. The kappa coefficient beyond chance was 0.396 which indicate fair agreement beyond chance between HTA using probe AF63 and nested PCR with the *MSP1* genes.

Table 13 The multiplicity of *P. falciparum* infection in AF samples detected by nested PCR (*MSP1*) and HTA

		Nested PCR (*MSP-1*)			Total
		1	2	3	
HTA (Probe AF63)	1	11	1	1	13
	2	6	10	2	18
	3	2	2	2	6
Total		19	13	5	37

Chi-Square Tests
P-value = 0.022

Kappa coefficient calculation

Kappa coefficient	=	0.396
Standard error of Kappa	=	0.118
P-value	=	0.001

6. HTA for Identification of Recrudescence and Re-infection in *P. falciparum* Infections.

In order to test the ability of HTA to differentiate between recrudescence and re-infection by the AF63 probe in MSP1-HTA, 17 patients were studied. All of them were treated with mefloquine and were identified as treatment failure during the 42 day of follow up. Paired primary and recurrent infection samples from these treatment failures in the GR study were analyzed by HTA using the AF63 probe. The block2 of *MSP1* were amplified from 17 paired of admission and recurrent infection samples. The PCR products were annealed with the AF63 probe labeled on one strand and heteroduplexes were separated by running paired samples side by side in the same gel.

Based on the criteria that the infection was considered to be recrudescence if the heteroduplex mobility pattern obtained from the recurrent infection sample (sample R) were fully or partially identical to the admission sample (sample A). And when the patterns of both samples were different, a new-infection could be assumed. These experiments revealed that the heteroduplex mobility pattern of the recurrent infection samples in 10 cases (GR001, GR004, GR008, GR010, GR014, GR031, GR037, GR045, GR046, and GR050) full or partially matched the pattern of the primary isolate. These 10 cases could be recrudescent infections resulted from treatment failure. The heteroduplex mobility patterns of recurrent infection samples in 7 cases (GR012, GR022, GR027, GR030, GR032, GR034, and GR041) contained novel sequence variants when compared to their admission samples. These 7 cases were most likely re-infections. However, the novel bands in these samples could also indicate an outgrowth of a minor parasite population that was originally undetected.

Since malaria infections are occasional composed of multiple clones of *P.falciparum* at the admission date (50), the heteroduplex mobility pattern from the admission samples frequently expressed one or more heteroduplex bands than the recurrent mobility pattern. The number of heteroduplex bands detected by HTA in 17 paired of admission and recurrent samples were summarized in Table 14.

The MOI of admission samples were ranged from 1 to 10 with the mean of 4.6 and the MOI in recurrent samples were ranged from 1 to 8 with the mean of 4.7. Only two cases (12%) were infected with one strain of *P.falciparum* (MOI =1) whereas 15 cases (88%) contained more than one strains. Within the multiclonal infections, 8 cases were identified as recrudescence while 7 cases were either recrudescence or re-infection. The number of days when parasites reappeared after mefloquine treatment was ranged from 7 to 44 days with the mode of 28 days.

Table 14. Coefficient of similarity scores (*F* score) of *MSP1* heteroduplex patterns of recurrent infection samples compared to their admission samples

Patient No	Days PT	Nx	Ny	Nxy	Nz	*F*	Result
GR001	29	6	2	2	0	0.5	R
GR004	29	1	1	1	0	1	R
GR008	28	6	6	6	0	1	R
GR010	42	1	1	1	0	1	R
GR012	21	5	6	5	1	0.91	R and N
GR014	35	6	6	6	0	1	R
GR022	35	4	4	2	2	050	R and N
GR027	8	5	4	3	1	0.66	R and N
GR030	35	2	6	2	4	0.50	R and N
GR031	28	4	4	4	0	1	R
GR032	42	10	8	6	2	0.67	R and N
GR034	44	3	2	1	1	0.40	R and N
GR037	20	6	6	6	0	1	R
GR041	7	2	6	1	5	0.25	R and N
GR045	28	8	8	8	0	1	R
GR046	8	7	7	7	0	1	R
GR050	7	3	3	3	0	1	R

The number of days post-treatment (Days PT) a second malaria infected blood sample was taken. Nx: the total number of heteroduplex bands in admission sample, Ny: the total number of heteroduplex bands in recurrent infection samples, Nxy: the total number of heteroduplex bands shared in paired samples, Nz: the number in excess of heteroduplex bands present in recurrent infection samples, *F* value of 1.0 indicates the Heteroduplex mobility pattern of a recurrent infection sample is identical to, or contained within that of the admission sample, R: Recudescence; N: New infection

6.2 The coefficient of similarity (*F* value)

The similarity of heteroduplex mobility patterns in 17 paired of admission and recurrent infection samples was reflected by the F score (coefficient of similarity) (Table 14). Each recurrent infection sample had a *F* score of 1.0 compared to its paired admission sample, suggesting the identity to primary infection while *F* score less than 1.0 represented non-identity. Figure 28 demonstrated the heteroduplex mobility patterns of three recurrent infection samples (GR004, GR008, and GR010) which were identical to their paired admission samples and the *F* score was equal to 1.0. These samples were identified as recrudescent infections. In contrast, the GR012, GR022, and GR027, had both matching bands and novel bands in the recurrent infection samples, and their *F* scores were 0.91, 0.50, and 0.66 respectively, suggesting that both recrudescence and new infections. However it was noted that the heteroduplex mobility pattern in GR 001 with *F* score 0.5 contained similar pattern with some parts of the paired admission sample, suggesting pure recrudescence.

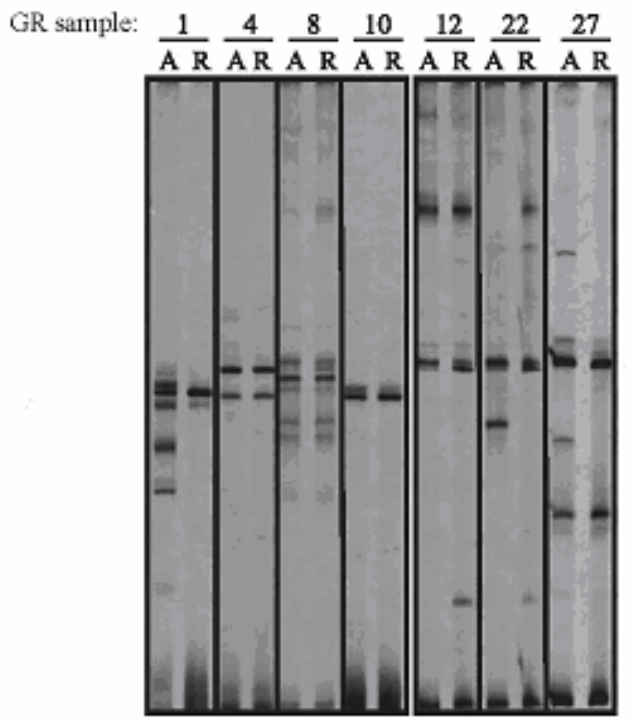


Figure 28. MSP1-HTA analysis of admission and recurrent infection samples using probe AF63. Samples are identified by a letter indicating which PCR product was used in each lane: A, admission sample; R, recurrent infection sample.

