การวิเคราะห์การกลายพันธุ์และแฮปโลทัยป์ของยีนสร้างกลูโคส -6- ฟอสเฟต ดีไฮโดรจีเนสในประชากรเอเชียตะวันออกเฉียงใต้

นางสาวชาลิสา หลุยเจริญ

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MUTATIONS AND HAPLOTYPE ANALYSIS OF THE *G6PD* LOCUS IN THE SOUTHEAST ASIA POPULATION

Miss Chalisa Louicharoen

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Medical Science

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ชาลิสา หลุยเจริญ : การวิเคราะห์การกลายพันธุ์และแฮปโปลทัยป์ของยีนสร้างกลูโคส -6- ฟอสเฟต ดีไฮโดรจีเนสในประชากรเอเชียตะวันออกเฉียงใต้ (MUTATIONS AND HAPLOTYPE ANALYSIS OF THE *G6PD* LOCUS IN THE SOUTHEAST ASIA POPULATION) อ. ที่ปรึกษา: รศ.นพ. อิศรางค์ นุช ประยูร. 88 หน้า. ISBN 974-17-4116-2.

ภาวะพร่องเอ็นซัยม์กลูโคส -6- ฟอสเฟต ดีไฮโดรจีเนส (G6PD) เป็นโรคทางพันธุกรรมที่เกิดจากความผิดปกติของเอ็นซัยม์ G6PD ซึ่งเป็นภาวะที่พบได้บ่อยในประชากรเอเชียตะวันออกเฉียงใต้ โดยมีสาเหตุมาจากการกลายพันธุ์ของยีน *G6PD* จากการศึกษาก่อนหน้านี้พบว่า G6PD เวียงจันทน์ (871G → A) เป็นการกลายพันธุ์ที่พบได้บ่อยที่สุดในประชากรเอเชียตะวันออกเฉียงใต้ในการทดลองนี้เราได้ศึกษาการกลายพันธุ์และ polymorphism ของประชากรเอเชียตะวันออกเฉียงใต้ เพื่อหาความสัมพันธ์ด้านพันธุกรรมของประชากรในภูมิภาคเอเชียตะวันออกเฉียงใต้ โดยการคัดกรองผู้ที่มีภาวะพร่อง G6PD จากตัวอย่างเลือดที่เก็บจากเด็กแรกเกิดและเด็กที่ตัวเหลืองจากโรงพยาบาลบุรีรัมย์จำนวน 172 และ 195 รายตามลำดับ, เด็กที่ตัวเหลืองจากโรงพยาบาลจุฬาลงกรณ์จำนวน 303 ราย นอกจากนี้ยังเก็บตัวอย่างเลือดจากแรงงานอพยพที่จังหวัดจันทบุรีจำนวน 195 รายและ 78 รายจากผู้ป่วยแรงงานพม่าที่โรงพยาบาลสมุทรสาคร หลังจากนั้นศึกษาการกลายพันธุ์และ polymorphisms ของยีน *G6PD* ในผู้ที่มีภาวะพร่องเอ็นไซม์ G6PD ด้วยวิธี PCR-RFLP

ผลการทดลองพบว่าความชุกของผู้ที่มีภาวะพร่องเอ็นซัยม์ G6PD ในชาวเขมร, ไทย และลาวคือ 18.5,21.4 และ 17.4% ตามลำดับ ซึ่งจัดว่าสูงกว่าในชาวพม่าที่มีความชุกของผู้ที่มีภาวะพร่องเอ็นซัยม์ G6PD เพียง 9.7% ส่วนความชุกของผู้ที่มีภาวะพร่องเอ็นซัยม์ G6PD ในเด็กที่ตัวเหลืองชาวเขมร, ไทย และลาวคือ 25.0, 16.0 และ 20.5% ตามลำดับ และเมื่อศึกษาการกลายพันธุ์ของยีน *G6PD* พบว่าชาวเขมร, ไทย และลาว มีการกลายพันธุ์แบบ G6PD เวียงจันทน์มากที่สุดคือ 82.4, 47.7 และ 46.2% ตามลำดับ แต่การกลายพันธุ์ดังกล่าวไม่พบเลยในชาวพม่า แต่จะพบการกลายพันธุ์แบบ G6PD มหิดลเป็นส่วนใหญ่ในชาวพม่า (62.5% ของชาวพม่าที่มีภาวะพร่องเอ็นซัยม์ G6PD ทั้งหมด) และพบเล็กน้อยในชาวไทย (4.5% ของชาวไทยมีภาวะพร่องเอ็นซัยม์ G6PD ทั้งหมด) แต่ไม่พบเลยในชาวเขมรและชาวลาว จากการศึกษา polymorphisms ของ G6PD เวียงจันทน์พบรูปแบบ haplotype 2 รูปแบบคือ -,-,+,+,+,+ (*Pvu* II: 611C → G, *Sca* I: 175C → T, *Bsp*H I: 163C → T, *Pst* I: 1116G → A, *Bcl* I: 1311C → T, *Nla* III: 93T → C) (46 จาก 49 ราย; 93.9%) ซึ่งพบได้ในคนปกติ (43 จาก 202 ราย; 21.3%)(p <0.0001) และ haplotype อีกรูปแบบหนึ่งคือ -,-,+,+,+,- (3 จาก 49 ราย; 6.1%) และเมื่อหาความสัมพันธ์ระหว่างตำแหน่งกลายพันธุ์และ polymorphism พบว่า G6PD เวียงจันทน์มีความสัมพันธ์กับ 1311T และ 93C อย่างมีนัยสำคัญทางสถิติ ขณะที่การกลายพันธุ์แบบอื่นๆจะมีรูปแบบ haplotype เพียงแบบเดียวคือ -,-,+,+,-,- ซึ่งพบได้เป็นส่วนใหญ่ของคนปกติ (141 จาก 202; 69.8%)

G6PD เวียงจันทน์เป็นการกลายพันธุ์ที่พบได้บ่อยในผู้ที่มีภาวะพร่องเอ็นซัยม์ G6PD ที่เป็นชาวเขมร, ไทย และลาว โดยมีความถี่ของ allele คือ 0.10, 0.06 และ 0.06 ตามลำดับ และพบว่าตำแหน่ง 1311T เป็น polymorphism บรรพบุรุษของ G6PD เวียงจันทน์

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต……...…….....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษา……...……...……

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KEY WORDS: GLUCOSE-6-PHOSPHATE DEHYDROGENASE / RFLP HAPLOTYPE / SOUTHEAST ASIAN

CHALISA LOUICHAROEN : MUTATIONS AND HAPLOTYPE ANALYSIS OF THE *G6PD* LOCUS IN THE SOUTHEAST ASIA POPULATION. THESIS ADVISOR: ASSOC. PROF. ISSARANG NUCHPRAYOON, M.D., PH.D. 88 pp. ISBN 974-17-4116-2.

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the common hereditary enzymopathy in Southeast Asian caused by mutation of *G6PD*. G6PD Viangchan (871G → A) is the most common mutation in Southeast Asian. We studied G6PD mutations and polymorphisms in Southeast Asian to clarify the genetic relationship between them.

Cord blood samples from 172 normal neonates and 195 jaundiced newborns at Buriram Hospital, 303 jaundiced newborns at King Chulalongkorn Memorial Hospital and peripheral blood samples from 195 migrant Cambodian, Laotian, and Myanmars laborers at Chunthaburi and 78 migrant Myanmars laborers admitted to the Samutsakhon Hospital were assayed for G6PD activity. The prevalence of G6PD deficiency was high in Cambodians (17 of 92 males; 18.5%), Thais (3 of 14 males) and Laotians (12 of 69 males; 17.4%) but was less in Myanmars (7 of 72 males; 9.7%). Among the jaundiced newborns, the prevalence of G6PD deficiency was very high in Cambodians (14 of 56 males; 25.0%), Thai (30 of 188 males; 16.0%) and Laotian (8 of 39 males; 20.5%). PCR-RFLP method was used to identify *G6PD* mutations in G6PD deficient samples and do haplotype analysis of the *G6PD* locus. G6PD Viangchan (871G>A) was the most common mutation among Cambodian (28 of 34; 82.4%), Thai (21 of 44; 47.7%) and Laotian (12 of 26; 46.2%) but not Myanmars (0 of 8) G6PD deficiency. G6PD Mahidol was the prevalent mutation in Myanmars (5 of 8; 62.5%), Thai (2 of 44; 4.5%) but not found in Cambodian or Laotian. The haplotype of G6PD Viangchan had 2 forms consisted of -,-,+,+,+,+ (*Pvu* II: 611C → G, *Sca* I: 175C → T, *Bsp*H I: 163C → T, *Pst* I: 1116G → A, *Bcl* I: 1311C → T, *Nla* III: 93T → C)(46 of 49; 93.9%),which similar to 21.3% (N = 202)($p < 0.0001$) of G6PD normal and another form is -,-,+,+,+,-(3 of 49; 6.1%). G6PD Viangchan associates with 1311T and 93C significantly. While haplotype of other G6PD mutations and 69.8% of normal was -,-,+,+,-,-.

G6PD Viangchan is the most common G6PD deficient mutation among Cambodian, Thai and Laotian with allele frequency of 0.10, 0.06 and 0.06 respectively. The 1311T allele is the ancestral polymorphism of G6PD Viangchan.

Field of study Medical Science Student's signature……
Academic year 2003 Advisor's signature……...

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LIST OF ABBREVIATIONS

ACD	acid – citrate – dextrose
bp	base pairs
°C	degree Celsius
cm	centimeter
DNA	deoxyribonucleic acid
dNTPs	dATP, dTTP, dGTP, dCTP
EDTA	ethylenediamine tetraacetic acid
F	forward primer
g	gram (s)
G6PD	glulose-6-phosphate dehydrogenase
Hb	hemoglobin
I.U.	International unit
IVS	intervening sequence
kb	kilobase
M	molar
min	minute
ml	millilitre
mM	millimolar
n	number
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometer
nt	nucleotide
OD	optical density
PCR	polymerase chain reaction
pmol	picomole
R	reward primer
RBC	red blood cell
RFLP	restriction fragment length polymorphism

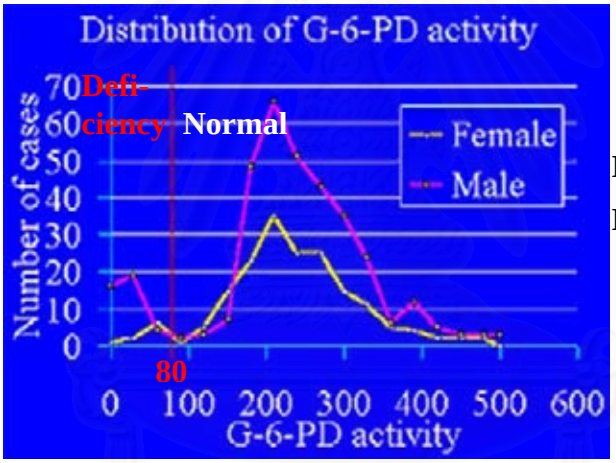
rpm	revolution per minute
sec	second
SD	standard deviation
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_m	temperature
Tris-HCl	tris-(hydroxymethyl)-aminoethane
U	unit
µg	microgram
µl	microlitre
µmol	micromole
UV	ultraviolet
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1. Background and Rationale

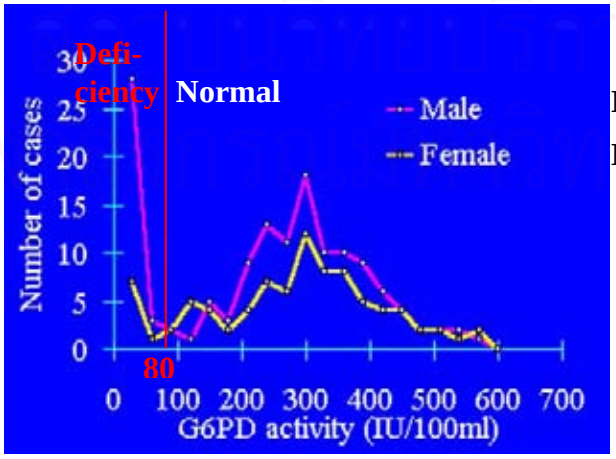
Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most frequent hereditary enzyme abnormalities[1]. Inherited deficiency of this enzyme cause hemolytic anemia[2,3]. G6PD deficiency is the major problem of public health[4]. The prevalence of G6PD deficiency is 11.1% in Thai male neonates and 5.8% in female neonates (figure 1). Among the neonates with hyperbilirubinemia, the prevalence of G6PD deficiency is 22.1% in males and 10.1% in females (figure 2)[5].



N= 179

N= 350

Figure 1 Prevalence of G6PD deficiency in Thai newborn (cord blood)[5].



N= 140

N= 89

Figure 2 Prevalence of G6PD deficient Thai hyperbilirubinemia newborn[5].

The distribution of G6PD deficiency is highly correlated with the distribution of current or past malaria endemicity (figure 3 and 4). This observation supported the hypothesis that G6PD deficiency confers reduced risk from infection by the *Plasmodium* parasite, in other words, G6PD is a selective advantage for people to resist malaria.

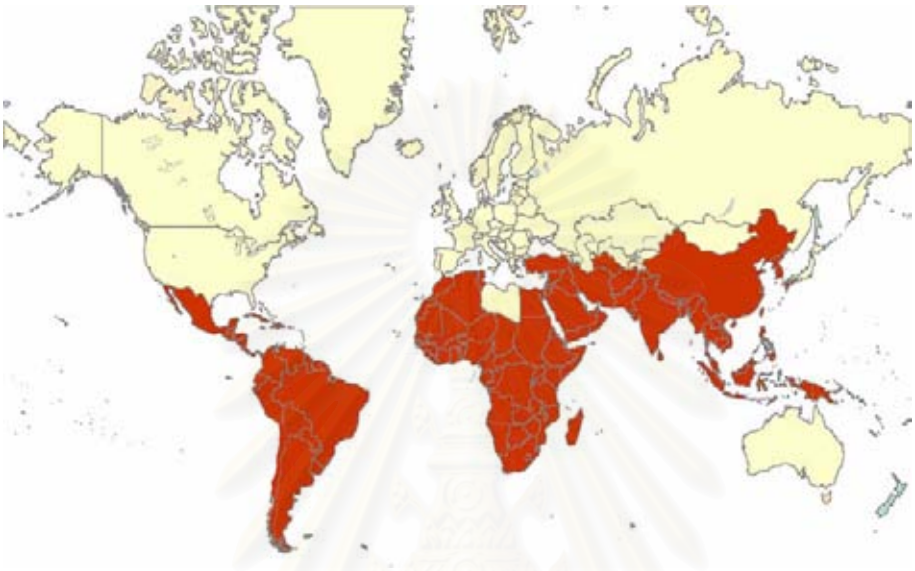


Figure 3 World map represented the population lives in countries (in gray on map) where malaria epidemics[6].

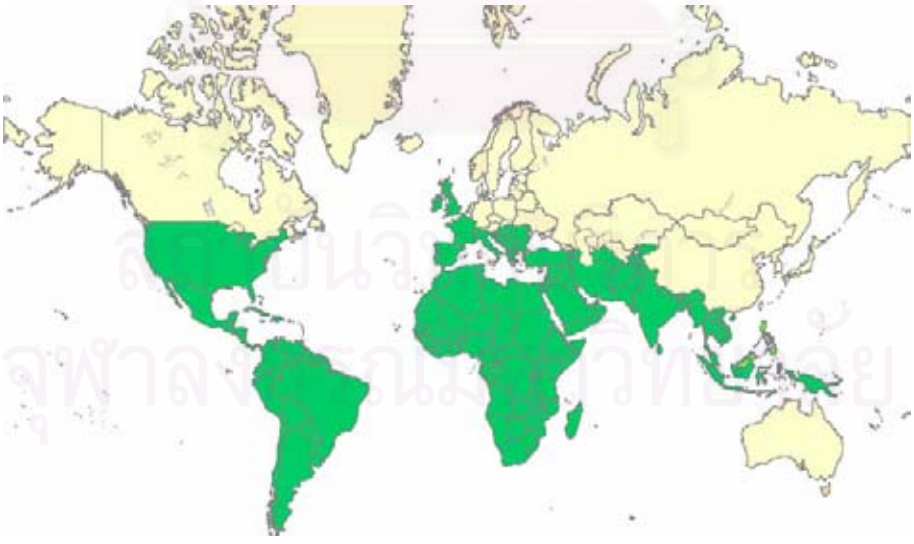


Figure 4 Distribution of G6PD deficiency in the world associated with the distribution of malaria shown in figure 3. Gray countries represent those in which more than 1 in 200 men have G6PD deficiency[6].