6.3 The effect of parasitaemia on the heteroduplex mobility patterns

Parasite density in each samples were significantly different Also parasitemia in the admission sample significantly higher than the parasite density of the recurrent infection samples (Table 4). The effect of parasite density to the heteroduplex pattern was examined. Four 10-fold serial dilutions of *P. falciparum* DNA from three-paired of admission and recurrent infection samples (GR001, GR022, and GR027) were made and analyzed by *MSP1*-HTA. The different parasite densities affect the appearance and intensity of the heteroduplex bands detected by HTA. When the same samples with different parasite densities were electrophoresed, weak bands transiently vanished in the samples with a lower parasite density but distinct bands were consistently visible (Figure 29).

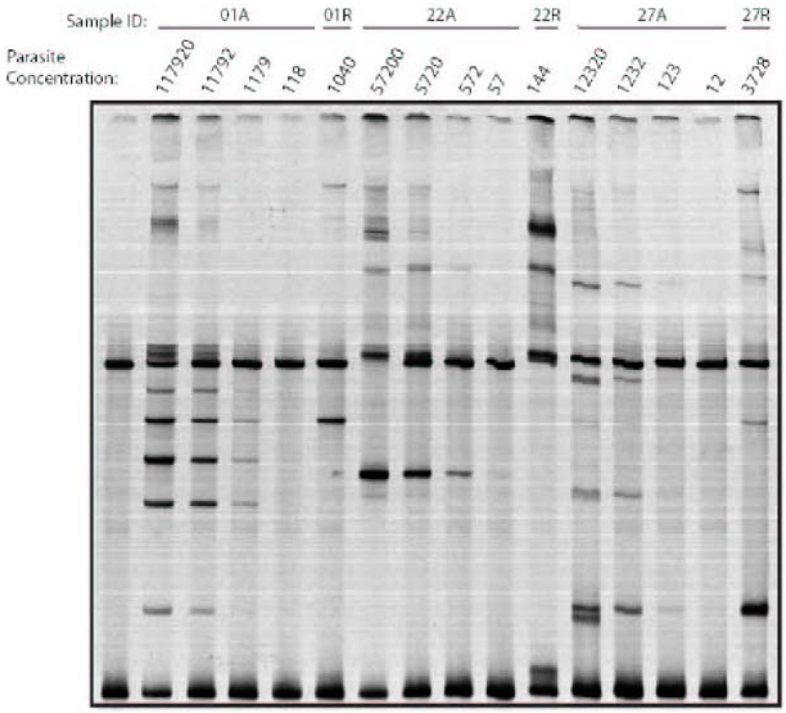


Figure 29. Autoradiogram of MSP1-HTA analysis using probe AF63. The probe was annealed with PCR products amplified from three paired admission and recurrent infection samples. The samples are identified by number which PCR products are used in each lane. The admission samples were diluted until reach to the parasitemia of their matched recurrent infection samples.

CHAPTER 6

DISCUSSION AND CONCLUSION

Malaria is an infectious disease transmitted by the bites of mosquitoes infected with any of four species of *Plasmodium* protozoa, namely *P.vivax, P. ovale, P. malariae* and *P. falciparum*. Among four species of *Plasmodium*, falciparum malaria can cause more serious problems, including heart, lung, kidney, or brain damage and possibly death (23). Falciparum malaria remains an important public health concern due to increasing resistance to common antimalarials and the high mortality rate (20). Clinical infections of *P. falciparum* are often composed of a mixture of parasite strains, especially in the endemic area (1). The mixture infection can be defined as the multiplicity of infection (MOI). Because a different strain of *P. falciparum* exhibits a unique genotype, therefore, MOI is resulting in the extent of genetic diversity in *P. falciparum* in each infection (5). The genetic diversity and the MOI represent one of the major problems of malaria pathogenesis and the acquisition of immunity and also of vaccine development (41, 42).

During malaria replication in the cells, a number of surface proteins of asexual stage merozoite display polymorphic forms (77). These proteins appear to play an important role in the process of red blood cell invasion (78). The major surface proteins include integral membrane proteins such as the merozoite surface protein 1 (*MSP1*) and the merozoite surface protein 2 (*MSP2*) (77). The glutamine rich protein (*GLURP*) is a peripheral membrane protein that associated with merozoite surface at the time of schizont rupture (77). Different strains of *P. falciparum* express different forms of these proteins. The gene encoding these three highly polymorphic proteins including MSP1, MSP2, and GLURP are the most frequently used as genetic markers to genotype field isolates of *P. falciparum* (5-8). These markers differ in the type of polymorphisms that they present.

In order to study molecular epidemiology of malaria infection, the researchers have to enumerate or to characterize the multiplicity of *P. falciparum* infections. Various molecular techniques based on polymerase chain reaction (PCR) have been employed to elucidate the genetic diversity of *P. falciparum* infections (79),(80). However, there are some limitations of using these techniques. For example, nested PCR and Restriction Fragment Length Polymorphism (RFLP) rely on electrophoretic migration of relatively small DNA fragments, which is only sensitive to changes of the fragment length. Moreover, fragments with a similar number of nucleotides but different sequences will co-migrate to the same distance, leading to genotype misinterpretations (12). Unlike nested PCR and RFLP, Single Strand Conformation Polymorphism (SSCP) can detect a single nucleotide difference, but it is technically demanding and is most effective only when fragment sizes are less than 150 bp (12). Although the direct DNA sequencing can be used for the detection of genetic variation, but it is expensive and requires additional cloning method (13). However, sequencing with or without cloning are tedious and time-consuming because some genotypes may exist in low level in the plasmid library. The low level of genetic variations is difficult to retrieve, particularly in population genetic studies, where large numbers of samples required analysis.

In contrast to the above techniques, heteroduplex tracking assay (HTA) can detect both clustered nucleotide mismatches and size polymorphisms. HTA is a screening method based on heteroduplex analysis (HDA), which designed to determine whether one or more variations of DNA sequence exist in a sample (12). HDA relies on the principle that heteroduplex molecules containing clusters of base mismatches or unpaired bases will be separated under particular electrophoretic conditions from almost identical molecule containing no mismatches (homoduplex). Heteroduplexes tend to migrate more slowly than homoduplexes in non-denaturing polyacrylamide gels. According to the characteristic of heteroduplexes, Delwart and colleagues (61) initially used HTA to analyze HIV-1 variation. Moreover, various HTAs have also been developed to study genetic diversity of other pathogenic microorganisms such as EBV (18), *Mycobacterium tuberculosis* (19).