Glucose-6-phosphate dehydrogenase (G6PD, MIM# 305900) is a housekeeping enzyme which catalyses the first step in the hexose monophosphate pathway (HMP) and provides reductive potential, in the form of NADPH, required for a variety of biosynthetic reaction[7] and for protection against oxidation of H_2O_2. Though G6PD deficiency affects every cell in the body, its primary effects are haematological because the red cell has only HMP pathway as the role source of NADPH[3]. When the G6PD deficient person exposes to oxidant chemicals or drugs, oxidant stress may induce haemolysis by damaging cell membrane and haemoglobin[8]. G6PD deficiency predisposes to a number of diseases, including acute haemolytic anemia, which induced by drugs or infections, to favism, severe chronic non-spherocytic haemolytic anemia and neonatal jaundice[9]. Infection- or drug-induced hemolysis may be very severe leading to hemoglobinuria, acute renal failure and death[10]. The G-6-PD deficiency is a common cause of neonatal hyperbilirubinemia, which leads to kernicterus in some[11]. The severity and pattern of clinical manifestations vary greatly both among individuals with G6PD deficiency and among people of different ethnic backgrounds. One of the reasons for these varieties is the difference in types of enzyme, which caused from different mutation in *G6PD.*

To date, at least 442 biochemical and 130 molecular variants of the G6PD enzyme have been identified in various populations, nearly all of which are single-base substitutions that cause an amino acid substitution[2]. The advances in molecular techniques have allowed the molecular characterization of the G6PD gene in any population to be carried out with ease[12]. The normal activity G6PD B variant is present worldwide, but other variants, particularly those resulting in enzyme deficiency, are restricted to specific geographic regions[13]. Previous studies have established the molecular abnormalities responsible for G6PD deficiency in several ethnic groups in Southeast Asia[1]. Epidemiological and molecular studies had previously shown that G6PD deficiency in Southeast Asia is heterogeneous. G6PD Viangchan (871G → A; Val 291 Met) seems to be the most common variant in Thais and Laotian[1,5]. As, G6PD Mahidol (487G → A; Gly 163 Ser) is the most variant in Myanmese[1] (figure 5). Nowadays, there has been no study on molecular variants in Cambodian. The purpose of this study has been to clarify the distribution and feature of G6PD mutations in various ethnic groups in Southeast Asia include Thai, Laotian, Myanmese and Cambodian.

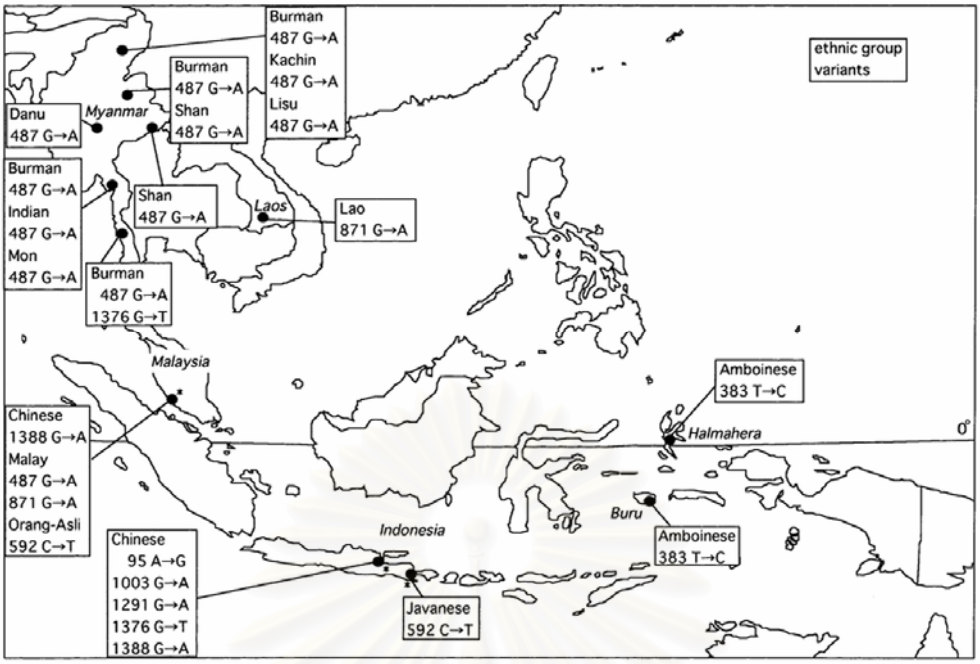


Figure 5 Distribution of G6PD mutations in Southeast Asia. Asterisks indicate the cases detected in a hospital-based study or case study; the others are from a population-based study[1].

Since DNA polymorphisms detected by restriction enzymes have become an attractive tool for estimating genetic distance between different populations[14] because only G6PD mutations as population markers is limited very few haplotypes have been identified[1], there have been many reports studied G6PD polymorphisms both coding region and intervening sequence of *G6PD.* For example, four polymorphisms in non-coding regions and two polymorphisms at a synonymous site of the *G6PD* gene have been identified in only African, European and Middle East[15-21]. After that, they used RFLP polymorphisms in *G6PD* to be a genetic marker in reconstructing the evolution history of populations in several ethnic groups in Africa, Europe and Middle East[13,15,16,22,23].

For Southeast Asian populations, there have been no studies in G6PD polymorphisms. Thus, in this study, we attempted to clarify the genetic relationship among Southeast Asian populations and to infer their history by using the information of polymorphic G6PD mutations in G6PD deficiency and single nucleotide polymorphisms (SNPs) in *G6PD* both in G6PD deficient and normal G6PD Thais, Laotians, Myanmars and Cambodians.

2. Research Questions

Primary Question

What is the most common G6PD deficiency variant in Cambodian?

Secondary Question

What are the diversity of *G6PD*'s RFLP haplotypes in Southeast Asian ethnic groups?

3. Objective of This Research

The aim of this study is

1. To analyze mutation of *G6PD* in Cambodian who have G6PD deficiency.

2. To analyze RFLP haplotypes of *G6P*D in Thai, Laos, Myanmars and Cambodian.

4. Hypothesis

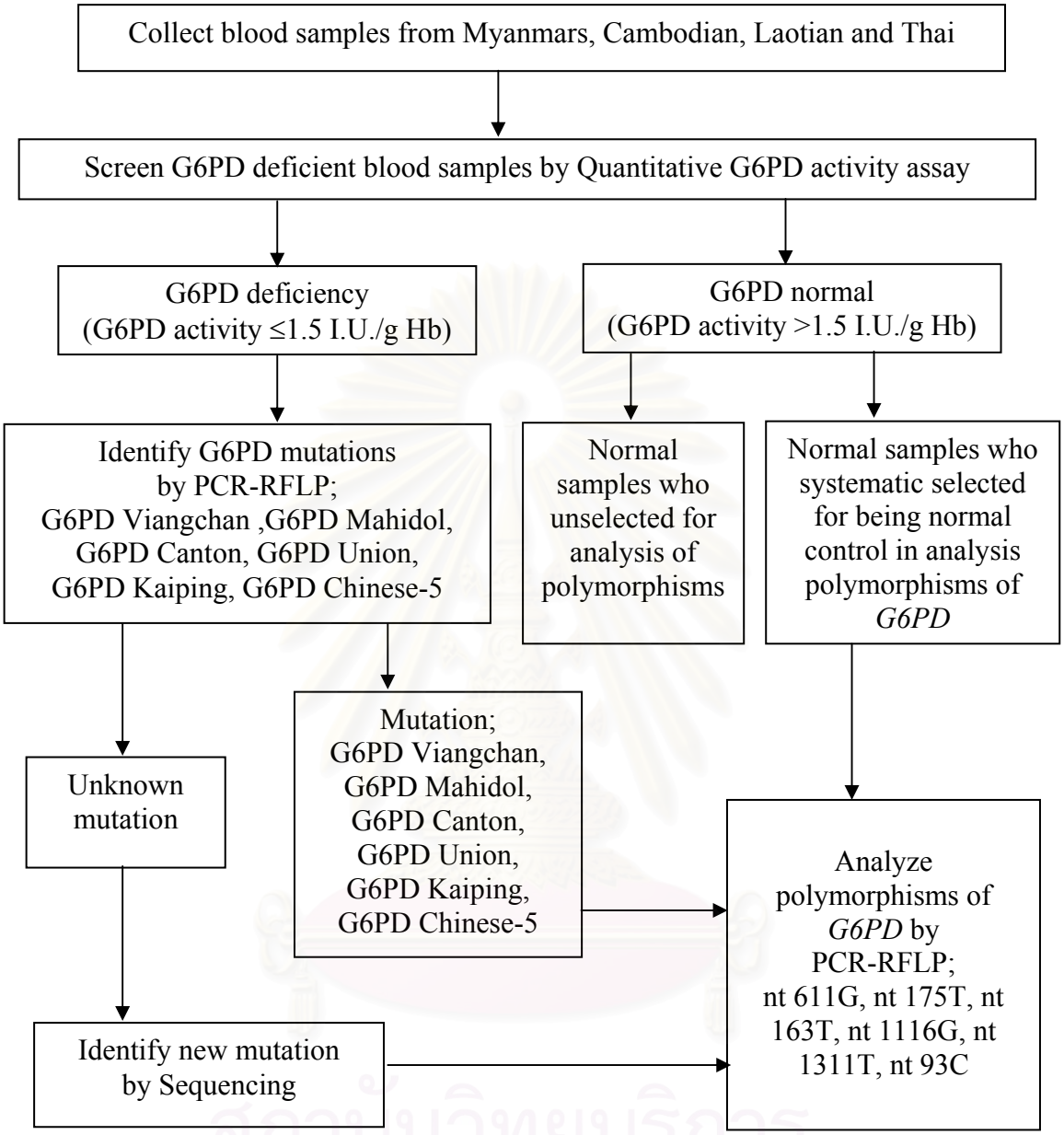
1. Mutation of *G6PD* in Cambodian who have G6PD deficiency is similar to mutation in Thai, Laotian (G6PD Viangchan).

2. Haplotype of G6PD Viangchan in Thai, Laotian & Cambodian are similar.

5. Keywords

Glucose-6-phosphate dehygrogenase
RFLP haplotype
Southeast Asian

6. Conceptual Framework



7. Expected Benefit and Application

1. Data base of population genetic information on *G6PD* of Southeast Asian populations.

2. Help to understand the evolution of the populations in Southeast Asia.

CHAPTER II

LITERATURE REVIEW

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme that accounts, in many cells, for about 0.03% of cellular protein[24]. The active form G6PD from mammalian cells functions as dimer of identical subunits (figure 6). The G6PD monomer consists of a 515 amino-acid subunit with a calculated molecular weight of 59 kilodalton[25,26]. Aggregation of inactive monomers into catalytically active dimers and higher forms requires the presence of NADP[27]. Thus, NADP appears to be bound to the enzyme both as a structural component and as one of the substrates of the reaction[25,28,29]. The binding sites for this coenzyme have not been identified at the structural level, but examination of mutant has suggested that amino acids 386 and 387, the basic amino acid lysine and arginine, respectively, seem to bind one of the phosphates of NADP[30]. The evidence that this is involved in the binding of NADP is as follows: (1) all mutants that rapidly lose activity at a 10 μmol/l NADP concentration, but are reactivated at high concentrations of NADP have been shown to have mutation in this region: (2) mutations in this region result in paradoxical electrophoretic migration of the enzyme as if it had become more positively charged, even when the amino acid change adds a negative charge, suggesting failure of binding of negatively charged NADP. The glucose-6-phosphate (G-6-P) binding site has been identified at amino acid 205 by locating a lysine at this position that is reactive with pyridoxal phosphate in competition with G-6-P[31].

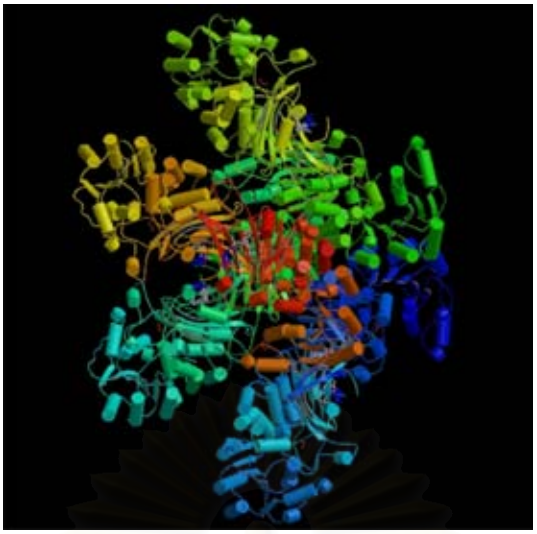


Figure 6 Three dimension structure of Glucose-6-phosphate dehydrogenase in tetramer form (www.rscb. org).

G6PD catalyzes the first step of the hexose monophosphate pathway (HMP) or pentose phosphate pathway (PPP) (figure 7), converting glucose-6-phosphate to 6-phosphogluconolactone and reducing the cofactor nicotinamide-adenine dinucleotide phosphate (NADP) to NADPH[4].

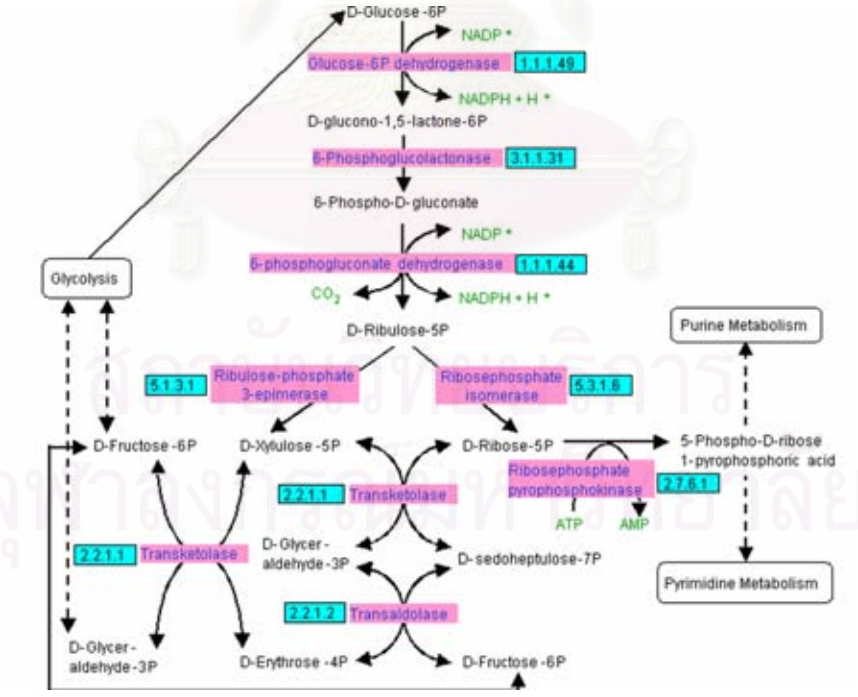


Figure 7 Hexose monophosphate pathway or Pentose phosphate pathway (PPP)(www.sites.huji.ac.il/malaria/maps/ppcpath.html).

The HMP is the only source of NADPH in the erythrocytes and it also serves to produce the ribose needed for synthesis of nucleotides in the salvage pathways. The main function of the pathway seems to be to protect the red blood cell (RBC) and its hemoglobin from oxidation in view of their role in oxygen transport. The -SH groups of several enzymes and of the β-chain of hemoblobin are particularly vulnerable to oxidation, with potentially serious consequences. Protection against oxidation is mediated by glutathione, which is actively synthesized and is present in high concentration in red cells, almost entirely in the reduced form (GSH). Reduced glutathione (GSH) serves as a substrate for glutathione peroxidase (GSHPx), which removes peroxide from the erythrocyte[32], becoming itself oxidized (to GSSG) in the process. NADPH is required for the reduction of oxidized glutathione and protein sulfhydryl groups by enzyme glutathione reductase, it is an essential factor in the chain of reactions that defends the RBC against peroxide (figure 8).

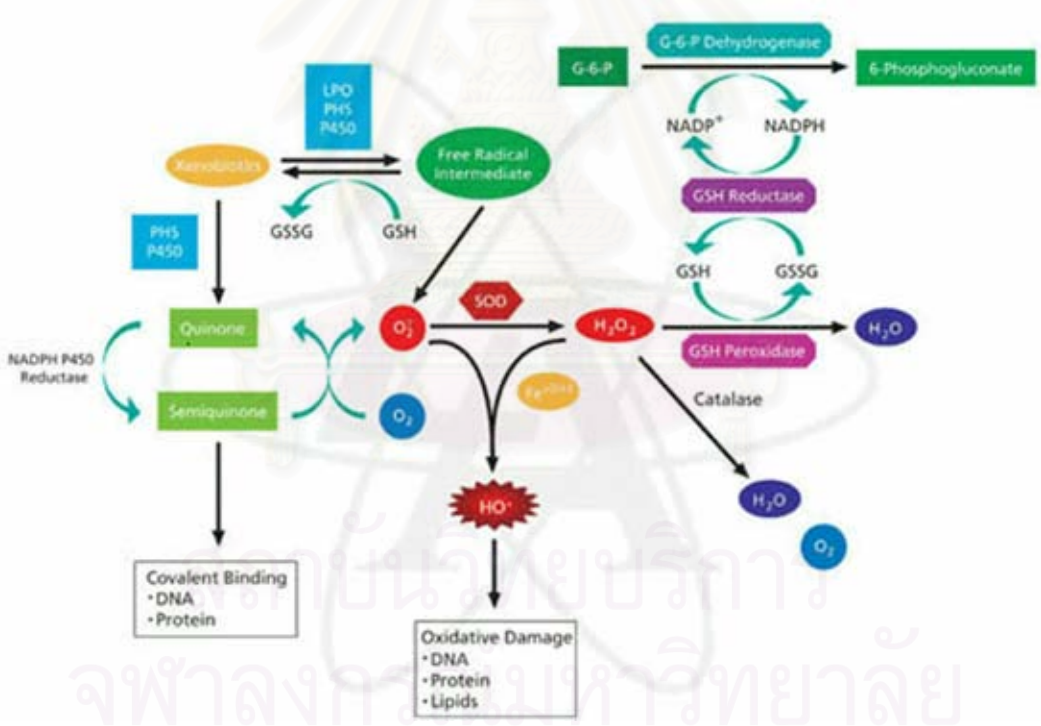


Figure 8 G6PD generates the NADPH which protects the red cell against peroxidates and superoxides generated by oxidative stresses (www.sigmaa/drich.com/img/assets/0460/oxidat_stress.gif).

In normal intact red cells NADP is mostly in the reduced form NADPH, and G6PD operates at only about 2 % of its theoretical maximum rate. This

is because, under normal circumstances, (1) the quantities of G-6-P and NADP are well below saturating levels, (2) NADPH and ATP inhibit the enzyme, and (3) most of the NADP present is not free but bound to catalase. Oxidative stress leading to increased oxidation of NADPH simultaneously releases enzyme inhibition and increases the level of NADP therefore, G6PD activity increases proportionately. Consequently, the normal red cell responds to oxidation by increasing its reducing capacity, and its large reserves allow it to deal with very significant levels of oxidative stress. This is why a major reduction in G6PD activity has little clinical effect under ordinary circumstances, but may become dramatically apparent in the presence of oxidative stress[4]. The activity of G6PD, like that of most other red cell enzymes, diminishes as the cell ages; in G6PD normal cells, lack of enzyme never endangers cell survival. However, in G6PD deficient cases the older red cells are even more deficient than younger ones, and G6PD eventually becomes a limiting factor. Though G6PD deficiency affects every cell in the body, its primary effects are hematological because the red cell has no alternative source of NADPH. Other more complex types of cells are protected by additional enzyme systems that can generate NADPH in the absence of adequate G6PD activity. Moreover, the red blood cells have long nonnucleated life span and they contain proteases that are more likely to degrade the mutant enzyme than are the proteases of other tissues[33]. This is probably caused of the associated mild hemolysis and reduces red cell life span.

Clinical Manifestation of Glucose-6-Phosphate Dehydrogenase Deficiency

G6PD deficiency is the most common human genetic disorder inherited in an X linked Mendelian transmission pattern[34]. G6PD deficiency is a globally important cause of neonatal jaundice, which can lead to kernicterus and death or spastic cerebral palsy. It can also lead to life-threatening hemolytic crises in childhood and in later ages by interacting with specific drugs, and with fava beans in the diet. The frequency and severity of these complications is heavily influenced by extrinsic and cultural factors and by other genetic tendencies[2].

Neonatal Jaundice

Neonatal jaundice (total bilirubin > 15 mg/dl) is one of the most life - and health - threatening consequences of G6PD deficiency, and kernicterus may occur in these infants[35,36]. The risks that develop to neonatal jaundice are as follows: the nature of the variant concerned, and the level of G6PD activity in the liver; the genetic background, exogenous factors such as the maturity of the infant and the method of feeding; exposure of the newborn to environment agents; the frequency of infectious causes of neonatal jaundice in newborns; the consumption of potentially hemolytic agents such as fava beans, drug, or herbal remedies by pregnant heterozygotes[4].

Favism

This term is used to describe the occurrence of an acute hemolytic reaction in a G6PD deficient individual following the ingestion of fava beans (*Vicia faba)* (figure 9).

Figure 9 Fava bean (*Vicia faba)*(www.oceanmist.com/favaProdSpec.htm) and (www. vc/bs.org/beansaboutbeans.htm).

Patients with favism are always G6PD deficient, but not all G6PD deficient individuals develop hemolysis when they ingest fava beans. Thus, G6PD deficiency is a necessary but not sufficient cause of favism. Presumably some other factor, probably also genetic[37] and very likely related to metabolism of the active ingredients in the beans, is involved. It occurs most frequently in children below 5 years of age, is relatively uncommon in adults[38], and can be fatal. It used to be common in Mediterranean areas where fava beans are often eaten, but not has not been describes in Africans. Fava bean contains vicine, convicine, ascorbate, and L-DOPA that have been considered candidate toxins. The most likely offenders are

vicine and convicine, β-glucosides of pyrimidine compounds that are converted by β-glucosidases to their aglycones, vicine and isouramil respectively. These compounds form reactive semiquinoid-free radicals and can generate active oxygen species. This results in the formation of ferrylhemoglobin, methemoglobin, and inactivation of various enzymes (figure 10). The reaction that occur are complex and varied and, therefore, largely unpredictable[39].

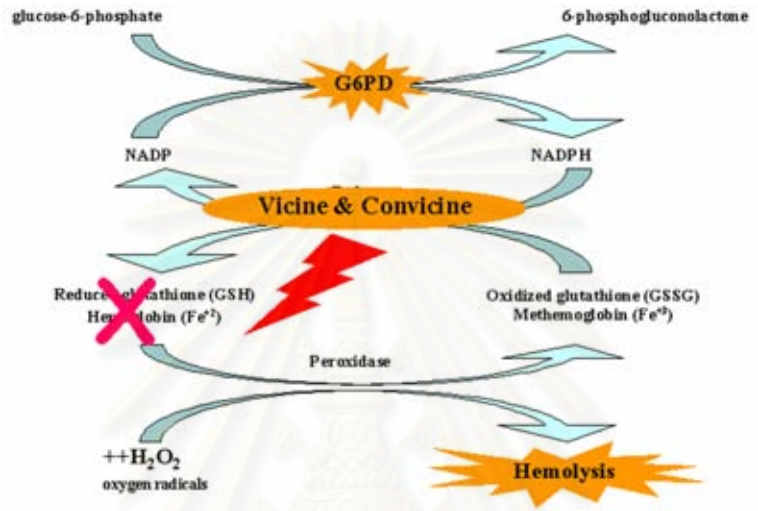


Figure 10 Oxidative stress by fava bean.

Acute Hemolytic Anemia

Drug-Induced Hemolysis. (Table 1) Primaquine is a drug that shortens RBC life span in G6PD-deficient persons[2] (figure 11). Acute hemolysis begins within 1 or 2 days of the administration of the drug. The mechanism of red cell destruction (figure 12 A and B) is denaturation and precipitation of hemoglobin to form Heinz bodies, adhered to the RBC membrane, appear in the early stages of drug administration and disappear as hemolysis progresses. Heinz bodies cause the red cells to become trapped in the spleen, where they are destroyed. The reaction may vary from transient mild anemia to rapidly progressing anemia with back and abdominal pain, jaundice and hemoglobinuria (figure 13), and transient splenomegaly. One of the most curious features of the acute hemolytic reaction is that it is erratic, in the sense that the same agent may cause hemolysis in one G6PD deficient person but not in another, and in the same person in the same time but not another. Differences between individuals may be determined by different G6PD variants and other genetic differences within the red cell, or the some other organ such as the liver. Genetic

factors affecting the rate of absorption and metabolism of the drugs may also be involved. However, genetic factors cannot be invoked to explain intra-patient variability. The differences must be due to something in the environment, involving more than one triggering factor[4].

Table 1 Drugs and Chemicals that should be avoided by persons with G6PD deficiency[2].

Acetanilid	Primaquine
Furazalidone	Sulfacetamide
Methylene Blue	Sulfamethoxazole
Nalidixic acid	Sulfanilamide
Naphthalene	Sulfapyridine
Niridazole	Thiazolesulfone
Isobutyl nitrite	Toluidine blue
Nitrofurantoin	Trinitrotoluene
Phenazopyridine	Urate oxidase

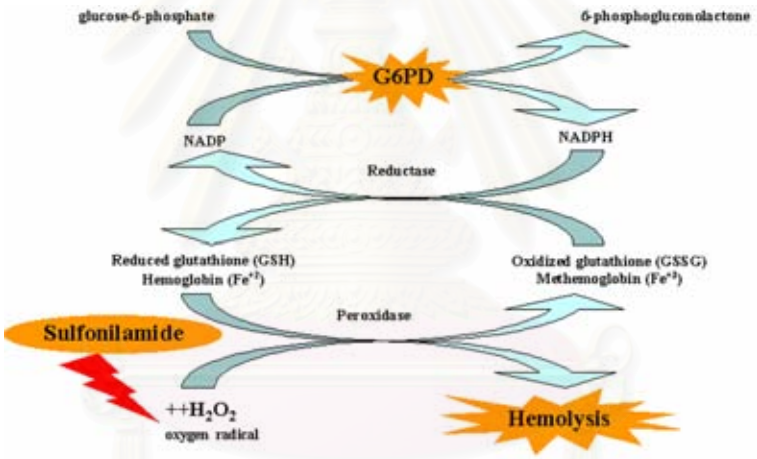


Figure 11 Oxidative stress by Sufanilamide.

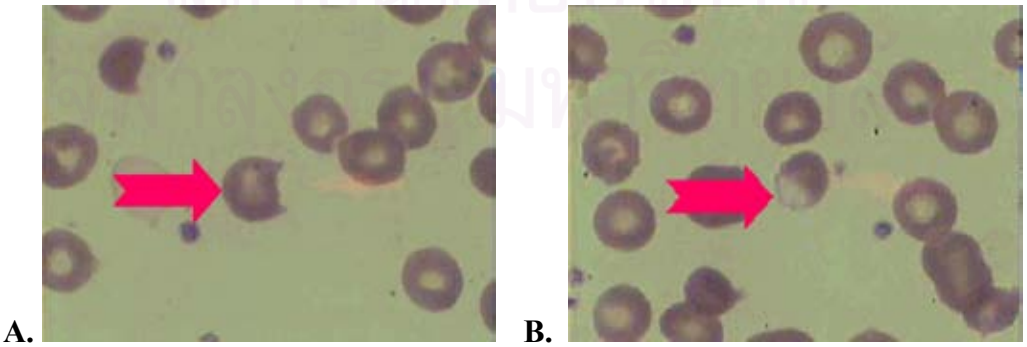


Figure 12 A. Hemolysis of red blood cell in form of bite cell and B. basket form.

Figure 13 Hemoglobinuria.

Infection-Induced Hemolysis. The mechanism by which this occurs is not clear, but an imaginative suggestion has been that during phagocytosis, leukocytes damage erythrocytes in their environment by discharging active oxygen species during phagocytosis[40].

Chronic Nonspherocytic Hemolytic Anemia. The syndrome occurs in persons who inherited rare mutations, designated class 1 (see below) because of their association with chronic hemolysis. Presumably class 1 variants produce chronic hemolysis because the functional severity of the defect is so great that the erythrocyte cannot even withstand the normal stresses that it encounters in the circulation. The RBCs of patients with class 1 variants may have residual G6PD activity as high as 35% of normal[41] when measured under standard conditions. The functional impairment that leads to the shortening of the RBC life span in these patients may include such factor as susceptibility to inhibition by NADPH[42] and in *vivo* lability[43]. Possibly the most consistent common feature of class 1 variants is the location of the mutation. In the great majority of cases, it is in the region of the putative NADP-binding or glucose-6-phosphate binding site of the molecule.

Classification of G6PD Variants

G6PD variants have been classified in 4 groups[44] by measurement the level of G6PD activity as follows:

Class 1 chronic nonspherocytic hemolytic anemia.

Class 2 severe enzyme deficiency (activity < 10 % of normal) such as G6PD Kaiping, G6PD Union, G6PD Viangchan, G6PD Jammu and G6PD Canton.

Class 3 mild-moderate enzyme deficiency (activity 10-60 % of normal) such as G6PD Mahidol.

Class 4 non-deficient variants (activity > 60 %) such as G6PD A. These variant were discovered incidentally but had normal G6PD enzyme activity.

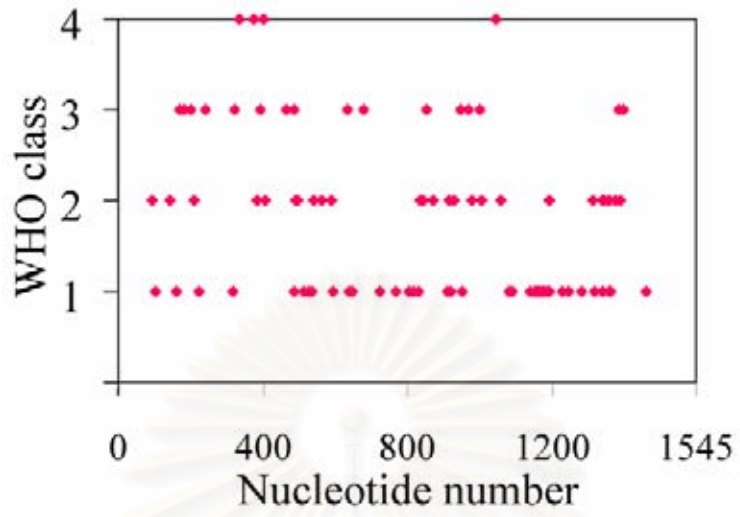


Figure 14 Distribution of G6PD mutations in *G6PD* gene.

Diagnosis and Screening

A WHO Scientific Group in 1967 recommended the method for diagnosis G6PD deficiency as follows:

Quantitation of G6PD Activity in Erythrocytes The assay is a standardized method for G6PD assay of hemolysates. Assays of G6PD commonly depend upon the increase in absorbance that occurs at 340 nm when NADP is reduced to NADPH. This reaction takes place when two electrons are transferred from G6P to NADP in the reaction catalysed by G6PD[44].

Screening for G6PD Deficiency. Methemoglobin is formed through the action of nitrite on the red cells. In the presence of methylene blue, methemoglobin is reduced primarily through the oxidative pathway, and the rate of reduction is therefore proportional to the G6PD activity of the cell[44].

In addition, a WHO Scientific Group in 1967 recommended that the biochemical characterizations should be used for identification of G6PD variants (1) red cell G6PD activity, (2) electrophoretic migration, (3) Michaelis constants (K_m's) for G6P, (4) relative rate of utilization of 2dG6P, (5) thermal stability and G6PD mutations were identified by molecular characterizations.

Geographical Distribution and Frequency

The frequency of G6PD deficiency is usually expressed as the proportion of a sample of males that is found to be hemizygous. The approximate frequencies of G6PD deficiency in the different countries of the world are summarized in Table 2.

Table 2 Global frequency of G6PD deficiency (assuming that birth incidence is approximately equal to adult prevalence).

Region	% of all births (male + female)				
	Male hemizygote	**Female homozygote**	**Female heterozygote**	**Total with 1 or 2 genes for G6PD deficiency**	**G6PD deficiency (Estimated total)***
Africa	5.60	0.90	9.40	15.9	7.4
Americas	1.40	0.09	2.40	3.9	1.7
Asia	2.30	0.20	4.20	6.7	2.9
Europe	0.34	0.02	0.67	1.0	0.4
Oceania	0.90	0.06	1.80	2.8	1.1
Total	2.6	0.3	4.6	7.5	3.4

* Figure obtained by adding % of male hemizygotes, % of female homozygote, and 10% of female heterozygote[4].

G6PD deficiency is a significant public health problem. A conservative estimate of the total number of individuals with G6PD deficiency of all types in the whole is about 100 million[4]. About 7.5% of the world population carry one or two genes for G6PD deficiency, the proportion ranging from a maximum of 35% in parts of Africa, to 0.1% in Japan and part of Europe. In Thailand, the prevalence of G6PD deficiency is high, 11.1% in neonatal males and 5.8% in females[5]. About 2.9% of the world population are genetically G6PD deficient. It is important to note that though X-linked disorders are usually thought to affect only males in this cases, because of the high frequency of the gene, homozygous females contribute about 10% of those genetically G6PD deficient[4]. In addition, perhaps 10 % of heterozygous females are also effectively G6PD deficient due to unequal inactivation of their X-chromosomes, so about 3.4% of the world population are at risk for complications of G6PD

deficiency. With about 130 million births annually, about 4.5 million G6PD deficient children particularly vulnerable to neonatal jaundice and acute hemolytic crises are born every year. Furthermore, owing to global migrations, the complications of G6PD deficiency may now occur at least certain population groups in most countries and regions of the world[4].

The distribution of G6PD deficiency is positively associated with that of falciparum malaria in the sense that all the areas where high incidences of G6PD deficiency of one type or another have been reported are or were areas of high malaria endemicity. Malaria, resulting from infection by the *Plasmodium falciparum, P. vivax, P. malariae, or P. ovale* parasites, is the leading cause of death in the global human population. During the course of human evolution in regions where malaria is prevalent, naturally occurring genetic defense mechanisms have evolved for resisting infection by *Plasmodium.*Most of the human genes that are thought to provide reduced risk from malarial infection are expressed in red blood cells[36]. The selective advantage conferred by resistance to malarial infection is counterbalanced by a selective disadvantage associated with the hemopathologies associated with enzyme deficiency. Thus, the genetic variability maintained at the classic examples of natural selection acting on the human genome.

Glucose-6-Phosphate Dehydrogenase Gene

G6PD gene is a gene on the distal long arm of the X chromosome (q28)(figure 15), and it consists of 13 exons and 12 introns distributed over approximate 18 kb of genomic DNA[45]. The first exon contains no coding sequence[2]. All introns are smaller than 300 bp, except intron 2,which is extraordinarily long, extending for about 11 kb. The coding region is in exon 2-13[7]. The protein coding region is divided into 12 segments ranging in size from 12-236 bp; an intron is present in the 5' untranslated region[19]. At the 5' end of the gene is a cytidine- guanine dinucleotide (CpG)-rich island[2].

Figure 15 Diagram of *G6PD* gene structure.

Mutation of *G6PD* Gene

More than 400 G6PD variants have been described in the past 33 years according to the standards for the biochemical characterization established by the WHO[18]. As the molecular characteristics of 130 different mutations in the *G6PD* gene, which result in enzyme deficiency have been described[16]. The variants are produced in a number of different ways. Many different biochemical variants may turn out to be caused by mutations of the same gene, or one variant may turn out to be the result of several gene mutations[33].