Since the merozoite surface protein 1 is a candidate for a malaria vaccine (81), in the present study the *MSP1* gene has been chosen to be a genetic marker for analysis

of *P.falciparum* genetic diversity and for estimation of the MOI. The most striking polymorphisms of *MSP1* are resulting from repetitive sequences, where types of repeat units including their number and arrangement can be simply characterize different *P.falciparum* strains (43).

In the present study, HTA has been developed to analyse the *P. falciparum* genetic diversity. This developed technique is based on four major steps 1) PCR amplification of the polymorphic *MSP1* gene, 2) annealing the PCR products with a radiolabeled probe, 3) separation of heteroduplexes containing a radiolabeled probe by electrophoresis, and 4) visualization of heteroduplexes by exposure to X-ray film.

In the first part of experiments, instead of nested PCR, which requires additional time and materials, resulting in increasing risk for cross contamination between samples, a conventional PCR with minor modifications were used. The block 2 of *MSP1* was amplified by using HotstarTaq polymerase under PCR cycle condition as shown in Figure 11. It was found that the modified conventional PCR could specifically amplify the block 2 of *MSP1* of *P.falciparum* (Figure 18).

The four recombinant clones containing three different allelic families were generated in order to develop as probes in HTA analysis. Sequences of the block 2 of *MSP1* that are highly variable among the three major allelic families are K1-, MAD20- and RO33 types. The three alleles differ extensively in both repetitive and non-repetitive regions. In repetitive region, variation can occur in the presence or absence of 9-bp repeats, the number of the repeat, and the sequences of repeating units (76). The repetitive nature of *MSP1* makes it difficult to interpret sequence alignments and the derived genetic relatedness provided by the Clustal-X software, which is widely used for multiple alignments and for preparing phylogenetic trees. Therefore, allelic family assignments of four recombinant clones have been interpreted carefully as described in Chapter 5, section 3. The plasmid DNAs from four recombinant clones were double digested with *BamH1* and *Pst1*. After labeling with ^{35}S as described in Chapter 5, section 5.1, the digested products were used as probes. Four radiolabeled probes were named as the AF63 probe, the AF42 probe, the AF22 probe, and the MHP1452 probe. The AF63 probe and the MHP1452 probe were K1-type whereas the AF22 probe and the MHP1452 probe were MAD-20 type and RO33-type, respectively (Table 8).

The developed HTA for analysis of malaria genetic variations was further validated using plasmid DNA from four recombinant clones. The clones were used as DNA templates to amplify four different *MSP1* known sequences (Figure 19). These known sequences were then analyzed by HTA. The 6% non-denaturing polyacrylamide gel at 17 mA/gel for 4.5 hr was used to separate these known sequences. An overnight exposure to the X-ray film at 25ºC was sufficient for visualization of heteroduplexes (Figure 24). Due to the nature of the *MSP1* sequence, it is difficult to enumerate base pair mismatches and unpaired bases within heteroduplexes (76). The number of mismatch bases and unpaired bases required for a mobility shift has not been empirically defined in this study. However, pairwise alignment between the AF63 probe and three *MSP1* known sequences (Figure 21, Figure 22, and Figure 23) displayed more than 5% base pair mismatches and unpaired bases, resulting in a mobility shift of heteroduplexes (Figure 24). This finding could be supported by previous report in HIV study that a 5% sequence divergence between HIV variants is the detection threshold of HTA (82).

In Figure 24, the AF63 probe (K1-type) annealed with MAD20-type (AF22) showed a very fast migration whereas a very slow migration with the same type (MHP1452) was observed. This observation could be biochemically described that pairwise alignment between AF63 and MHP1452 displayed the large, centrally located insertion in MHP1452. The larger insertion, resulting in the kink formation in heteroduplex between AF63 and MHP1452 could have a more drastic affect on the heteroduplex migration than the smaller, more distally located insertion in AF22. Factors that affect on the mobility of heteroduplex are 1) the position of base pair mismatches and unpaired bases, and 2) the type of structural changes in heteroduplex (82). The degree of mobility change appears to be more influenced when base pair mismatches and unpaired bases occur in the middle of heteroduplex. Unpaired bases due to insertions/deletions that cause the formation of a kink in heteroduplex have grater effect than mismatched bases due to base substitutions that cause the formation of a bubble in heteroduplex (82). Within the mismatches, the degree of mobility change is likely not linear and appears to be influenced by type of mismatched base (82). Pyrimidine-pyrimidine mismatches effect on the mobility of heteroduplex more than purine-purine and purine-pyrimidine.

The AF63 probe showed a weak signal (faint band) at the position of MHP1452 in lane of a mixture of equal amounts of AF22, AF42, and MHP1452, whereas the probe showed a clear signal in lane of MHP1452 only (Figure 24). The weak signal had band intensity similar to background level of that found at several positions including the AF42 position in lane of MHP1452. The weak signal could be actually minor genotypes in patient blood or artifact bands. The experiment repeated by using the purified amplicons, which most PCR artifacts should be eliminated, showed that the MHP1452 band is still much fainter than the other bands. Additional experiment included an autoradiogram of the MHP1452 amplicon bound to the AF22 probe results in a similarly diffuse band (Figure 24, lane 10). Based on this evidence, it could be concluded that the diffuse band with low intensity is in fact the MHP1452 heteroduplex. The diffuse band might be due to the low homology between probe and target sequences.

In an initial analysis, 17 AF samples were selected to estimate for the MOI by HTA using four probes. The results showed that different probes displayed different heteroduplex mobility patterns (Figure 25) and different numbers of MOI (Table 9). The most likely source of the different number of MOI detected by the probes is the biochemistry of heteroduplex: the mobility patterns differ by the actual differences between probe and target. For example, subtle differences between probe and target, such as a single base-pair substitution or a mismatch, may not change the migration of the heteroduplexes, leading to the co-migration of multiple different sequences as a single band. Thus, the sequence of probe relative to the target sequence will effect to the estimation of MOI. Theoretically differences could originate from error during PCR amplification although PCR with the appropriate polymerase might reduce any artifact bands.

In the present study, the number of genotypes per infected person (MOI) was estimated from the number of distinct bands with at least 10% band intensity. The cut-off level of band intensity was used in order to either standardize HTA or exclude artifact bands. However, this criterion may lead to underestimate MOI since minor genotypes might not be included. Competing with major genotypes, which are responsible for clinical symptom, minor genotypes may not be successful amplified due to template competitions in PCR. Also, the amplification of all samples in

duplicate and running duplicate reactions side by side in PAGE were used to control for error during PCR step. All duplicates gave identical results (Figure 26). However, in future study suggestion of trivial blood collection should be considered in order to reduce this error.