The mutations of the *G6PD* gene can be classified in 2 classes:

1. Mutation in coding region: there is many G6PD variants can be grouped as follow:

1.1. Base substitutions: all of mutations in *G6PD* gene are single base substitutions in the coding region[46,47,48] or missense mutation. The amino acid substitutions affect the three-dimensional structure of G6PD, especially in the NADP-binding site or glucose-6-phosphate binding site. For example: the variants that produced chronic hemolytic anemia have been found to be clustered either between nt 563 and nt 844, which includes the putative glucose-6-phosphate binding site or between nt 1003 and 1376, which contains the putative NADP binding site[20]. The other mutation is nonsense mutation in which a base substitution at nt 1,284 C → A created a stop codon, "G6PD Georgia"[18].

1.2. Deletion: the mutations have been found less than the base substitution. From reports found 3 mutations: deletions of 3 bases in exon 2 "G6PD Stony Brook", "G6PD Sunderland"[47,49]. Deletion 6 bases of nts 724 to 729 in exon 7 affect deletion of 242 glycine and 243 threonine[18]. And "G6PD Nara" has been found deletion of 24 bases[50]. Significantly, all of the deletions that have been found occur in the multiples of 3, resulting in the triple codon deletion rather than a frameshift.

In 1995, 3' acceptor splice site mutation in G6PD gene was repeated[18]. The deletion of the invariant dinucleotide ApG at the 3' acceptor splice site in the highly conserved sequence between intron 10 and exon 11 "G6PD Varnsdoft". They didn't know this mutation affect which position on polypeptide chain but this mutation produced chronic hemolytic anemia[18].

Mutations in the coding region associated with an altered enzyme phenotype are all potentially subject to biological selection, and some of them are known to have undergone selection by *P. falciparum* malaria[48].

Distributions of G6PD mutations are found among the people of various ethnic groups. G6PD B variant is present world wide, but other mutations, particularly those resulting in enzyme deficiency, are restricted to specific geographic regions[36]. In Asia, G6PD Canton (1376 G → T) has been found to be the most common variant among the Chinese in Taiwan[9,51,52], China, Malaysia[53] and Singapore[54]. The another of G6PD variants have been found in Chinese are G6PD Kaiping (1388G → A), G6PD Union (1360 C → T), G6PD Chinese-5 (1024 C → T)[1,5,9,18,51,54]. As G6PD Viangchan (871 G → A) has been found to be the most common variant among the Laotian in Laos[1,20], Thai in Thailand[5]. Moreover G6PD Viangchan has been found among Lao in Hawaii, Chinese in South China[18,55]. In Southeast Asia have many G6PD variants such as G6PD Mahidol (487G → A) have been found in many ethnic groups of Myanmar, Malay in Malaysia[1], Thai in Thailand[5,11]. G6PD variants that have been found are summarized in table 3.

Table 3 Variants of G6PD in East and Southeast Asian.

Variant	Nucleotide Substitution	Amino acid Substitution	WHO Class	Ethnic Group	Reference
Normal B	-	-	-	various	4
Viangchan	G 871 A	Val 291 Met	2	Laotian, Thai	1,5,33,55
Jammu				Indian	
Mahidol	G 487 A	Gly 163 Ser	3	Myanmars, Thai, Taiwanese	2,5,33,56
Chinese-5	C 1024 T	Leu 342 Phe	?	Chinese	5,57
Union	C 1360 T	Arg 454 Cys	2	Chinese,Thai	5,58,59
Canton	G 1376 C	Arg 459 Leu	2	South Chinese,	5,9,33,54,60
Taiwan-Hakka				Thai,Taiwanese	
Kaiping	G 1388 A	Arg 463 His	2	Chinese,Thai,Taiwanese	5,9,33,54,61

2. Mutations in noncoding regions may not have an altered phenotype and may therefore be neutral[48]. The restriction fragment length polymorphisms

(RFLPs) have been shown to be in marked in linkage disequilibrium with the polymorphic mutations in G6PD variants[17].

There are many polymorphic mutations have been found (figure 16). One of these is the intervening sequence 5 (IVS5) nt 611 C → G mutation, previously identified as that responsible for a *Pvu* II polymorphism in the African population (100% of G6PD A-, 20% of G6PD A)[15,17,18,21,22]. This allele has not been found in the European, Asia and Middle Eastern populations[22]. The second is an IVS7 nt 175 C → T mutation. The former does not create or destroy a restriction enzyme recognition sequence and so, for its detection, a mutagenic primer was designed matching the sequence of intron 7 and create a *Sca* I site when there is a T at IVS7 nt 175 but not when there is a C at this position. This mutation has been found in African (100% of G6PD A-, 20% of G6PD A)[17]. The third is an IVS8 nt 163 C → T mutation, which destroys a *Bsp*H I recognition sequence. This mutation has been found in African (30% of G6PD B, 10% of G6PD A)[17]. The fourth is an exon10 nt 1116 G → A mutation was always cleavable with the enzyme *Pst* I, demonstrating the presence of a G residue at this position[15,16,17,18,21]. The fifth is an exon11 nt 1311 C → T mutation was cleavable with enzyme *Bcl* I have been found in G6PD Viangchan[5,16,18], G6PD Mediterranean[3,19], some case in G6PD B[3,11,15,17,62] and G6PD Canton[18]. The 1311 mutation is now available as a polymorphic marker of the *G6PD* gene, independent of G6PD deficiency[3]. The sixth is an IVS11 nt 93 T → C mutation was cleavable with enzyme *Nla* III have been found in G6PD B and G6PD Viangchan[15,16,18].

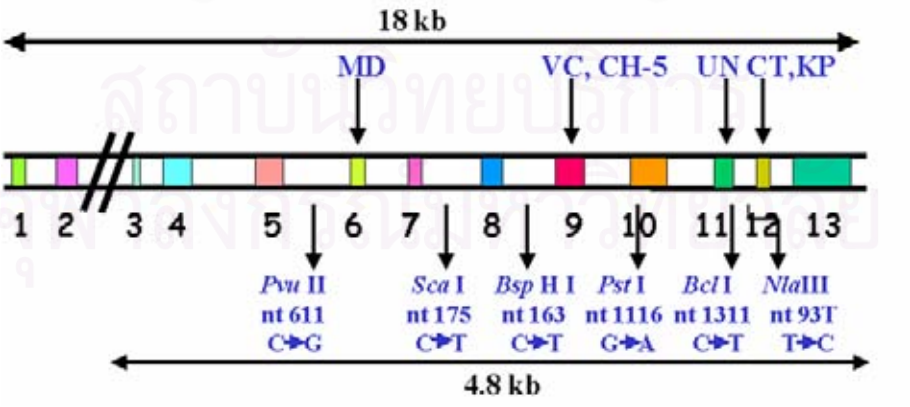


Figure 16 Diagram of G6PD gene structure showing the location of the mutations and RFLPs. MD, VC, CH-5, UN, CT and KP represent Mahidol, Viangchan, Chinese-5, Union, Canton and Kaiping respectively.

Table 4 Association of G6PD variants with different RFLP haplotypes in various ethnic.

Variant	Nucleotide Substitution	Ethnic origin	Haplotype						Reference
			Pvu II	*Sca* I	*BspH* I	*Pst* I	*Bcl* I	*Nla* III	
G6PD B	-	African	-	-	+	+	-	NR	17
G6PD B	-	African	-	-	+	+	+	NR	17
G6PD B	-	African	-	-	+	-	-	NR	17
G6PD B	-	White, Black Amazonian	-	NR	NR	+	NR	-	16,21
G6PD B	-	Black	-	NR	NR	-	NR	NR	21
G6PD B	-	Mexican	-	NR	NR	+	+	-	15
G6PD B	-	Mexican	-	NR	NR	+	+	+	15
G6PD B	-	Mexican	+	NR	NR	+	-	-	15
G6PD B	-	Mexican	+	NR	NR	+	-	+	15
G6PD A	376G	African	-	-	+	+	-	NR	17
G6PD A	376G	African	-	-	-	+	-	NR	17
G6PD A	376G	African	+	+	-	+	-	NR	17
G6PD A	376G	Black	-	NR	NR	+	NR	NR	21
G6PD A	376G	Mexican	-	NR	NR	+	+	+	15
G6PD A	376G	Mexican	+	NR	NR	+	-	+	15
G6PD A-	202A/376G	African	+	+	-	+	-	NR	17
G6PD A-	202A/376G	Black, Mexican Puerto Rican White US, Spanish	+	NR	NR	+	NR	NR	21
G6PD A-	202A/376G	Canary Island	+	NR	NR	+	-	+	15,18
G6PD A-	376G/680T	Black	+	NR	NR	+	NR	NR	21
G6PD A-	376G/968C	Spanish, Black	+	NR	NR	+	NR	NR	21
G6PD A-	376G/968C	Canary Island	-	NR	NR	+	-	+	15
G6PD A-	376G/968C	Canary Island	-	NR	NR	+	-	-	18

Table 4 Association of G6PD variants with different RFLP haplotypes in various ethnic (continued).

Variant	Nucleotide Substitution	Ethnic origin	Haplotype						Reference
			***Pvu* II**	***Sca* I**	***BspH* I**	***Pst* I**	***Bcl* I**	***Nla* III**	
G6PD Viangchan	871A	Laotian,Chinese	-	NR	NR	+	+	+	18,20
G6PD Jammu	871A	Indian	NR	NR	NR	NR	-	NR	20
G6PD Ananindeua	871A/376G	Amazonian	-	NR	NR	+	-	+	16
G6PD Canton	1376T	South Chinese	-	NR	NR	+	-	-	18
G6PD Med	563T	Mediterranean	NR	NR	NR	NR	+	NR	3,19
G6PD Med	563T	Mediterranean	NR	NR	NR	NR	-	NR	19

Genetic analysis of G6PD deficiency may help to clarify the genetic relationship among Southeast Asian populations and to infer their history movement[1]. In previous study, Allelic frequency data for DNA polymorphisms have been used for estimating genetic distance between different populations[14]. However, the use of only G6PD mutations as population markers is limited, because very few haplotypes have been identified[1]. Thus, the information of polymorphic G6PD mutations and single nucleotide polymorphisms are necessary in reconstructing the evolutionary history of the *G6PD* gene in Southeast Asian population.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1. Population Study

In this study volunteers from each ethnic group who concented for blood sampling were included. People blood samples with normal G6PD activity were excluded from mutation analysis.

Thai's blood samples were collected from newborns. Three hundreds and three umbilical cord blood samples of neonatal jaundices were collected from June 2001 - March 2002 at King Chulalongkorn Memorial Hospital, Bangkok, Thailand. While seven cord blood samples of neonatal jaundices that normal delivered and twenty three cord blood samples from neonates delivered by caesarean section were collected from April – May 2003 at Buri ram Hospital, Buri ram, Thailand.

Myanmars's blood samples were collected from Myanmars immigrant laborers. Fifty three peripheral blood samples from March 2002 at Chanthaburi province and seventy eight blood samples of Myanmars patients who visited a Samutsakhon Hospital from February – June 2003.

One hundred and eight peripheral blood samples of Cambodian immigrant laborers were collected from March 2002 at Chanthaburi province. While fifty six cord blood samples from neonates delivered by caesarean section and fifty one cord blood samples from normal delivered neonatal jaundice that mother speaks Cambodian were collected from April – May 2003 at Buri ram Hospital.

Thirty four peripheral blood samples of Laos immigrated laborers were collected from March 2002 at Chanthaburi province. Among ninety three cord blood samples from neonates delivered by caesarean section and thirty five cord blood samples from normal delivered neonatal jaundice that mother speaks Laos were collected from April – May 2003 at Buri ram Hospital.

Normal blood samples from screening G6PD activity were randomly selected about 2 fold (2n) of G6PD deficient blood samples (n) to be normal control

by a systematic sampling. In identification of nationality, the volunteers were interviewed with translator observe their language.

1.2. Collecting Specimen

About 3.75 ml of blood were collected in 5 ml of Vacutainer™ tube containing 1.25 ml of acid – citrate – dextrose (ACD) for G6PD assay of hemolysates standardized method. And 2.7 ml of blood were collected in 3 ml of Vacutainer™ tube containing 0.3 ml of ethylene diaminetetraacetic acid (EDTA) for DNA extraction. Blood samples were stored at 4°C until used.

1.3. DNA Extraction

QIAamp® DNA Blood Mini Kit was purchased from QIAGEN, Germany.

1.4. Identification of G6PD Mutations and Polymorphisms

1.4.1. Synthetic Oligonucleotides (or primers)

Oligonucleotides used in this study were purchased from Bio Service Unit, NSTDA, Thailand.

Table 5 Oligonucleotdes and their descriptions[5,9,17,52].

Name	Sequence (5'-3')	T_m (°C)	Description
871F	TGGCTTTCTCTCAGGTCTAG	60	Oligonucleotides for PCR amplification of a fragment of G6PD Viangchan.
9R	GTCGTCCAGGTACCCTTTGGTGG	74	
487F	GCGTCTGAATGATGCAGCTCTGAT	72	Oligonucleotides for PCR amplification of a fragment of G6PD Mahidol.
487R	CTCCACGATGATGCGGTTCAAGC	72	
1360F	ACGTGAAGCTCCCTGACGC	62	Oligonucleotides for PCR amplification of a fragment of G6PD Union and IVS nt 93 T → C .
1360R	GTGAAAATACGCCAGGCCTTA	62	
1376F	The same as for nt 1360F		Oligonucleotides for PCR amplification of a fragment of G6PD Canton.
1376R	The same as for nt 1360R		
1388F	The same as for nt 1360F		Oligonucleotides for PCR amplification of a fragment of G6PD Kaiping.
1388R	GTGCAGCAGTGGGGTGAACATA	68	

Table 5 Oligonucleotdes and their descriptions (continued).

Name	Sequence (5'-3')	T_m (°C)	Description
1024F	GTCAAGGTGTTGAAATGCATC	60	Oligonucleotides for PCR amplification of a fragment of G6PD Chinese-5.
1024R	CATCCCACCTCTCATTCTCC	62	
G6P5F	TATGTGGCTGGCCAGTACGATG	68	Oligonucleotides for PCR amplification of a fragment of IVS5 nt 611 C → G.
G6P6R	AGCCGGTCAGAGCTCTGCAGGT	72	
R1G7	TGGACCCCTACACAGCCAAGTAC	72	Oligonucleotides for PCR amplification of a fragment of IVS7 nt 175 C → T.
R1S	GGCATGCTCCTGGGGACTGCT	70	
G6P 8F	TGATGCAGAACCACCTACTGCA	66	Oligonucleotides for PCR amplification of a fragment of IVS8 nt 163 C → T.
G6P 9R	GTCGTCCAGGTACCCTTTGGTGG	74	
G6P9F	AGGTGCAGGCCAACAATGTGGT	68	Oligonucleotides for PCR amplification of a fragment of exon 10 nt 1116 G → A.
G6P10R	TCAGGTCCAGCTCCGACTCCTC	72	
R3F	TGTTCTTCAACCCCGAGGAG	62	Oligonucleotides for PCR amplification of a fragment of exon 11 nt 1311 C → T.
R3Md	AAGACGTCCAGGATGAGGTGATC	70	
G6P3F	AGGATGATGTAGTAGGTCG	56	Oligonucleotides for PCR amplification of a fragment of exon3,4.
G6P4R	CCGAAGTTGGCCATGCTGGG	66	
G6P4F	GTGGCTGTTCCGGGATGGCCTTC	76	Oligonucleotides for PCR amplification of a fragment of exon4,5.
G6P5R	TTCTTGGTGACGGCCTCGTAGA	68	
G6P6F	AACCGCATCATCGTGGAGAAG	64	Oligonucleotides for PCR amplification of a fragment of exon6-8.
G6P8R	CCATGGCCACCAGACACAGCAT	70	
G6P10F	GAAGCCGGGCATGTTCTTCAAC	68	Oligonucleotides for PCR amplification of a fragment of exon10-13.
G6P13R	CCAGGGCTCAGAGCTTGTG	62	

Note T_m was calculated from the formula 2(A+T) + 4(G+C)

_ represent mutagenic site.

1.4.2. Enzymes

Taq DNA polymerase	Fermentas, Promaga
Restriction endonucleases	Biolabs, Fermentas

Table 6 Restriction enzymes with their recognition sites, recommended buffer and manufacturer[5,17,52].

Enzymes	Recognition sequence	Buffer	Manufacturer
Xba I	T^CTAGA	Buffer 2 Buffer Y^+/Tango™	Biolabs Fermentas
*Hin*d III	A^AGCTT	Buffer 2	Biolabs
Hha I	GCG^C	Buffer 4	Biolabs
Afl II	C^TTAAG	Buffer 2	Biolabs
Nde I	CA^TATG	Buffer 4	Biolabs
Mbo II	GAAGA $(N)_8$^	Buffer 2	Biolabs
Pvu II	CAG^CTG	Buffer B	Biolabs
Sca I	AGT^ACT	Buffer U Buffer *Sca* I^+	Biolabs Fermentas
BspH I*	T^CATGA	Buffer 4	Biolabs
Pag I*	T^CATGA	Buffer O^+	Fermentas
Pst I	CTGCA^G	Buffer H	Biolabs
Bcl I	T^GATCA	Buffer 3	Biolabs
Nla III	CATG^	Buffer 4	Biolabs
Hsp 92 II	CATG^	Buffer K	Promaga

Note ^ represent the cleavage site of restriction enzyme.

* represent the isoschizomer.

1.4.3. Positive Control for Restriction Enzymes Activity

pTrcHisA (Invitrogen), a vector containing *Pvu* II and *Sca* I sites, were digested by *Pvu* II and *Sca* I respectively. Its physical map was shown in figure 17. Number of cuts and their positions were presented in table 7.