In order to demonstrate that HTA was used to estimate the MOI in field samples, 39 samples were tested by HTA using the AF63 probe, which is more sensitive than other probes (Table 9). It was found that mean of MOI was 1.92 $\pm$ 0.13. Thirteen samples (33.3%) had one genotype, 18 samples (46.2%) had two genotypes, 6 samples (15.4%) had three genotypes, and 2 samples (5.1%) had four genotypes. Since falciparum infections are often composed of a mixture of *P.falciparum* strains (50, 83), the unexpected low MOI in AF samples (MOI = 1.9) comparing to the MOI in GR samples (MOI = 4.6) may occur due to in vitro cultivation. Some parasite strains may over grow (bias selection) and others may be lethal during in vitro cultivation (clone loss). Fewer strains are expected to be present in parasite culturing as compared to fresh blood samples. In addition to this limitation, PCR–HTA is also potentially limited by the sensitivity of PCR for detecting all genotypes present in an infection, especially when one strain is a minor component of a multiclonal infection.

Statistical analysis can be used to determine whether nested PCR and HTA do not give the same results, but it can't indicate that HTA is better than nested PCR because of lacking of the gold standard for estimation of MOI. In a direct comparison between HTA and nested PCR, HTA documented a greater amount of MOI than nested PCR. HTA allows direct analysis of various parasite strains in clinical isolates with or without in vitro culture. It is interesting to note that HTA only requires a single reaction of the amplification per sample, while the nested PCR requires six reactions of the amplification per sample indicating that nested PCR requires additional time and materials and leads to a high potential for cross-contamination between samples. Therefore, even if HTA and nested PCR show identical results in terms of MOI observed, the reduction in the number of reactions per sample makes HTA a more desirable and practical method to characterize *P.falciparum* genotype within an isolate than the nested PCR method. The assay can also detect the presence of minor parasite strains within infected patients in a manner more sensitive than an exhaustive and impractical DNA-sequencing.

In order to assess the agreement of the MOI estimated by HTA using the AF63 probe and nested PCR, the kappa coefficient (*k*) (72) was calculated by the SPSS statistical package. The results showed that the MOI estimated by HTA using the AF63 probe are not in agreement with that estimated by nested PCR (k = 0.262-0.396) (Table 12 and Table 13). One of the possible explanations is that nested PCR seems to underestimate the MOI because alleles with identical size but differing in sequence may not be distinguished and alleles present at a very low density in a patient blood with low parasitemia are likely to remain undetectable (84). In contrast to nested PCR, HTA seems to overestimate the MOI because of the higher sensitive radiolabeled probes.

Malaria is one of the most fatal infectious diseases. The major problems of malaria widespread resulted from many factors ranged from host factor, parasite genetic diversity and antimalarial drug resistance. All of these factors might result in treatment failure. Recurrent infection after antimalarial treatment could be true re-infection, or recrudescence from undetectable former parasite hidden somewhere in the body. Efficacy studies of antimalarial drugs rely mainly on the results of *in vivo* studies that assess clinical and parasitologic outcomes after treatment. These studies are limited by their inability to determine whether disease that recurs after treatment is due to recrudescence of resistant *P. falciparum* (parasites) or re-infection with new *P. falciparum* strains. Molecular genotyping methods such as nested PCR (85), restriction fragment length polymorphism (RFLP) (5, 10) or single strand conformation polymorphism (SSCP) (11) have been used increasingly to help in the elucidation of antitmalarial drug efficacy studies. Infecting of malaria parasites can be “fingerprinted” through PCR amplification of high polymorphic genes. The “fingerprint” patterns of the isolates obtained from before treatment and after parasite reappearance have been widely used to distinguish recrudescence from re-infection in falciparum infections.

Although molecular genotyping such as RFLP, SSCP has been used to differentiate recrudescence from re-infection in falciparum infections in many studies, the methodology and interpretation of the results have not been standardized (20). In particular, the definitions used to classify treatments as recrudescence or re-infections. *P.falciparum* infections are usually composed of a mixture of parasite strains. Due to

sequestration, strain competition, and clearance affect that related to either strain-specific immune response or treatment, disappearance or reappearance of *P.falciparum* called as clone fluctuation is observed during treatment (86) (87). Clone fluctuation causes difficulties in assessing polymorphisms and in distinguishing re-infection from recrudescence. A portion of parasite populations that sequestered in deep vascular tissues at the first sampling would appear in the peripheral blood in the following day. In this study, the samples were collected at two occasions; at the time of admission (A) and re-admission (R), failure of collecting blood samples on two consecutive days after admission (Day0, Day1, Day2) is critical for distinguishing recrudescence from re-infection. However, studies designed in this manner for such study to distinguish recrudescence from re-infection are costly and impractical.

In theory, the appearance of new genotypes could be either the result of re-infection or the detection of a minority parasite strain initially present earlier at an undetectable level (87). In contrast, the appearance of identical genotype was identified as either recrudescence or new infection with the same genotype as present in the admission day. Regardless to failure of repeated blood collection on two consecutive days after admission, a readmission sample contained the new genotypes; the reappearing parasites were considered a re-infection. When an infection was considered recrudescent if the heteroduplex mobility pattern obtained from readmission blood sample was identical to or contained within the admission pattern.

In GR samples collected from Kanchanaburi Province, a low transmission area where patients received less than 1 infective bite per year and could be expected to harbor close to 1 genotype per infection. The MOI with the mean of 4.6 is higher than expected. The possible explanation for the high MOI is that patients were infected with a single bite with a mixture of parasite strains. High level of drug resistance in the study area leads to a long duration of infection, remaining resistant parasites. Despite a small sample size of 17, the data presented here suggest that HTA is a less labor intensive and potentially more sensitive method to differentiate re-infection from recrudescence in falciparum infections.

The results suggest that HTA can effectively describe or resolve complex mixtures of *P. falciparum* strains and genetic relationships between *P. falciparum* strains that can exist sequentially and /or simultaneously within *P. falciparum* infected

patients can be rapidly evaluated. The power of a resolution to detect the number of genotypes per sample may be increased if a single locus of *MSP2* is used because *MSP2* seems more polymorphic than *MSP1* (88). Finally, the number of HTA probes used to genotype *P. falciparum* strains needs to be considered. If the experimental goal is to determine MOI, using multiple probes on each sample is recommended. However, based on the data presented herein, if the scientific goal is to determine if paired samples of before treatment and after parasite reappearance contain a similar complement of parasites, a single probe is sufficient.

In conclusion**,** HTA has been applied for falciparum malaria. The MSP1-HTA is a simple screening method with a high resolution for detecting and estimation of genetic divergence between *P. falciparum* strains. The assay is on the basis of the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gel proportional to their degree of divergence. The HTA is reproducible and sensitive to detect *P.falciparum* strains in patient blood samples, which contain multiple strains. Furthermore, the use of HTA to distinguish recrudescence from re-infection in *P. falciparum* infections will provide the useful information for the study of antimalarial drug efficacy.