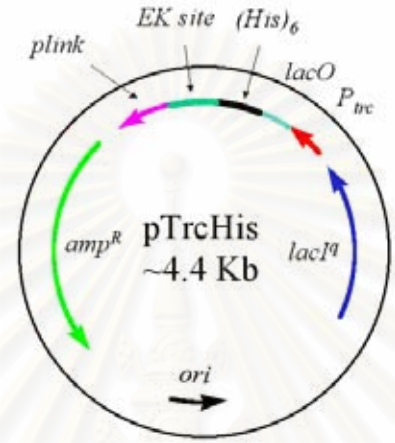


Figure 17 Map of the pTrcHisA vector.

Table 7 Restriction map of pTrcHisA vector.

Restriction enzyme	Number of cuts	Positions
Pvu II	3	538, 4244, 4337
Sca I	1	526

1.4.5. Polyacrylamide Gel Electrophoresis

Mini-PROTEAN®3 Electrophoresis Cell was purchased from BIO-RAD Laboratories, USA.

1.5. Identification of Unknown Mutation

1.5.1. Synthetic Oligonucleotides (or primers)

Oligonucleotides used in this study were purchased from Bio Service Unit, NSTDA, Thailand (table 4).

1.5.2. DNA Purification from Gel Slice

QIAquick® Gel Extraction Kit was purchased from QIAGEN Inc., USA.

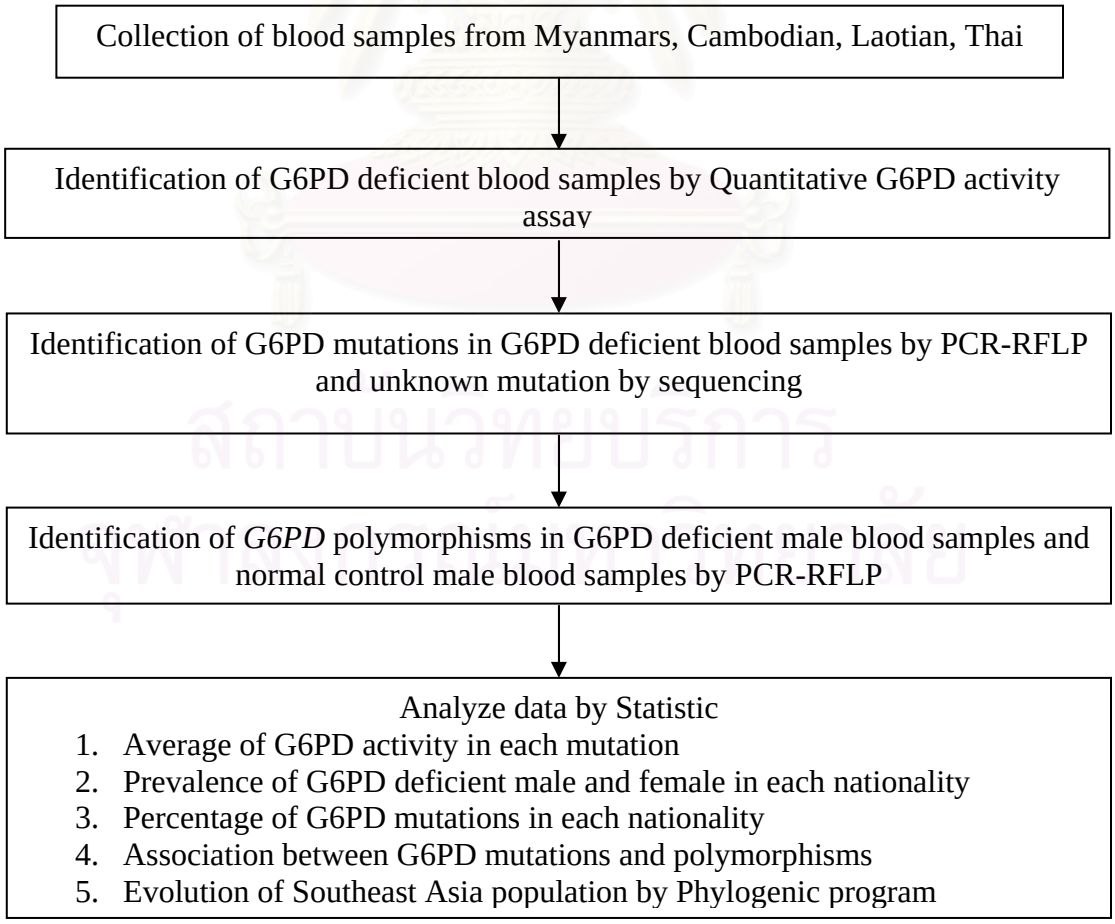
1.5.3. DNA Sequencing Reaction

ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit Version 3.0, was purchased from Applied Biosystems, USA.

1.6. Chemicals

All other chemicals used in this work were either analytical or molecular biology grades purchased from many suppliers (Sigma; BIO-RAD; USB; Mallinckrodt; Eiken chemical and Merck).

Work Outline



2. Methods

2.1. Standardized Method for G6PD Assay of Haemolysates

The G6PD activity method was modified from technical report series of World Health Organization[44]. The whole blood was centrifuged at 3,000 rpm for 1 minute and the plasma and buffy coat removed by aspiration. The cells were washed three times in at least five volumes of 0.9% saline with removal of any residual buffy coat after each washing by centrifuged at 3,000 rpm for 1 minute. The washed blood cells were screened G6PD activity were measured amount hematocrit and hemoglobin by an automated complete blood count (CBC) with differentials (Technicon H*3, Bayer, New York, USA). After that 50 µl of washed blood was added into the new 5 ml tube that contained 950 µl of distilled water, then mixed well. The new tube was freezed at -20^{o}C for 10 minutes, then centrifuged at 8,000 rpm for 20 minutes. The supernate fluid was haemolysate for next step. Fifty microliters of haemolysate was added to 2.5 ml cuvette and mixed with TPN in buffer (2 mM NADP, 1 M Tris-HCl pH 8.0, 1 M $MgCl_2$) 850 µl. The mixture was incubated at room temperature for 5 minutes. Next 6 mM G-6-P 100 µl was added in the mixture to start the reaction in spectrophotometer for 5 minutes. The optical density is followed in cuvette with a 1-cm light path at 340 nm and at 22^{o}C in calculation of results international units were used in expressing enzymatic activity. One unit of G6PD shall consist of quantity of enzyme, which reduces 1 µmol of NADP per minute. One µmol/ml of reduced NADP has an absorbance of 6.22 in a light path of one centimeter.

$$\text{Activity (IU)/g Hb} = \frac{1\text{x O.D./min}}{6.22} \times \text{dilution} \times \frac{100}{0.05} \times 1.66 \qquad \text{---(equation 1)}^{44}$$

1 : amout of assay mixture

6.22 : of NADP, NADPH

dilution : 0.1 ml : 1.9 ml = 20

100 : expressed as I.U./100 ml RBC

0.05 : amout of hemolysate used

1.66 : connection for temperature factor 22^{o}C

Normal male 7.39 ± 2.57 I.U./ g Hb

Normal female 6.94 ± 2.51 I.U./g Hb

G6PD deficiency < 1.5 I.U./ g Hb[44]

2.2. Extraction of Genomic DNA from Blood Sample

Genomic DNA was extracted from blood sample by using QIAamp® DNA blood mini kit (QIAGEN, Germany). The method was modified from Mini kit handbook. Twenty microliters of protease was pipeted into 1.5 ml microtube, added 200 µl of AL buffer to the sample, then mixed by pulse vortexing for 15 seconds. The mixture was incubated at 56°C for 15 minutes. The mixture was removed to spin column (in a 2 ml collection tube) and centrifuged at 8,000 rpm for 1.5 minutes. The spin column was placed in clean 2 ml collection tube and discarded the tube containing the filtrate. Five hundreds microliters of AW1 buffer was added into the spin column, then centrifuged at 8,000 rpm for AW2 buffer was added into spin column, then centrifuged at 12,000 rpm for 5 minutes. Two hundreds of AE buffer was added into the spin column, incubated at room temperature for 5 minutes, and then centrifuged at 8,000 rpm for 1.5 minutes.

2.3. Identification of G6PD Mutations

2.3.1. Primers

2.3.1.1. 871F/9R Primers

871F and 9R primers have been previously described[2]. These primers were designed from the database of the *G6PD* sequence available online (Accession X55448 GI:450527). 871F primer was designed mutagenic site at 3' created to be restriction recognition site for *Xba* I to detect mutation G6PD Viangchan at 871G → A.

2.3.1.2. 487F/487R Primers

487F-487R primers have been previously described[52]. 487R primer was designed mutagenic site at 3' created to be restriction recognition site for *Hin*d III to detect mutation G6PD Mahidol at 487G → A.

2.3.1.3. 1360F/1360R Primers

1360F-1360R primers have been previously described[52]. 1360R primer was designed mutagenic site at 3' created to be restriction recognition site for *Hha* I to detect mutation G6PD Union at 1360C → T.

2.3.1.4. 1376F/1376R Primers

1376F-1376R primers are the same as 1360F-1360R primers[52]. The PCR product of these primers contains restriction recognition site for *Afl* II to detect mutation G6PD Canton at 1376G → T.

2.3.1.5. 1388F/1388R Primers

1388F primer is the same as 1360F primer[52] but 1388R primer was designed containing restriction recognition site for *Nde* I to detect mutation G6PD Kaiping at 1388G → A.

2.3.1.6. 1024F/1024R Primers

1024F-1024R primers have been previously described[52]. The PCR product of these primers contains restriction recognition site for *Mbo* II to detect mutation G6PD Chinese-5 at C → T.

2.3.2. PCR Conditions

The typical PCR reaction was carried out in a 25 μl reaction containing 1X PCR buffer, 1.25 U of Taq polymerase (Promaga, Fermentas), 50 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs and approximate 300 ng DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 1 minute of denaturation, 56°C for 1 minute of annealing and 72°C for 1 minute of extension. The final amplification cycle included an addition of a 15 minutes extension at 72°C.

2.3.3. Digestion of Restriction Endonucleases

Ten microliters of PCR product was digested with 5 U of each restriction enzyme (The type of restriction enzyme was used in digestion depend on G6PD mutation that represent in table) according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided) and sterile distilled water added to a final volume of 20 μl. The digestion was incubated at 37°C 2-4 hours.

Table 8 G6PD mutations, restriction enzyme and the results for G6PD mutation.

G6PD mutations	Restriction enzymes	Result (bp)
G6PD Viangchan	*Xba* I	N 126 M 106+20
G6PD Mahidol	*Hin*d III	N 104 M 82+22
G6PD Union	*Hha* I	N 142+45+27 M 187+27
G6PD Canton	*Afl* II	N 214 M 194+20
G6PD Kaiping	*Nde* I	N 227 M 206+21
G6PD Chinese-5	*Mbo* II	N 187 M 150+37

Note bp represent base pair (size of PCR product).
N represent normal digestion result.
M represent mutant digestion result.

2.3.4. Polyacrylamide Gel Electrophoresis

Six percentage of polyacrylamide gel was freshly prepared as follows:

6% of Polyacrylamide Gel (1 page)

40% Acrylamide:Bisarylamide	0.750 ml
5% TBE (Maniatis)	1.000 ml
Distilled water	3.250 ml
10% ammonium persulphate	0.050 ml
TEMED	0.004 ml

After gel setting, ten microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (w/v) sucrose in water) and loaded into gel slots in a submarine condition. Electrophoresis was performed at 100 volts for 1 hour. The DNA bands in the gel were visualized by staining with 2.0 μg/ml ethidium bromide and photographed under UV light at 302 nm.

2.4. Identification of G6PD Polymorphisms

2.4.1. Primers

2.4.1.1. G6P5F/G6P6R Primers

G6P5F-G6P6R primers have been previously described[9]. The PCR product of these primers contains restriction recognition site for *Pvu* II to detect variation IVS5 nt 611 C → G.

2.4.1.2. R1G7/R1S Primers

R1G7-R1S primers have been previously described[17]. R1G7 primer was designed mutagenic site at 3' created to be restriction recognition site for *Sca* I to detect variation IVS7 nt 175 C → T.

2.4.1.3. G6P8F/G6P9R Primers

8F-9R primers have been previously described[9]. The PCR product of these primers contains restriction recognition site for *Bsp*H I or *Pag* I to detect variation IVS8 nt 163 C → T.

2.4.1.4. G6P9F/G6P10R Primers

G6P9F-G6P10R primers have been previously described[9]. The PCR product of these primers contains restriction recognition site for *Pst* I to detect variation exon 10 nt 1116 G → A.

2.4.1.5. R3F/R3Md Primers

R3F-R3Md primers have been previously described[17]. R3Md primer was designed mutagenic site at 3' created to be restriction recognition site for *Bcl* I to detect variation exon 11 nt 1311 C → T.

2.4.1.6. 1360F/1360R Primers

1360F/1360R primers have been previously described[52]. The PCR product of these primers contains restriction recognition site for *Nla* III to detect variation IVS11 nt 93 T → C.

The sequence of each primer shown in table 4 and their positions relative to the coding regions of the gene is seen in figure 18.

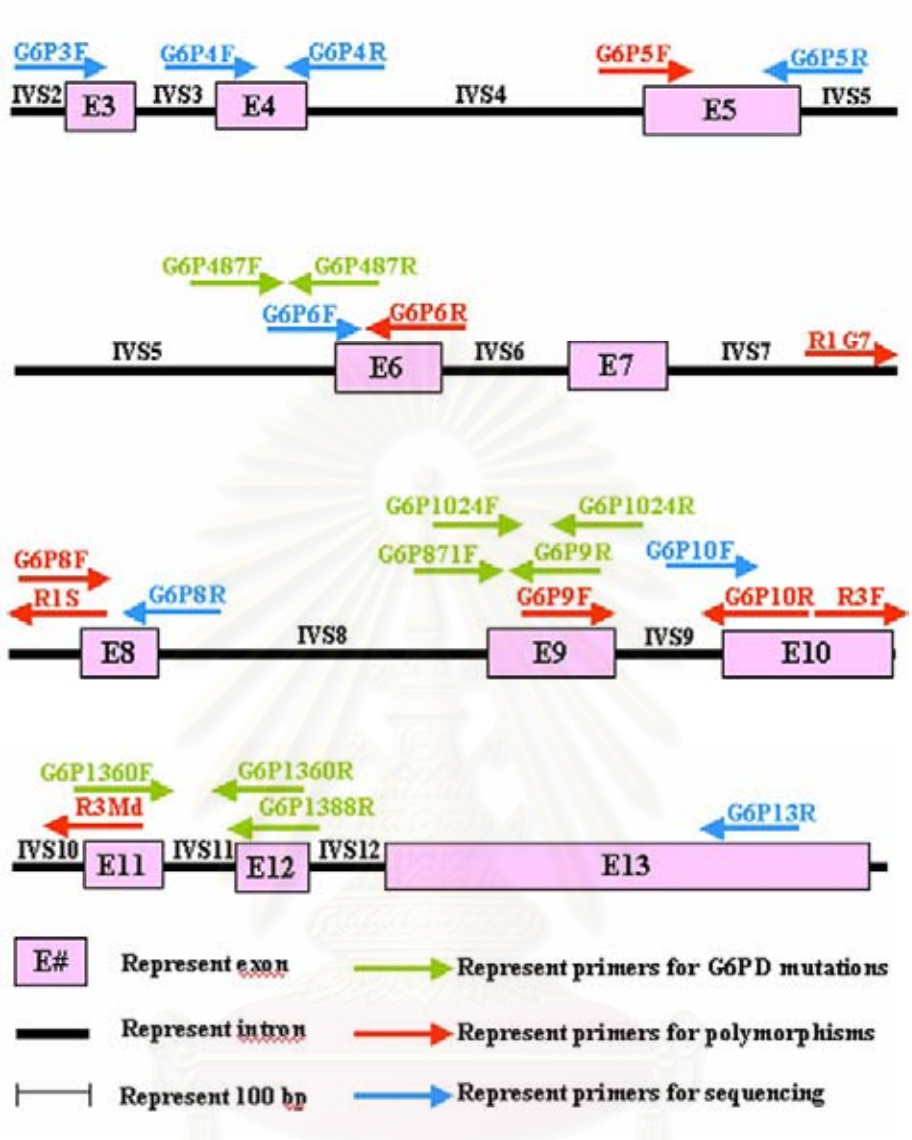


Figure 18 Primers and their positions relative to the regions of the *G6PD*.

2.4.2. PCR Conditions

2.4.2.1.For G6P5F/G6P6R, G6P8F/G6P9R, G6P9F/G6P10R

The typical PCR reaction was carried out in a 25 µl reaction containing 1X PCR buffer, 1.25 U of Taq polymerase (Promaga, Fermentas), 50 ng of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs and approximate 300 ng DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 1 minute of

denaturation, 60°C for 1 minute of annealing and 72°C for 1 minute of extension. The final amplification cycle included an addition of a 15 minutes extension at 72°C.

2.4.2.2. For R1G7/R1S

The typical PCR reaction was carried out in a 25 μl reaction containing 1X PCR buffer, 1 U of Taq polymerase (Promaga, Fermentas), 50 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs and approximate 300 ng DNA template. After incubation at 94 °C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 1 minute of denaturation, 63°C for 1 minute of annealing and 72°C for 1 minute of extension. The final amplification cycle included an addition of a 15 minutes extension at 72°C.

2.4.2.3. For R3F/R3Md, 1360F/1360R

The typical PCR reaction was carried out in a 25 μl reaction containing 1X PCR buffer, 1.25 U of Taq polymerase (Promaga, Fermentas), 50 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTPs and approximate 300 ng DNA template. After incubation at 94°C for 5 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters; 94°C for 1 minute of denaturation, 56°C for 1 minute of annealing and 72°C for 1 minute of extension. The final amplification cycle included an addition of a 15 minutes extension at 72°C.