REFFERNCES

1. Druilhe P, Daubersies P, Patarapotikul J, Gentil C, Chene L, Chongsuphajaisiddhi T, et al. A Primary Malarial Infection Is Composed of a Very Wide Range of Genetically Diverse but Related Parasites. J. Clin. Invest. 1998;101(9):2008-16.
2. Egan A, Chappel J, Burghaus P, Morris J, McBride J, Holder A, et al. Serum antibodies from malaria-exposed people recognize conserved epitopes formed by the two epidermal growth factor motifs of MSP1(19), the carboxy-terminal fragment of the major merozoite surface protein of *Plasmodium falciparum*. Infect. Immun. 1995;63(2):456-66.
3. Jouin H, Rogier C, Trape JF, Mercereau-Puijalon O. Fixed, epitope-specific, cytophilic antibody response to the polymorphic block 2 domain of the *Plasmodium falciparum* merozoite surface antigen MSP-1 in humans living in a malaria-endemic area. Eur J Immunol 2001;31(2):539-50.
4. Ekala M-T, Jouin H, Lekoulou F, Issifou S, Mercereau-Puijalon O, Ntoumi F. *Plasmodium falciparum* merozoite surface protein 1 (MSP1): genotyping and humoral responses to allele-specific variants. Acta Tropica 2002;81(1):33-46.
5. Brockman A, Paul R, Anderson T, Hackford I, Phaiphun L, Looareesuwan S, et al. Application of genetic markers to the identification of recrudescent *Plasmodium falciparum* infections on the northwestern border of Thailand. Am J Trop Med Hyg 1999;60(1):14-21.
6. Eisen D, Saul A, Fryauff D, Reeder J, Coppel R. Alterations in *Plasmodium falciparum* genotypes during sequential infections suggest the presence of strain specific immunity. Am J Trop Med Hyg 2002;67(1):8-16.

7. Cattamannchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G. Distinguishing Recrudescence from Reinfection in a longtitudinal antimalarial drug efficacy study: comparison of results based on Genotyping of MSP-1, MSP-2, and GLURP. Am J Trop Med Hyg 2003;68(2):133-139.
8. Aubouy A, Migot-Nabias F, Deloron P. Polymorphism in two merozoite surface proteins of Plasmodium falciparum isolates from Gabon. Malar J 2003;2(1):12.
9. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol 1986;51 Pt 1(Related Articles, Links):263-73.
10. Ohrt C, Mirabelli-Primdahl L, Karnasuta C, Chantakulkij S, Kain K. Distinguishing Plasmodium falciparum treatment failures from reinfections by restrictions fragment length polymorphism and polymerase chain reaction genotyping. Am J Trop Med Hyg 1997;57(4):430-7.
11. Kain KC, Craig AA, Ohrt C. Single-strand conformational polymorphism analysis differentiates Plasmodium falciparum treatment failures from re-infections. Mol Biochem Parasitol 1996;79(2):167-75.
12. Gasser RB. Mutation scanning methods for the analysis of parasite genes. Int J Parasitol 1997;27(12):1449-63.
13. Snounou G, Beck H. The Use of PCR Genotyping in the Assessment of Recrudescence or Reinfection after Antimalarial Drug Treatment. Parasitology Today 1998;14(11):462-467.
14. Snounou G, Viriyakosol S, Zhu X, Jarra W, Pinheiro L, do Rosario V, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993;61(2):315-20.
15. Delwart E, Sheppard H, Walker B, Goudsmit J, Mullins J. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. J. Virol. 1994;68(10):6672-83.
16. Nelson J, Fiscus S, Swanstrom R. Evolutionary variants of the human immunodeficiency virus type 1 V3 region characterized by using a heteroduplex tracking assay. J. Virol. 1997;71(11):8750-8.

17. Kitrinos KM, Hoffman NG, Nelson JAE, Swanstrom R. Turnover of env Variable Region 1 and 2 Genotypes in Subjects with Late-Stage Human Immunodeficiency Virus Type 1 Infection. J. Virol. 2003;77(12):6811-22.
18. Sitki-Green D, Edwards RH, Webster-Cyriaque J, Raab-Traub N. Identification of Epstein-Barr Virus Strain Variants in Hairy Leukoplakia and Peripheral Blood by Use of a Heteroduplex Tracking Assay. J. Virol. 2002;76(19):9645-56.
19. Williams D, Waguespack C, Eisenach K, Crawford J, Portaels F, Salfinger M, et al. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob Agents Chemother 1994;38(10):2380-6.
20. Bloland P. Drug Resistance in Malaria: WHO/CDS/CSR/DRS/2001.4; 2001.
21. RBM. What is Malaria?: World Health Organization Roll Back Malaria.
22. McCutchan T, Dame J, Miller L, Barnwell J. Evolutionary relatedness of Plasmodium species as determined by the structure of DNA. Science 1984;225(4664):808-11.
23. Markell EK, Voge M, John DT. Medical Parasitiology. 6th ed. Philadelphia: W.B. Saunders company; 1986.
24. Wirth DF, Cowman A. Mechanisms of drug resistance in protozoan parasites. In: Smith DF, Parsons M, editors. Molecular biology of parasite protozoa. Newyork: Oxford University press; 1996. p. 181-202.
25. WHOSEA. Malaria: Issues and Challenges:World Health Organization Southeast Asian Region; 2000.
26. Olliaro P. Mode of action and mechanisms of resistance for antimalarial drugs. Pharmacol Ther 2001;89(2):207-19.
27. Warhurst D. Antimalarial interaction with ferriprotoporphyrin IX monomer and its relationship to activity of blood schizonticides. Biochem Pharmachol 1987;30(24):3323-7.
28. Meshnick S. Artemisinin and related endoperoxides. In: Rosenthal P, editor. Antimalarial Chemotherapy: Mechanisms of Action, Modes of Resistance, and New Directions in Drug Development. Totowa, NJ: Humana Press; 2001. p. 191-201.

29. Baird K. Reurgent malaria at the millenium: control strategies in crisis. Drugs 2000;59:719-743.

30. Miller L, Hoffman S. Research towards vaccines against malaria. Nat.Med 1998;4:520-4.

31. Wongsrichanalai C, Pickard A, Wernsdorfer W, Meshnick S. Epidemiology of drug-resistant malaria. Lancet Infect Dis 2002;2(4):209-18.

32. Trape J, Pison G, Preziosi M, Enel C, Desgrees DLA, Delaunay X, et al. Impact of chloroquine resistance on malaria mortality. CR Acad Sci III 1998;321(8):689-97.

33. Sangster NC. Managing parasiticide resistance. Veterinary Parasitology 2001;98(1-3):89-109.

34. WHO. Monitoring Antimalarial Drug Resistance. Geneva: WHO consultation; 2001.

35. Rieckmann K, Campbell G, Sax L, Mrema J. Drug sensitivity of plasmodium falciparum. An in-vitro microtechnique. Lancet 1978;1(8054):22-3.