2.4.3. Restriction Fragment Length Polymorphism (RFLP)

Ten microliters of PCR product was digested with 5 U of each restriction enzyme (The type of restriction enzyme was used in digestion depend on G6PD polymorphisms that represents in table) according to manufacturer's protocols (New England Biolabs), 1x reaction buffer (provided) and sterile distilled water added to a final volume of 20 μl. The digestion was incubated at 37°C 2-4 hours.

Table 9 Polymorphisms, restriction enzymes and the results for G6PD variant.

Polymorphisms	Restriction enzymes	Result (bp)
IVS5 nt 611 C → G	*Pvu* II	N 901 M 776+125
IVS7 nt 175 C → T	*Sca* I	N 133 M 112+21
IVS8 nt 163 C → T	*Bsp*H I or *Pag* I	N 397+250 M 647
Exon 10 nt 1116 G → A	*Pst* I	N 366+150 M 516
Exon 11 nt 1311 C → T	*Bcl* I	N 207 M 184+23
IVS11 nt 93 T → C	*Nla* III	N 214 M 172+42

Note bp represent base pair (size of PCR product).
N represent normal digestion result.
M represent mutant digestion result.

2.4.4. Test Activity of Enzyme

2.4.4.1 RFLP in pTrcHisA Vector as a Positive Control

pTrcHisA vector, which has *Pvu* II and *Sca* I sites were digested by *Pvu* II and *Sca* I. It can confirm the activity of there restriction enzymes. Ten microliters of pTrcHisA with PCR buffer (same condition to PCR product) was digested with 5 U of each restriction enzyme according to manufacturer's protocols (New England Biolabs), 1x reaction buffer (provided) and sterile distilled water added to a final volume of 20 μl. The digestion was incubated at 37°C 2-4 hours.

2.4.4.2. Sequencing

The PCR sequencing was performed by using BigDye™ Terminator Cycle Sequencing Ready Reaction kit. The PCR reaction was carried out in a 10 μl reaction containing 4.0 μl of terminator ready reaction mix, 3.2 pmol of sequencing primer and 500 ng DNA template. After incubation at 95°C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 sec of denaturation, 50°C for 5 sec of annealing and 60°C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 μl of 70% ethanol and air dried. The DNA pellet was resuspended in 10 μl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

2.4.5. Electrophoresis

2.4.5.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect G6PD variants that their result produced large DNA fragments as follows: IVS5 nt 611C → G, IVS8 nt 163C → T, exon 10 nt 1116 G → A. Agarose gel of 1.5% was prepared by completely dissolving the gel powder upon heating in 1X TAE (89 mM Tris-HCl pH 7.4, 89 mM acetic acid, 2.5 mM EDTA). The solution was boiled in a microwave oven until it was completely dissolved and allowed to cool to around 50°C before pouring into an electrophoresis chamber set, with comb inserted. Twenty microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (w/v) sucrose in water) and loaded into gel slots in a submarine condition. Electrophoresis was performed at 100 volts for 1 hour. The DNA bands in the gel were visualized by staining with 2.0 μg/ml ethidium bromide and photographed under UV light at 302 nm.

2.4.5.2. Acrylamide Gel Electrophoresis

Acrylamide gel electrophoresis was used to detect G6PD variants that their result produced DNA fragments different less than 20 bp as follows: IVS7 nt 175 C → T, exon 11 nt 1311 C → T, IVS11 nt 93 T → C. Six percentage of polyacrylamide gel was freshly prepared as follows:

6% of Polyacrylamide Gel (1 page)

40% Acrylamide:Bisarylamide	0.750 ml
5% TBE	1.000 ml
Distilled water	3.250 ml
10% ammonium persulphate	0.050 ml
TEMED	0.004 ml

After gel setting, ten microliters of digestion's product was mixed with 1/6 volume of Loading Dye (0.25% bromophenol blue, 40% (w/v) sucrose in water) and loaded into gel slots in a submarine condition. Electrophoresis was performed at 100 volts for 1 hour. The DNA bands in the gel were visualized by staining with 2.0 μg/ml ethidium bromide and photographed under UV light at 302 nm.

2.5. Identification of Unknown Mutation

2.5.1. Primers

2.5.1.1. G6P3F/G6P4R, G6P4F/G6P5R, G6P6F/G6P8R, G6P10F/G6P13R Primers

G6P3F/4R, 4F/5R, 6F/8R, 10F/13R primers have been previously described[9].

2.5.2. Sequencing

The PCR sequencing was performed by using BigDye™ Terminator Cycle Sequencing Ready Reaction kit. The PCR reaction was carried out in a 10 μl reaction containing 4.0 μl of terminator ready reaction mix, 3.2 pmol of sequencing

primer and 500 ng DNA template. After incubation at 95°C for 2 min, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 sec of denaturation, 50°C for 5 sec of annealing and 60°C for 4 min of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at -20°C for 1 hour. After centrifugation at 12,000 rpm for 10 min, the pellet was washed with 500 μl of 70% ethanol and air dried. The DNA pellet was resuspended in 10 μl Template Suppression Reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

2.5.3. Alignment and Computational Searching Sequences Analysis

The nucleotide sequences from the PCR products were compared against nucleotide sequences in online database by using ClustalX multiple alignment program for finding novel mutation.

2.6. Data Collection

Data were collected secretly in Excel program.

2.7. Data Analysis

2.7.1. Calculate Prevalence of G6PD Deficient Male and Female in Each Nationality

Prevalence of G6PD deficient male and female in each nationality was calculated by SPSS program.

Prevalence of G6PD deficient male in each nationality

$$= \frac{\text{Number of G6PD deficient male in each nationality}}{\text{All of males in each nationality}} \quad \text{----(equation 2)}$$

Prevalence of G6PD deficient female in each nationality

$$= \frac{\text{Number of G6PD deficient female in each nationality}}{\text{All of females in each nationality}} \quad \text{----(equation 3)}$$

2.7.2. Calculate Prevalence of G6PD Deficient Jaundice Male and Female in Each Nationality

Prevalence of G6PD deficient jaundice male and female in each nationality was calculated by SPSS program.

Prevalence of G6PD deficient jaundice male in each nationality

$$= \frac{\text{Number of G6PD deficient jaundice male in each nationality}}{\text{All of jaundice males in each nationality}} \quad \text{---(equation 4)}$$

Prevalence of G6PD deficient jaundice female in each nationality

$$= \frac{\text{Number of G6PD deficient jaundice female in each nationality}}{\text{All of jaundice females in each nationality}} \quad \text{-(equation 5)}$$

2.7.3. Calculate Mean of G6PD Activity in Each Mutations

Amount of G6PD activity in each nationality and each ~~-G~~6PD mutations were calculated by SPSS program. Statistic was used is mean (X) and standard deviation (SD).

2.7.4. Calculate Percentage of Each G6PD Mutation in Each Nationality

Percentage of each G6PD mutation in each nationality was calculated by SPSS program.

Percentages of each G6PD mutations in each nationality

$$= \frac{\text{Number of each G6PD mutations in each nationality} \times 100}{\text{All of G6PD mutation in each nationality}} \quad \text{----(equation 6)}$$

2.7.4. Analysis of Association

2.7.4.1. Association Between Pairs of Polymorphic Sites

Allelic frequencies for the polymorphic site were determined by directly counting alleles in population. A more accurate assessment of linkage desequilibrium is obtained by statistical analysis of the distributions of pairwise

haplotypes. The coefficients of linkage disequilibrium *D*, the maximum or minimum possible value for *D* ($D_{max\ or\ min}$) at given allelic frequencies, and the normalizes coefficient *D** were calculated for each pairwise haplotype[64]. The absolute coefficients of linkage disequilibrium were calculated first according to the definition

$$D = (++ \times --) - (+- \times -+) \quad \text{----(equation 4)}^{64}$$

and normalized according to two different methods as

$$Y = D/(++ \times --) + (+- \times -+) \quad \text{----(equation 5)}^{64}$$

or $$D^* = D/D_{max\ or\ min} \quad \text{----(equation 6)}^{64}$$

where $D_{max\ or\ min}$ is, according to the sign of *D*, either

$$D_{max} = p(1 - q) \quad \text{----(equation 7)}^{64}$$

$$D_{min} = -pq \quad \text{----(equation 8)}^{64}$$

X^2-test calculated the significant degree of linkage disequilibrium between all of the pairwise combinations.

2.7.4.2. Association between Haplotypes and G6PD Mutations

2.7.4.2.1 Association between Haplotype II (+,-; 1311T, 93T) in G6PD Viangchan of Cambodian and Non-Cambodian

Hypothesis

H_0 : +,- (1311T, 93T) doesn't associate with G6PD Viangchan of Cambodian.

H_a : +,- (1311T, 93T) associates with G6PD Viangchan of Cambodian.

Fisher's exact test was used to prove the association. Statistic: *p-value* (confidence interval 95%) from 2 x 2 table

Table 10 2 x 2 table for Fisher's exact test.

Haplotypes	G6PD mutation	Normal control	Total
Test	a	b	e
control	c	d	f
Total	g	h	n

$$p\text{-}value = \frac{e!f!g!h!}{n!a!b!c!d!} \quad \text{----(equation 9)}^{63}$$

2.7.4.2.2 Association between Haplotype I (+,+; 1311T, 93C) of G6PD Viangchan and Normal Control

Hypothesis

H_0 : +,+ (1311T, 93C) doesn't associate with G6PD Viangchan.

H_a : +,+ (1311T, 93C) associates with G6PD Viangchan.

2.7.5. Phylogenetic Reconstruction of G6PD Mutations

To reconstruct the evolutionary history of G6PD deficiency mutations, MIX that is a maximum parsimony program is a program in cluster of PHYLIP (the PHYLIP package of phylogeny inference programs), was chosen to analyze restriction site data[65]. The method of this program follows by the documentation file for the discrete-characters programs (www.umanitoba.ca/afs/plant_science/psgendb/doc/Phylip/mix.asc).

The method of reconstruction phylogenetic tree from restriction site by maximum parsimony is[65]

1. Built the matrix shown binary characters (0,1) for all of OTUs (operational taxonomic units)

```
 16 14
B 00000000001100
BA 00000000001110
BB 00000000001101
BC 00000000001111
BD 0000000000100?*
A  1000000000110?
AA 1000000000010?
AB 1000000011010?
AC 1100000011010?
VA 00100000001111
VB 00100000001110
MD 00010000001100
CT 00001000001100
UN 00000100001100
KP 00000010001100
CH 00000001001100
```

* ? represents never been reported

The first line contains the number of species and the number of characters. Next come the species and character data in separate lines. The characters are representing of restriction sites follow as: nt 376, nt 202, nt 871, nt 487, nt 1376, nt 1360, nt 1388, nt 1024, nt 611C → G, nt 175C → T, nt 163 C → T, nt 1116 G → A and nt 1311 C → T. 0 and 1 represent was not digested and digested with restriction enzyme respectively.

2. Analyzed data by the MIX program from http://bioportal.bic.nus.edu.sg/phylip/mix.html (figure 19).

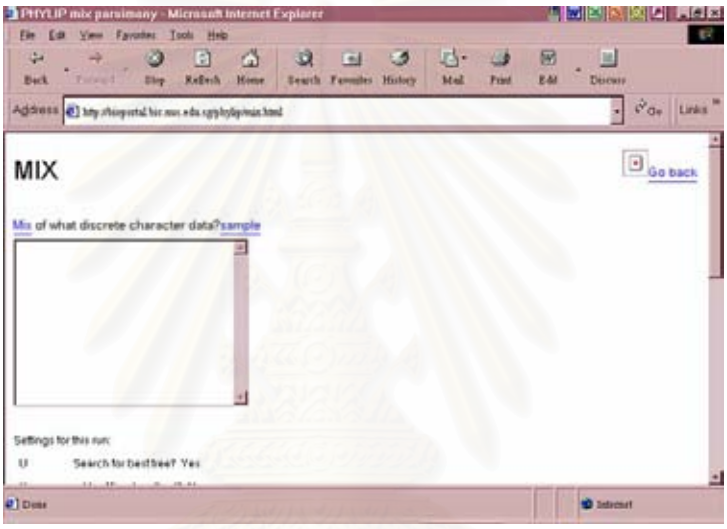


Figure 19 Phylip mix parsimony.

CHAPTER IV

RESULTS

Phenotype data from all of samples were analyzed. Averaged age of Myanmars, Cambodian and Laotian immigrant laborers is 26 ± 8.6, 27 ± 8.3, 26 ± 5.5 years old respecitively. For Thai blood samples and some of Cambodians and Laotians come from newborn.

1. Identification of G6PD Deficient Patients

All of blood samples were identified by quantitative G6PD activity assay. Blood samples had amount of G6PD activity less than 1.5 I.U./g Hb were identified to be G6PD deficiency. Prevalence of G6PD deficient patients in each nationality was presented in table 11. As prevalence of G6PD deficiency in neonatal jaundices in each nationality was presented in table 12.

Table 11 Prevalence of G6PD deficiencies in each nationality.

Nationality	Cases (N)	Sex (N)	G6PD deficient cases (N)	G6PD level (IU/g Hb)(SD)	Prevalence
Myanmars	131	M 72	7	0.8 (0.43)	0.10
		F 59	1	1.1	0.02
Cambodian	164	M 92	17	0.4 (0.29)	0.19
		F 72	3	0.3 (0.56)	0.04
Laotian	127	M 69	12	0.7 (0.29)	0.17
		F 58	5	0.3 (0.44)	0.09
Thai	23	M 14	3	1.0 (0.15)	0.11
		F 9	1	1.4	0.06

In Myanmars laborers, prevalence of G6PD deficiency was found 7 (9.72%) of 72 males and 1 (1.69%) in 59 females. As in Cambodian, prevalence of G6PD deficiency was found 17 (18.48%) of 92 males and 3 (4.16%) in 72 females. For Laotian, prevalence of G6PD deficiency was found 12 (17.39%) of 69 males and

5 (8.62%) in 58 females. In Thai, prevalence of G6PD deficiency was found 3 of 14 males and 1 in 9 females.

Table 12 Prevalence of G6PD deficient jaundice in each nationality.

Nationality	Cases (N)	Sex (N)	G6PD deficient cases (N)	G6PD level (IU/g Hb)(SD)	Prevalence
Cambodian	51	M 27 F 24	14 0	0.7 (0.43) -	0.52
Laotian	35	M 15 F 20	8 1	0.8 (0.34) 1.2	0.53 0.05
Thai	310	M 184 F 126	30 10	0.2 (0.27) 0.5 (0.53)	0.16 0.08

In neonatal jaundice Cambodian, prevalence of G6PD deficiency was found 14 (51.85%) of 27 males but in G6PD deficient females were not found. As in neonatal jaundice Laotian, prevalence of G6PD deficiency was found 8 (53.33%) of 15 males and 1 (5.00%) in 20 females. In neonatal jaundice Thai, prevalence of G6PD deficiency was found 30 (16.30%) of 184 males and 10 (7.94%) in 126 females. G6PD deficient neonatal jaundice Myanmars was not studied.

2. Identification of G6PD Mutations

G6PD Viangchan was the first mutation was identified in all G6PD deficiencies except G6PD deficient Myanmars that G6PD Mahidol was identified before the other mutations. In identification G6PD Viangchan, 871F and 9R primers amplified PCR product size 126 bp. In digestion of PCR product with *Xba* I, the product of G6PD Viangchan mutation was 106 and 20 bp but the product of normal was 126 bp. As, female heterozygous product was both 126 and 106 bp (figure 20).

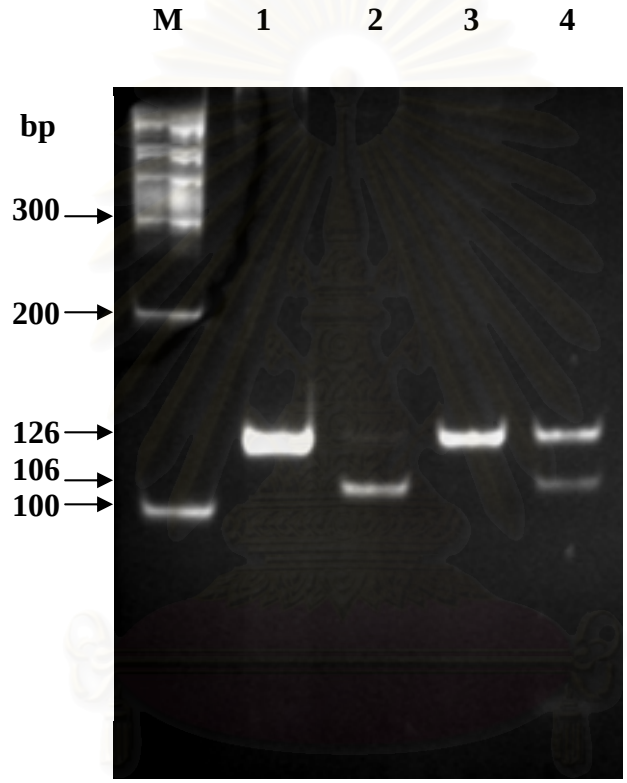


Figure 20 PCR-RFLP with *Xba* I for G6PD Viangchan. Lane M, 100 bp ladder; lane 1, undigested G6PD Viangchan showed a 126 bp band; lane 2, digested G6PD Viangchan that reduced to 106 bp after *Xba* I digestion; lane 3, digested normal shown 126 bp band can not digested with *Xba* I; lane 4, digested female heterozygote shown both 126 and 106 bp after *Xba* I digestion.