36. Basco LK, Ringwald P. Molecular epidemiology of malaria in Yaounde, Cameroon. VII. Analysis of recrudescence and reinfection in patients with uncomplicated falciparum malaria. Am J Trop Med Hyg 2000;63(5-6):215-21.

37. Magesa S, Mdira K, Farnert A, Simonsen P, Bygbjerg I, Jakobsen P. Distinguishing Plasmodium falciparum treatment failures from re-infections by using polymerase chain reaction genotyping in a holoendemic area in northeastern Tanzania. Am J Trop Med Hyg 2001;65(5):477-83.

38. Wernsdorfer W. Epidemioligy of drug resistance in malaria. Acta. Trop 1994;56:143-56.

39. Conway D, Rosario V, Oduola AMJ, Salado LA, Greenwood B, McBride JS. Plasmodium falciparum: intragenic recombination and nonrandom associations between polymorphic domains of the precursor to the major merozoite surface antigens. Exp Parasitol 1991;73:469-80.

40. Miller LH, Roberts T, Shahabuddin M, McCutchan TF. Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP 1). Mol Biochem Parasitol 1993;59(1):1-14.

41. Beck S. Multiplicity of *Plasmodium falciparum* infections in pregnancy. Am J Trop Med Hyg 2001;65:631-6.

42. Smith T. Age dependence of the multiplicity of *Plasmodium falciparum* infections and of other malariological indices in an area of high endemicity. Trans R Soc Trop Med Hyg 1999;93:15-20.

43. Tanabe K, Mackay M, Gaman M, Scaife J. Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. J. Mol. Biol. 1987;195(2):273-87.

44. Jiang G, Daubenberger C, Huber W, Matile H, Tanner M, Pluschke G. Sequence diversity of the merozoite surface protein 1 of Plasmodium falciparum in clinical isolates from the Kilombero District, Tanzania. Acta Trop 2000;74(1):51-61.

45. Hoffmann E, Ribolla P, Ferreira M. Genetic relatedness of Plasmodium falciparum isolates and the origin of allelic diversity at the merozoite surface protein-1 (MSP-1) locus in Brazil and Vietnam. Malaria Journal 2003;2(1):24.

46. Ferreira M, Ribeiro W, Tonon A, Kawamoto F, Rich S. Sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-1 (MSP-1) of Plasmodium falciparum. Gene 2003;304:65-75.

47. Cavanagh DR, Elhassan IM, Roper C, Robinson VJ, Giha H, Holder AA, et al. A Longitudinal Study of Type-Specific Antibody Responses to Plasmodium falciparum Merozoite Surface Protein-1 in an Area of Unstable Malaria in Sudan. J Immunol 1998;161(1):347-359.

48. Saul A, Lord R, Jones G, Geysen H, Gale J, Mollard R. Cross-reactivity of antibody against an epitope of the *Plasmodium falciparum second merozoite surface antigen*. Parasite Immunol 1989;11:593-601.

49. Saul A, Lord R, Jones G, Spencer L. Protective immunization with invariant peptides of the Plasmodium falciparum antigen MSA2. J Immunol 1992;148:208-11.

50. Snewin VA, Herrera M, Sanchez G, Scherf A, Langsley G, Herrera S. Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in Plasmodium falciparum wild isolates from Colombia. Mol Biochem Parasitol 1991;49(2):265-75.

51. Felger I, Tavul L, Kabintik S, Marshall V, Genton B, Alpers M, et al. Plasmodium falciparum: extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. Exp Parasitol 1994;79(2):106-16.

52. Prescott N. Plasmodium falciparum genetic diversity can be characterised using the polymorphic merozoite surface antigen 2 (MSA-2) gene as a single locus marker. Mol Biochem Parasitol 1994;63:203-12.

53. Smythe J, Coppel R, Brown G, Ramasamy R, Kemp D, Anders R. Identification of two integral membrane proteins of Plasmodium falciparum. Proc Natl Acad Sci U S A 1988;85(14):5195–99.

54. Smythe J, Peterson M, Coppel R, Saul A, Kemp D, Anders R. Structural diversity in the 45-kilodalton merozoite surface antigen of Plasmodium falciparum. Mol Biochem Parasitol 1990;39(2):227-34.

55. Borre M, Dziegiel M, Hogh B, Petersen E, Rieneck K, Riley E, et al. Primary structure and localization of a conserved immunogenic Plasmodium falciparum glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. Mol Biochem Parasitol 1991;49(1):119-31.

56. Stricker Kd, Vuust J, Jepsen S, Oeuvray C, Theisen M. Conservation and heterogeneity of the glutamate-rich protein (GLURP) among field isolates and laboratory lines of Plasmodium falciparum. Mol Biochem Parasitol 2000;111(1):123-30.

57. Snounou G. Genotyping of Plasmodium spp., Nested PCR. In: Doolan DL, editor. Malaria Methods and Protocols. New Jersey: Human Press Inc.; 2002. p. 103-16.

58. Van Ha N, Dyk Dao L, Rabinovich SA. Use of nested PCR for differential diagnosis of falciparum malaria reinfection and relapse in drug-resistant patients. Bull Exp Biol Med 2002;134(4):379-81.

59. Sanger F, Nicklen S, Coulson A. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463-7.

60. Maxam AM, Gilbert W. Sequencing End-Labeled DNA with Base Specific Chemical Cleavages. Meth. Enzymol 1980;65:499.

61. Delwart E, Shpaer E, Louwagie J, McCutchan F, Grez M, Rubsamen-Waigmann H, et al. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science: 1993;262(5137):1257-61.

62. Barlow K, Green J, Clewley J. Viral genome characterisation by the heteroduplex mobility and heteroduplex tracking assays. Rev Med Virol 2000;10(5):321-35.

63. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, et al. Resistance to Antimalarials in Southeast Asia and Genetic Polymorphisms in pfmdr1. Antimicrob. Agents Chemother. 2003;47(8):2418-23.

64. Trager W, Gensen J. Human malaria parasites in continuous culture. Science 1976;193(4254):673-75.

65. Kamwendo D, Dzinjalamala F, Snounou G, Kanjala M, Mhango C, Molyneux M, et al. Plasmodium falciparum: PCR detection and genotyping of isolates from peripheral, placental, and cord blood of pregnant Malawian women and their infants. Trans R Soc Trop Med Hyg 2002;96(2):145-9.

66. Ferreira M, Liu Q, Kaneko O, Kimura M, Tanabe K, Kimura E, et al. Allelic diversity at the merozoite surface protein-1 locus of Plasmodium falciparum in clinical isolates from the southwestern Brazilian Amazon. Am J Trop Med Hyg 1998;59(3):474-80.