G6PD Mahidol was the second mutation was identified in G6PD deficiency that did not found G6PD Vaiangchan in the first test. For G6PD deficient Myanmars were identified G6PD Mahidol before. In identification G6PD Mahidol, 487F and 487R primers amplified PCR product size 104 bp. In digestion of PCR product with *Hin*d III, the product of G6PD Mahidol mutation was 82 and 22 bp but the product of normal was 104 bp (figure 21).

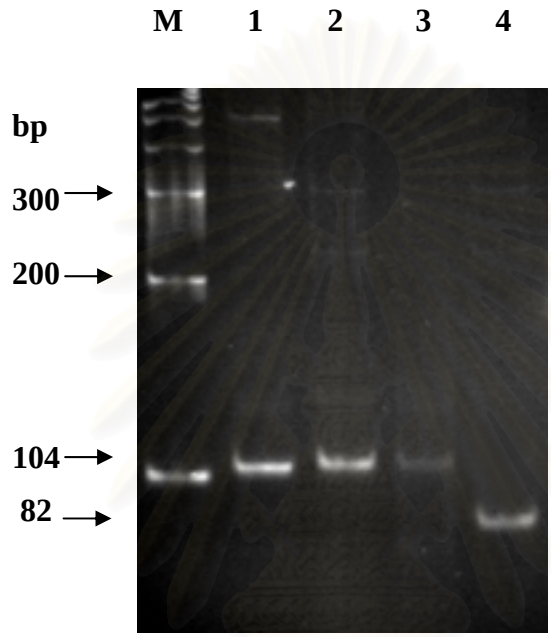


Figure 21 PCR-RFLP with *Hin*d III for G6PD Mahidol. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 104 bp band; lane 2-3, digested normal shown 104 bp band can not digested with *Hin*d III; lane 4, digested G6PD Mahidol that reduced to 82 bp after *Hin*d III digestion.

G6PD Canton was the third mutation was identified in G6PD deficiencies. In identification G6PD Canton, 1360F and 1360R primers amplified PCR product size 214 bp. In digestion of PCR product with *Afl* II, the product of G6PD Canton mutation was 194 and 20 bp but the product of normal was 214 bp. As female heterozygous product both 214 and 194 bp after digested with *Afl* II (figure 22).

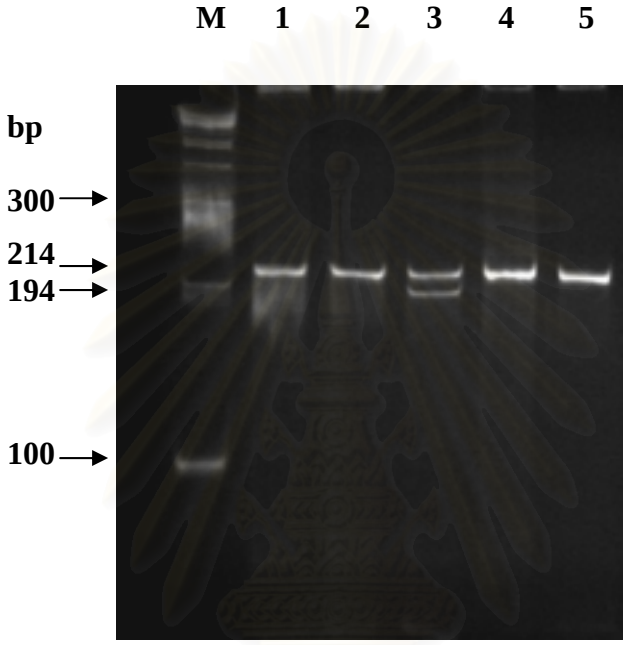


Figure 22 PCR-RFLP with *Afl* II for G6PD Canton. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 214 bp band; lane 2,4-5, digested normal shown 214 bp band can not digested with *Afl* II; lane 3, digested female heterozygote shown both 214 and 194 bp after *Afl* II digestion.

G6PD Union was the forth mutation was identified in G6PD deficiencies. In identification G6PD Union, 1360F and 1360R primers amplified PCR product size 214 bp. In digestion of PCR product with *Hha* I, the product of G6PD Union mutation was 187 and 27 bp but the product of normal was 142, 45 and 27 bp (figure 23).

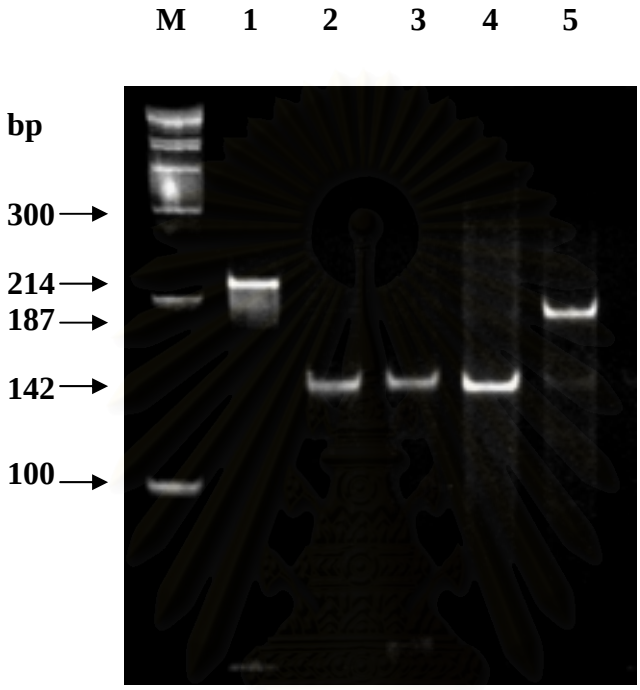


Figure 23 PCR-RFLP with *Hha* I for G6PD Union. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 214 bp band; lane 2-4, digested normal shown 142, 45 and 27 bp band after *Hha* I digestion; lane 5, G6PD Union that reduced to 187 and 27 bp after digested with *Hha* I.

G6PD Kaiping was the fifth mutation was identified in G6PD deficiencies. In identification G6PD Kaiping, 1360F and 1388R primers amplified PCR product size 227 bp. In digestion of PCR product with *Nde* I, the product of G6PD Kaiping mutation was 206 and 21 bp but the product of normal was 227 bp (figure 24).

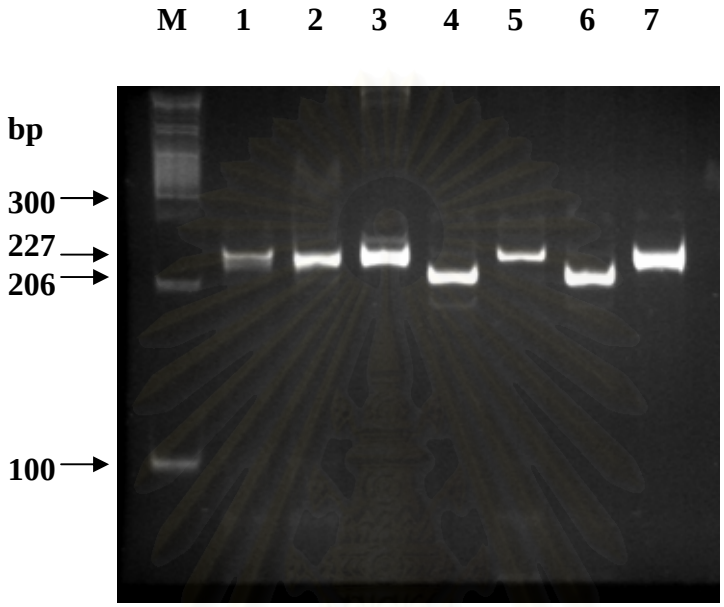


Figure 24 PCR-RFLP with *Nde* I for G6PD Kaiping. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 227 bp band; lane 2-3,5,7 digested normal shown 227 bp band can not digested with *Nde* I; lane 4,6, digested G6PD Kaiping that reduced to 206 and 21 bp after *Nde* I digestion.

G6PD Chinese-5 was the sixth mutation was identified in G6PD deficiencies. In identification G6PD Chinese-5, 1024F and 1024R primers amplified PCR product size 187 bp. In digestion of PCR product with *Mbo* II, the product of G6PD Chinese-5 mutation was 150 and 37 bp but the product of normal was 187 bp (figure 25).

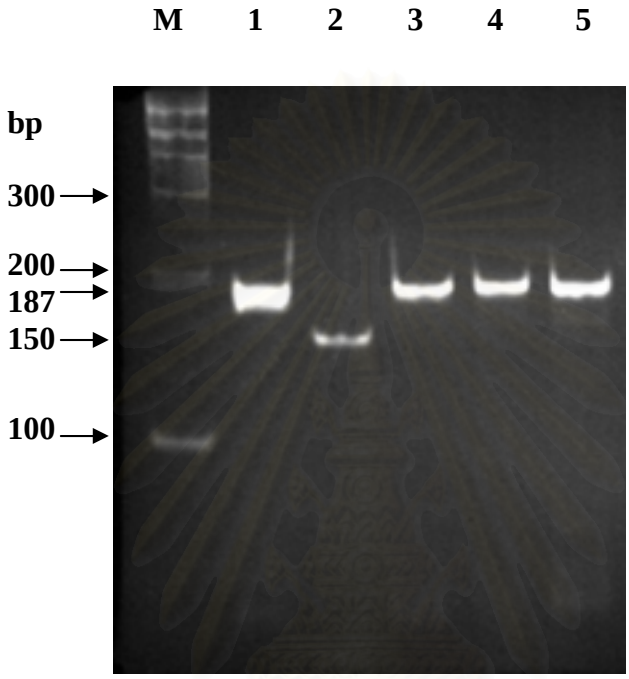


Figure 25 PCR-RFLP with *Mbo* II for G6PD Chinese-5. Lane M, 100 bp ladder; lane 1, undigested G6PD Chinese-5 showed a 187 bp band; lane 2, digested G6PD Chinese-5 that reduced to 150 and 37 bp after *Mbo* II digestion; lane 3-5, digested normal shown 187 bp band can not digested with *Mbo* II.

In identification of G6PD mutations, G6PD Mahidol was the most G6PD mutation was found in Myanmars and was not found G6PD Viangchan. As in Cambodian, Laotian and Thai, G6PD Viangchan was the most found G6PD mutation. G6PD Kaiping, G6PD Mahidol, G6PD Canton, G6PD Union and G6PD Chinese-5 were found decreasingly respectively (table 13).

Table 13 G6PD mutations and enzyme activity in different national population.

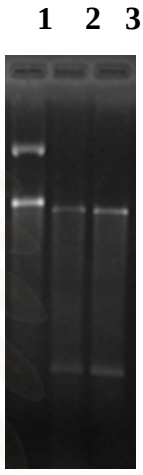
Mutation	Nucleotide substitution	Cases (N (%))	Population (N(%))				Sex (N)	G6PD level (IU/g Hb)(SD)
			Myanmars	Cambodian	Laotian	Thai		
Viangchan	871 A	61 (54.5)	0	28(82.4)	12(46.2)	21(47.7)	M 49 F 12	0.42 (0.120) 0.51 (0.592)
Mahidol	487 A	7 (6.3)	5(62.5)	0	0	2 (4.5)	M 6 F 1	0.62 (0.454) 1.10
Canton	1376 T	6 (5.4)	1(12.5)	0	4(3.8)	1(2.3)	M 5 F 1	0.56 (0.449) 0.62
Union	1360 T	2 (1.8)	0	1(2.9)	1(3.8)	0	M 2 F 0	0.72 (0.318) -
Kaiping	1388 A	9 (8.0)	0	0	2(7.7)	7(15.9)	M 8 F 1	0.22 (0.314) 0.00
Chinese-5	1024 T	1 (0.9)	0	0	0	1(2.3)	M 1 F 0	0.95 -
Unknown		26 (23.2)	2(25)	5(14.7)	7(26.9)	12(27.3)	M 20 F 6	0.43 (0.454) 0.59 (0.564)
Total		112 (100)	8(100)	34(100)	26(100)	44(100)	M 91 F 23	

3. Identification of G6PD polymorphisms

In identification IVS5 nt 611 C → G, 5F and 6R primers amplified PCR product size 901 bp. In digestion of PCR product with *Pvu* II, the product of nt 611 C → G was 776 and 225 bp but the product of nt 611 C was 901 bp (figure 27). In usually, *Pvu* II cannot digest PCR product of 5F and 6R primers. To prove *Pvu* II activity, pTrcHisA vecter has *Pvu* II site was used to test activity of *Pvu* II (figure 26).

Figure 26 *Pvu* II activity in pTrcHisA.

Lane 1, undigested pTrcHisA shown 2 bands contain circular form and super coil form; lane 2, pTrcHisA was digested with *Pvu* II within PCR buffer condition; lane 3, pTrcHisA was digested with *Pvu* II without PCR buffer condition.



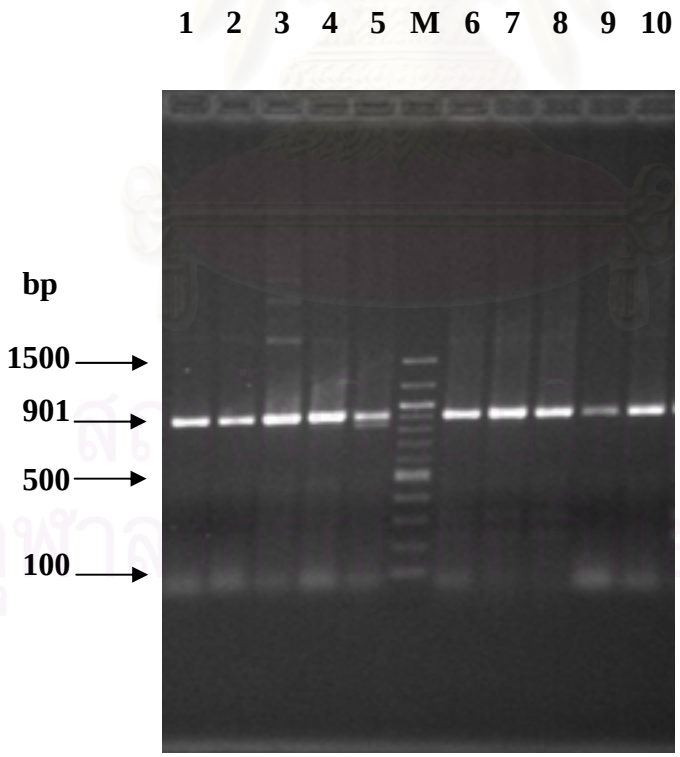


Figure 27 PCR-RFLP with *Pvu* II for nt 611 C → G. Lane M, 100 bp ladder; lane 1,10, undigested PCR product showed a 901 bp band; lane 2-5,6-9, digested nt 611 C shown 901 bp after *Pvu* II digestion.

PCR product of 5F and 6R primers was confirmed does not have *Pvu*II site by sequencing (figure 28).

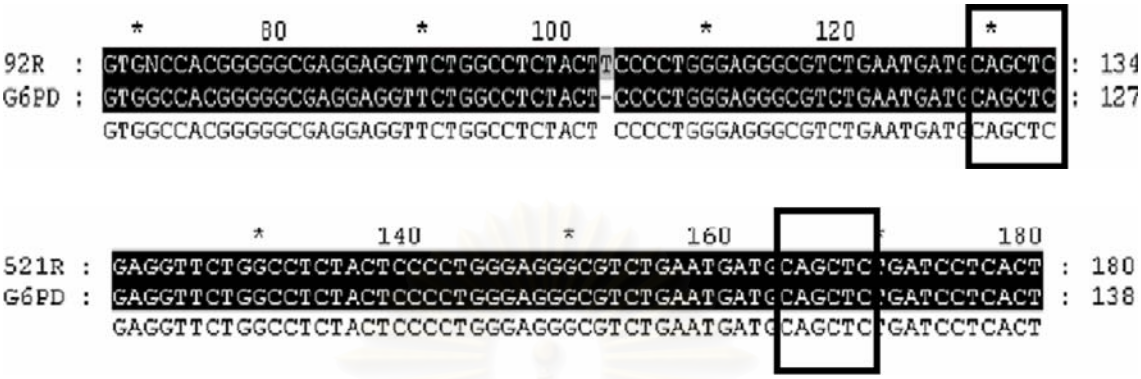


Figure 28 Sequence of 6R PCR product of 92R and 521R samples shown does not have *Pvu* II site (CAG^CTG) in black box.

In identification IVS7 nt 175 C → T, R1G7 and R1S primers amplified PCR product size 133 bp. In digestion of PCR product with *Sca* I, the product of nt 175 C → T was 112 and 21 bp but the product of nt 175 C was 133 bp (figure 30). In usually, *Sca* I cannot digest PCR product of R1G7 and R1S primers. To prove *Sca* I activity, pTrcHisA vector has *Sca* I site was used to test activity of *Sca* I (figure 29).

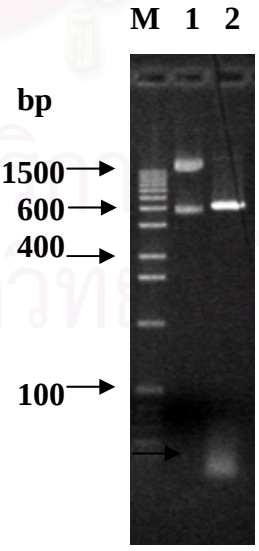


Figure 29 *Sca* I activity in pTrcHisA.

Lane M, 100 bp ladder; lane 1, undigested pTrcHisA shown 2 bands contain circular form and super coil form; lane 2, pTrcHisA was digested with *Sca* I within PCR buffer condition.