67. Sambrook J, Fitsch E, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Press; 1989.

68. Mwapasa V, Rogerson S, Molyneux M, Abrams E, Kamwendo D, Lema V, et al. The effect of Plasmodium falciparum malaria on peripheral and placental HIV-1 RNA concentrations in pregnant Malawian women. AIDS. 2004;18(7):1051-9.

69. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. Multiple sequence alignment with Clustal X. Trends in Biochemical Sciences 1998;23(10):403-5.

70. Nicholas KB, Nicholas HBJ, Deerfield DWI. GeneDoc: Analysis and Visualization of Genetic Variation. EMBNEW.NEWS 1997;4:14.

71. Perriere G, Gouy M. WWW-query: An on-line retrieval system for biological sequence banks. Biochimie 1996;78(5):364-369.

72. Cohen J. A coefficient of agreement for nominal scales. Educ Psychol Meas 1960;20:37-46.

73. Landis J, Koch G. The measurement of observer agreement for categorical data. Biometrics 1977;33:153-174.

74. Nei M, Li W. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci U S A 1979;76(10):5269–73.

75. Kimura E, Mattei D, di Santi S, Scherf A. Genetic diversity in the major merozoite surface antigen of Plasmodium falciparum: high prevalence of a third polymorphic form detected in strains derived from malaria patients. Gene 1990;91(1):57-62.

76. Hartl DL, Volkman SK, Nielsen KM, Barry AE, Day KP, Wirth DF, et al. The paradoxical population genetics of Plasmodium falciparum. Trends in Parasitology 2002;18(6):266-72.

77. Berzins K, Anders R. The Malaria Antigens. In: Wahlgren M, Perlmann P, editors. Malaria: Molecular and Clinical Aspects. Amsterdam: harwood academic publishers; 1999. p. 181-216.

78. Chitnis EC, Sinnis P, Miller LH. The sporozoite, the merozoite, and the infected red cell: parasite ligands and host receptors. In: Wahlgren M, Perlmann P, editors. Malaria: Molecular and Clinical Aspects. Amsterdam: harwood academic publishers; 1999. p. 249-285.

79. Peyerl-Hoffmann G, Jelinek T, Kilian A, Kabagambe G, Metzger WG, von Sonnenburg F. Genetic diversity of Plasmodium falciparum and its relationship to parasite density in an area with different malaria endemicities in West Uganda. Trop Med Int Health 2001;6(8):607-13.

80. Nzila AM, Mberu EK, Nduati E, Ross A, Watkins WM, Sibley CH. Genetic diversity of Plasmodium falciparum parasites from Kenya is not affected by antifolate drug selection. Int J Parasitol 2002;32(12):1469-76.

81. Rogers WO, Hoffman SL. Malaria Vaccines. In: Wahlgren M, Perlmann P, editors. Malaria: Molecular and Clinical Aspects. Amsterdam: harwood academic publishers; 1999. p. 439-493.

82. Upchurch DA, Shankarappa R, Mullins JI. Position and degree of mismatches and the mobility of DNA heteroduplexes. Nucleic Acids Res 2000;28(12):E69.

83. Daubersies P, Sallenave-Sales S, Magne S, Trape J, Contamin H, Fandeur T, et al. Rapid turnover of Plasmodium falciparum populations in asymptomatic individuals living in a high transmission area. Am J Trop Med Hyg 1996; 54:18-26.

84. Chaparro J, Rojas MO, Wasserman M. Plasmodium falciparum: underestimation of dihydrofolate reductase and dihydropteroate synthase polymorphism in field samples: a technical shortcoming of nested PCR assays with mutation-specific primers. Exp Parasitol 2001;99(3):115-22.

85. Snounou G, Singh B. Nested PCR analysis of Plasmodium parasites. Methods Mol Med 2002;72:189-203.

86. Farnert A, Snounou G, Rooth I, Bjorkman A. Daily dynamics of Plasmodium falciparum subpopulations in asymptomatic children in a holoendemic area. Am J Trop Med Hyg 1997;56(5):538-47.

87. Jafari S, Le Bras J, Bouchaud O, Durand R. Plasmodium falciparum clonal population dynamics during malaria treatment. J Infect Dis 2004;189(2):195-203.

88. Paul REL, Brockman A, Price RN, Luxemburger C, White NJ, Looareesuwan S, et al. Genetic analysis of Plasmodium falciparum infections on the north-western border of Thailand. Trans. Roy. Soc. Trop. Med. Hyg. 1999;93(6):587-93.

APPENDIX

APPENDIX

1. *In vitro* cultivation of *Plasmodium falciparum* strain 3D7

1.1 Materials

1.1.1 Incomplete RPMI 1640 medium

The medium consisted of 10.43 g RPMI 1640, 5 g HEPES buffer, 2 g sodium bicarbonate, 50 mg hypoxanthine and 2 g glucose in 1000 ml of deionized water and the pH adjusted to 7.2. The medium was passed through Millipores filter (0.22 μm) and kept at 4°C before use.

1.1.2 Human Sera

The AB-positive clotted blood from several bags are pooled and centrifuged to remove any red cells. The serum is heat-inactivated at 56^{o}C for 1 hour before being aliquoted into 40ml falcon tubes and stored at -70^{o}C. Sterile technique during preparation is very important.

1.1.3 Complete Culture Medium (CCM)

The media was prepared by mixing 90 ml of incomplete RPMI 1640 medium and 10 ml RPMI 1640 medium and 10 ml of heat inactivated AB-positive human serum and Gentamycin solution (Sigma) was added to give a final concentration of 10-μgml^{-1}.

1.1.4 Human red blood cells

The group “O-positive” red blood cells are obtained from the whole blood. The blood was washed to remove the anticoagulant before use.

1.2 Recultivation of *Plasmodium falciparum*

The cryogenic *Plasmodium falciparum* strain 3D7 will be removed from the liquid nitrogen tank and placed upright in water bath at 37^{o}C. The suspension will be

immediately transferred into a 50 ml sterile tube. NaCl, 12%, will be added by slow dropwise using a 1 ml syringe with 18 G needle to the volume of 0.2 x volume of thawing blood. The suspension will be left for 5 min and then 1.6 % NaCl will be added by fast dropwise using 10 ml syrinnge and 18 G needle to 10 x volume with 0.9 % NaCl by slow dropwise. Centrifugation at 1500 g for 10 minutes to remove the supernatant and the cells will be suspended with an equal volume of RPMI complete media media containing 10% human AB-serum to make a 50% suspension. *In vitro* continuous culture (candle jar Method) will be performed.

1.3 In vitro continuous culture of *Plasmodium falciparum* (candle jar Method)

The infected blood suspensions obtained from recultivation of *Plasmodium falciparum* strain 3D7 was diluted with RPMI complete media at ratio 1:5. The culture materials were dispensed in 4-ml volume into 30-mm sterile petri-dishes. The dishes were placed in an airtight candle jar containing a lighted paraffin candle. The lid of candle jar was closed well (sealed with silicone grease) while the stopcock was opened. As soon as the candle went out and the atmosphere inside the candle jar is approximately 17% O_2, 3% CO_2 and 80% N_2, the stopcock was closed. The candle jar was incubated at 37^{o}C. Every day the culture medium was changed by gently tipping the dish and pipetting off the medium with a sterile Pasteur pipette. The RPMI complete media was added back to each dish. The cells were resuspened by swirling the dish gently and placed in the candle jar.

Thin films were prepared and stained using 5% Giemsa in phos-phate buffer (pH = 7.2) for 30 min and examined to determine the growth and parasite density of the cultures every 48 hr. When the parasitemia exceeded 4%, fresh non-infected erythrocytes were used to dilute the cultures to be 1% parasitemia. The continuous culture will be performed as described above.

2. Blunt Cloning

2.1 Carbenicilin solution (100 μg/ml)

Dissolve 1g-Carbenicilin (Sigma) in 10ml water. Filter sterilize and aliquot in 1ml amounts. Store frozen at -20^{o}C.

2.2 Tetracycline solution (50μg/ml)

Dissolve 1g-Tetracycline (Sigma) in 10ml water. Filter sterilize and aliquot in 1ml amounts. Store frozen at -20^{o}C.

2.3 LB Broth containing Carbenicilin and Tetracycline

Ten grams of tryptone (Gibco), five grams of yeast extract (Gibco), and ten grams of sodium chloride were completely dissolved in one liter of distilled water. The media was sterilized in an autoclave at 15 lb/inch^2 (psi) at 121°C for 15 minutes. Let it cool to 50•C. Add 100 μg/ml of Carbenicilin solution and 50μg/ml of Tetracycline solution per liter sterile LB. Proper aliquots were made and kept at 4°C until used.

2.4 LB Agar containing Carbenicilin and Tetracycline

Ten grams of tryptone (Gibco), five grams of yeast extract (Gibco), ten grams of sodium chloride, and fifteen grams of agar were completely dissolved in one liter of distilled water. Dissolve agar in microwave and sterile in an autoclave at 15 lb/inch^2 (psi) at 121°C for 15 minutes. Let it cool to 50•C. Add 100 μg/ml of Carbenicilin solution and 50μg/ml of Tetracycline solution per liter sterile LB agar. The medium was approximately poured into sterile petridishes, dried, and kept at 4°C until used.

2.5 IPTG (Isopropyl-β-D-thiogalactopyranoside) (20% w/v, 0.8 M)

Dissolve 2 g-IPTG (Sigma) in 8 ml of distilled water. Adjust the volume of the solution to 10 ml with H_2O and sterilize by passing through a 0.22-μm disposable filter. Dispense the solution into 1-ml aliquots and store them at 20^{o}C.

2.6 X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (2% w/v)

Dissolve X-gal (Sigma) in dimethylformamide at a concentration of 20 mg/ml solution. Use a glass or polypropylene tube. Wrap the tube containing the solution in aluminum foil for light protection and store at 20^{o}C.

3. Probe labeling

3.1 Tris-HCl (1M, pH 8.0)

Dissolve 60.55 g of Tris base in 400 ml of triple distilled water. Adjust the pH to 8.0 by adding concentrated HCl and add triple distilled water to 500 ml. Sterilize by autoclaving and store the solution at room temperature.

3.2 Tris EDTA (TE) buffer

TE buffer was prepared from the stock Tris-HCl (pH 8.0) and EDTA (pH 8.0) to the final concentrations of 10 mM and 1 mM, respectively. The solution was kept at room temperature.

3.3 Dithiothreitol (DTT, 1M)

Dissolve 3.09 g-DTT (Sigma) in 20 ml of 0.01 M sodium acetate (pH 5.2) and sterilize by filtration. Dispense the solution into 1-ml aliquots and store them at 20^{o}C.

3.4 dGTT (10mM)

Dissolve 10 mg (Boehringe Mannheim)) in 1804 μl of TE (pH 7.0). Dispense the solution into 50 μl aliquots and store them at 20^{o}C.

3.5 Disodium Ethylenediaminetetraacetate (EDTA, 0.5M, pH 8.0)

Dissolve 186.12 g of EDTA in 800 ml of distilled water. Adjust the pH to 8.0 and add distilled water to 1 liter. Sterilize by autoclaving and store the solution at room temperature.

4. PCR-HTA for *P. falciparum*

4.1 Tris-Borate-EDTA (TBE, 10X)

Dissolve 108 g of Tris base, 55 g of Boric acid and 9.3 g of EDTA in 1 liter of distilled water. No adjustment required to the pH 8.3. Filter and store the buffer at room temperature.

4.2 Tris-HCl (1M, pH7.4)

Dissolve 121.1 g of Tris base in 800 ml of distilled water. Adjust the pH to 7.4 by adding 70 ml of concentrated HCl and add distilled water to 1 liter. Sterilize by autoclaving and store the solution at room temperature.

4.3 Annealing buffer (10X)

Dissolve 0.584 g of NaCl in 5 ml of distilled water. Add 1 ml of Tris-Cl (1M, pH7.4), and 0.4 ml of EDTA (0.5M pH 8.0). Adjust the volume of the solution to 10 ml with distilled water. Filter sterilize and store in aliquots at 20^{o}C.

4.4 DNA Loading Buffer (6X)

0.25% (w/v) Bromophenol Blue

0.25% (w/v) Xylene Cyanol FF

30% (v/v) Glycerol in H_2O

Store the solution at 4^{o}C.

4.5 Ethidium Bromide Stock Solution (10 mg/ml)

Dissolve 100 mg of ethidium bromide in 10 ml sterile ddH2O and dilute to 0.5μg/ml when using. Wrap tube in aluminum foil and store at 4^{o}C.

BIOGRAPHY

NAME	Miss. Warunee Ngrenngarmlert
DATE OF BIRTH	December 11, 1963
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTION ATTENDED	Mahidol University, 1982-1986 B.Sc. (Med. Tech.) Mahidol University, 1991-1993 M.Sc. (Trop. Med.) Mahidol University, 2001-2005 Ph.D. (Med. Tech.)
REREARCH GRANTS	The scholarship under The Commission on Higher Education Staff Development Project Partially supported by NIH grants (R23 AI054590) Supported in part by the Thesis Grant, Faculty of Graduate Studies, Mahidol University
POSITION&OFFICE	Department of Parasitology, Faculty of Medical technology, Mahidol University, Bangkok, Thailand (Tel. 02 4197170) Position: Lecturer E-mail: waruneeng@yahoo.com
HOME ADDRESS	104/8 Wuttakard Rd., Bangkaw, Jomthong, Bangkok, Thailand (Tel. 02 4688449)