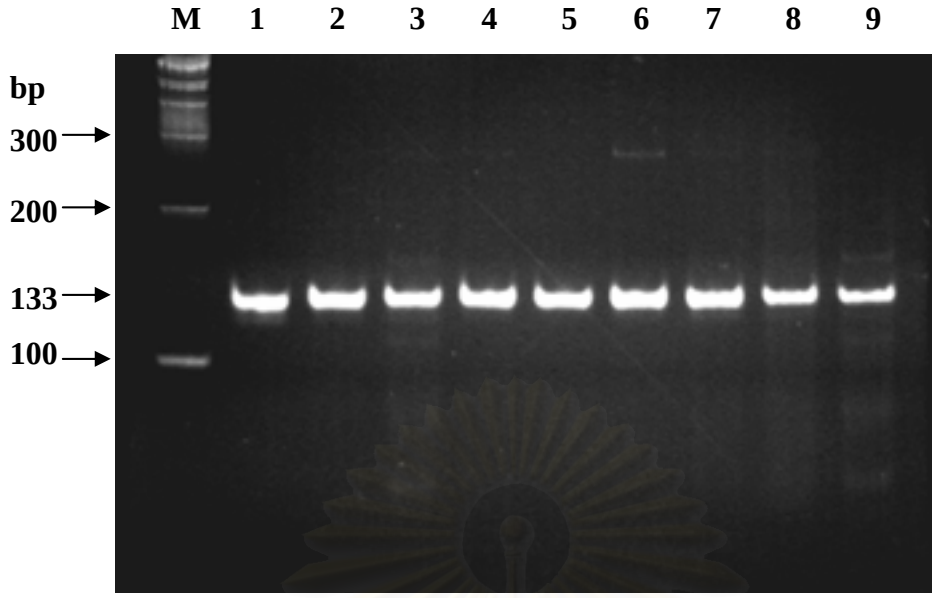


Figure 30 PCR-RFLP with *Sca* I for nt 175 C → T. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 133 bp band; lane 2-9, digested nt 175 C shown 133 bp after *Sca* I digestion.

PCR product of R1G7 and R1S primers was confirmed does not have *Sca* I site by sequencing (figure 31).

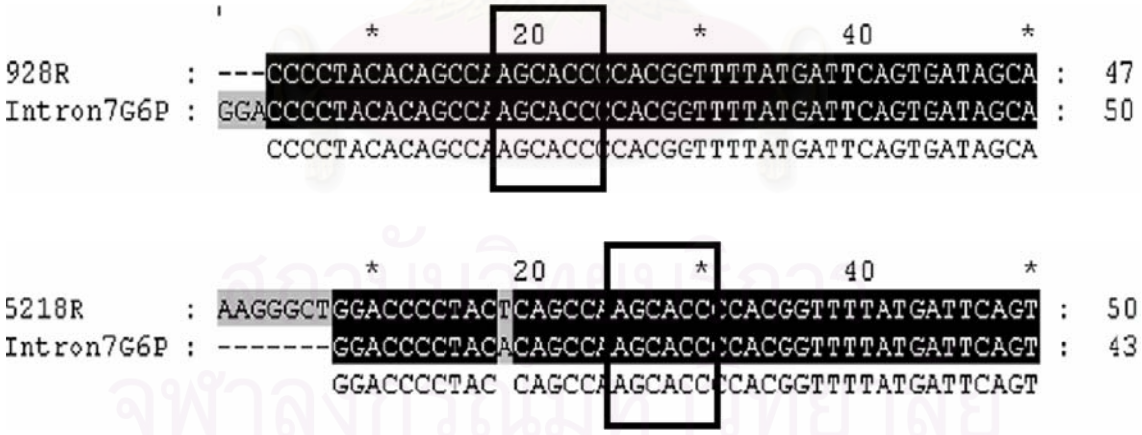


Figure 31 Sequence of 8R PCR product of 92 and 521 samples shown does not have *Sca* I site (AGT^ACT) in black box.

In identification IVS8 nt 163 C → T, 8F and 9R primers amplified PCR product size 647 bp. In digestion of PCR product with *Bsp*H I, the product of nt 163 C was 397 and 250 bp but the product of nt 163 C → T was 647 bp (figure 32).

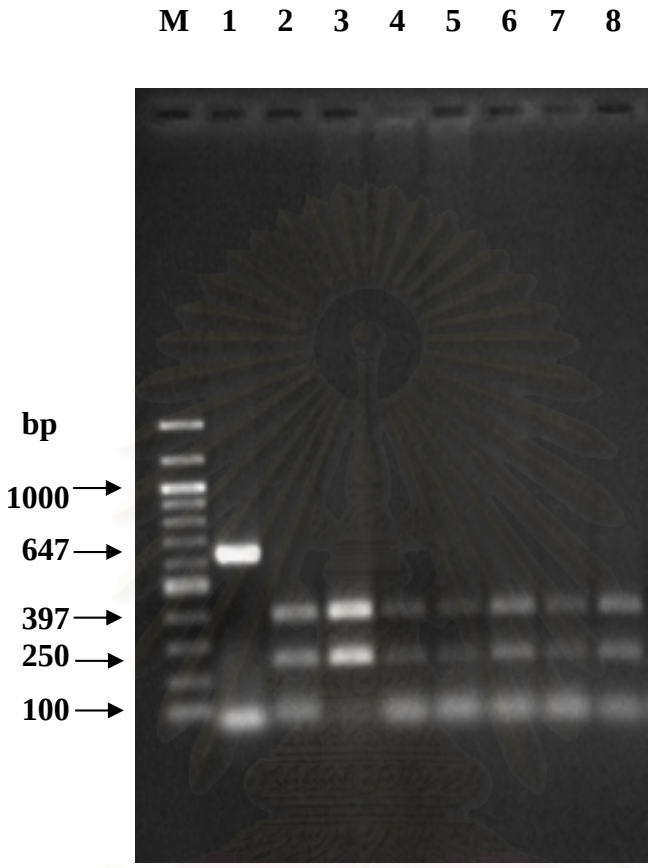


Figure 32 PCR-RFLP with *Bsp*H I for nt 163 C → T. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 647 bp band; lane 2-8, digested nt 163 C shown 397 and 250 bp after *Bsp*H I digestion.

In identification exon10 nt 1116 G → A, 9F and 10R primers amplified PCR product size 516 bp. In digestion of PCR products with *Pst* I, the product of nt 1116 G was 366 and 150 bp but the product of nt 1116 G → A was 516 bp (figure 33).

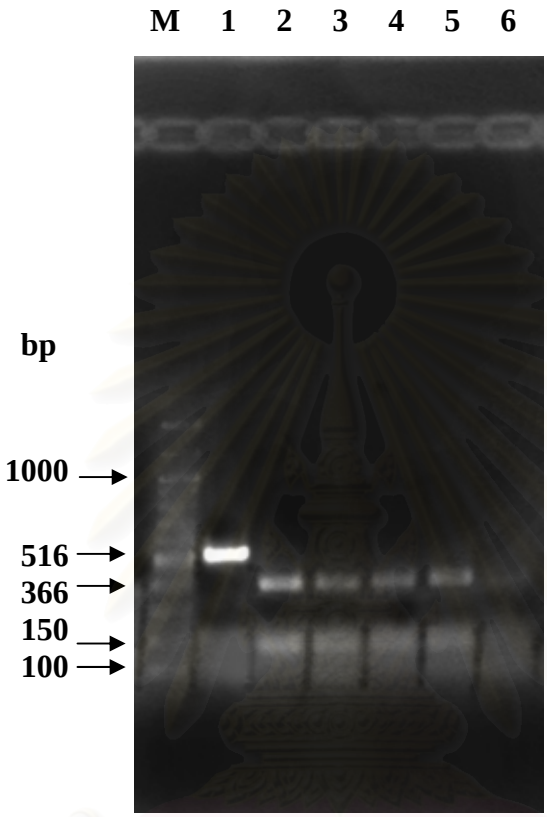


Figure 33 PCR-RFLP with *Pst* I for nt 1116 G → A. Lane M, 100 bp ladder; lane 1, undigested PCR product showed a 516 bp band; lane 2-6, digested nt 1116 G shown 366 and 150 bp after *Pst* I digestion.

In identification exon11 nt 1311 C → T, R3Md and R3F primers amplified PCR product size 207 bp. In digestion of PCR product with *Bcl* I, the product of nt 1311 C was 207 bp but the product of nt 1311 C → T was 184 and 23 bp (figure 34).

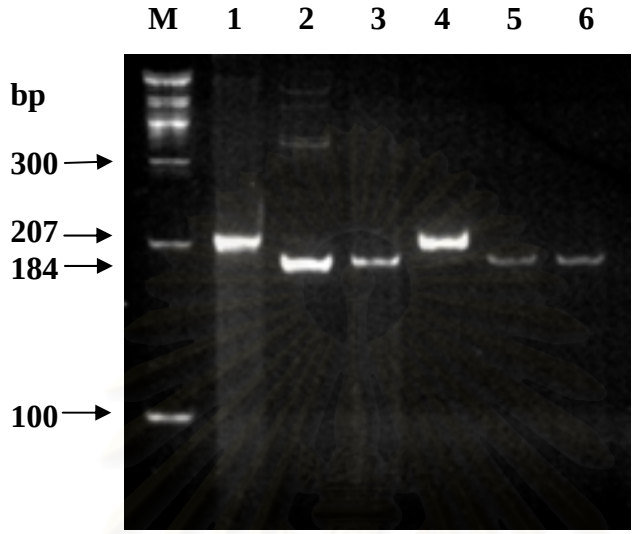


Figure 34 PCR-RFLP with *Bcl* I for nt 1311 C → T. Lane M, 100 bp ladder; lane 1, undigested nt 1311 C → T showed a 207 band; lane 2-3,5-6, digested nt 1311 C → T shown 184 and 23 bp after *Bcl* I digestion; lane 4, digested nt 1311 C shown 207 bp.

In identification IVS11 nt 93 T → C, 1360F and 1360R primers amplified PCR product size 214 bp. In digestion of PCR product with *Nla* III, the product of nt 93 T was 214 bp but the product of nt 93 T → C was 172 and 42 bp (figure 35).

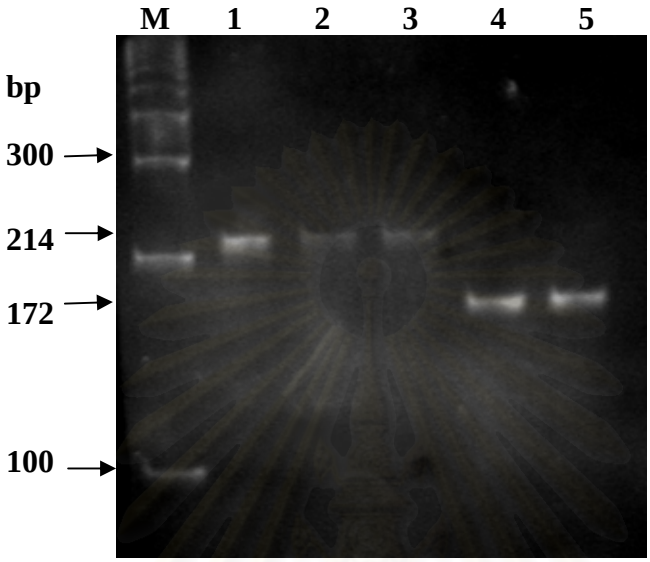


Figure 35 PCR-RFLP with *Nla* III for nt 93 T → C. Lane M, 100 bp ladder; lane 1, undigested nt 93 T → C showed a 214 band; lane 2-3, digested nt 93 T shown 214 bp; lane 4-5, digested nt 93 T → C shown 172 bp after *Nla* III digestion.

For identification of G6PD polymorphism, 6 polymorphic sites were analyzed in all male G6PD deficiencies (n) and male normal control samples (about 2n). Haplotypes of polymorphic sites in each G6PD mutation were summarized in table 14.

Table 14 Haplotypes of the G6PD mutations in Southeast Asia population.

Mutation	Haplotype	Polymorphic site						N	%
		611G	175T	163T	1116G	1311T	93 C		
		Pvu II	*Sca* I	*Bsp*HI	*Pst* I	*Bcl* I	*Nla III*		
G6PD B	I	-	-	+	+	+	+	43	21.3
G6PD B	II	-	-	+	+	+	-	5	2.5
G6PD B	III	-	-	+	+	-	+	13	6.4
G6PD B	IV	-	-	+	+	-	-	141	69.8
G6PD Viangchan	I	-	-	+	+	+	+	46	93.9
G6PD Viangchan	II	-	-	+	+	+	-	3	6.1
G6PD Mahidol	IV	-	-	+	+	-	-	6	
G6PD Canton	IV	-	-	+	+	-	-	5	
G6PD Union	IV	-	-	+	+	-	-	2	
G6PD Kaiping	IV	-	-	+	+	-	-	8	
G6PD Chinese-5	IV	-	-	+	+	-	-	1	
Unknown mutation	I	-	-	+	+	+	+	9	47.4
Unknown mutation	IV	-	-	+	+	-	-	9	47.4
Unknown mutation	III	-	-	+	+	-	+	1	5.3

Note: Identification of G6PD polymorphisms was studied only males or hemizygous.
N: The number of chromosomes having the haplotype of a given line.
%: Percentage of each haplotype in all chromosomes of each mutation.

There are 4 different haplotypes are detected, with haplotype IV accounting for 70% of all the chromosomes of G6PD B. Haplotype I was the most frequency of G6PD Viangchan chromosome.

4. Analysis of Association

4.1. Association between Pairs of Polymorphic Sites

To test for association between alleles in the population samples, the data in table 14 were first analyzed in terms of two sites (pairwise haplotypes) (table 15).

Table 15 Distribution of pairwise haplotypes.

		Total (N)
1311C → T *Bcl* I	93T → C *Nla* III	292
+	+	98
+	-	8
-	+	14
-	-	172
Viangchan 871 G → A *Xba* I	1311C → T *Bcl* I	292
+	+	49
+	-	0
-	+	57
-	-	186
Viangchan 871 G → A *Xba* I	93T → C *Nla* III	292
+	+	46
+	-	3
-	+	66
-	-	177

Note: The other pairwises were not presented in table 15 because they had only one haplotype that cannot calculate linkage disequilibrium. There are 3 pairwises had diversity can calculate linkage disequlibrium.

For more accurate assessment of linkage disequilibrium is obtained by statistic analysis of the distributions of pairwise haplotypes. The absolute coeffients of linkage disequilibrium were calculated first according to the definition D = (++ x --) - (+- x -+) (table 16) and normalized according to two different methods as $Y = D/$(++ x --) + (+- x -+). These values are shown in table 4, together with the physical distance between the polymorphic sites and the calculated X^2.

Table 16 Statistics of association between pairs of polymorphic sites.

		Kp[a]	***D***[b]	***Y***[c]	X^{2} [d]
1311C → T *Bcl* I	93T → C *Nla* III	0.15	0.20	0.99	202.96
Viangchan 871 G → A *Xba* I	1311C → T *Bcl* I	0.68	0.11	1.00	108.03
Viangchan 871 G → A *Xba* I	93T → C *Nla* III	0.70	0.10	0.95	78.40

[a] Physical distance between two loci in kilobases.
[b] *D*: Coefficient of linkage disequilibrium = (++ x --) – (+- x -+).
[c] *Y*: Normalized coefficient of linkage disequilibrium = *D*/(++ x --) + (+- x -+).
[d] The probability of null hypothesis (linkage disequilibrium) was calculated from the tables with 1 *df* and found to be $P<0.05$ for all the cases in the column.

The results demonstrated a significant degree of linkage disequilibrium between all of the pairwise combinations.

4.2. Association between G6PD Mutations and Polymorphisms

4.2.1. Association between Haplotype II (+,-; 1311T, 93T) in G6PD Viangchan of Cambodian and Non-Cambodian

When compared between haplotype II (+,-; 1311T, 93T) and haplotype I (+,+; 1311T, 93C) in G6PD Viangchan of Cambodian and Non-Cambodian, haplotype II (+,-; 1311T, 93T) did not associate with G6PD Viangchan of Cambodian($P>0.05$).

4.2.2. Association between Haplotype I (+,+; 1311T, 93C) in G6PD Viangchan and Normal Control of all ethnic groups

When compared between haplotype I (+,+; 1311T, 93C) and non-haplotype I (haplotype II, haplotype III and haplotype IV) in G6PD Viangchan and normal control of all ethnic groups, haplotype I (+,+; 1311T, 93C) associated with G6PD Viangchan in all ethnic groups ($P<0.05$).

4.2.3 Association between Haplotype IV (-,-; 1311C, 93T) in G6PD Mutations and Normal Control of all ethnic groups.

When compared between haplotype IV (-,-; 1311C, 93T) and non-haplotype IV (haplotype I, haplotype II and haplotype III) in G6PD Mahidol, G6PD Canton, G6PD Union, G6PD Kaiping and G6PD Chinese-5 of all ethnic groups and normal control, haplotype IV (-,-; 1311C, 93T) did not associate with all of G6PD mutation in all ethnic groups (P>0.05).

5. Phylogenetic reconstruction of G6PD mutations

When 3 polymorphic sites are found to be closely associated, it is possible to reconstruct the pattern of evolution of G6PD mutations. Mix program of phylip program reconstructed phylogenetic tree of G6PD mutations in 100 trees. The most possible tree (figure 36) was chosen from base on reference of evolution of G6PD mutations in African[17], percentage of each form reconstructed in 100 trees and phylogenetic map of human populations bases on genetic distances[66].

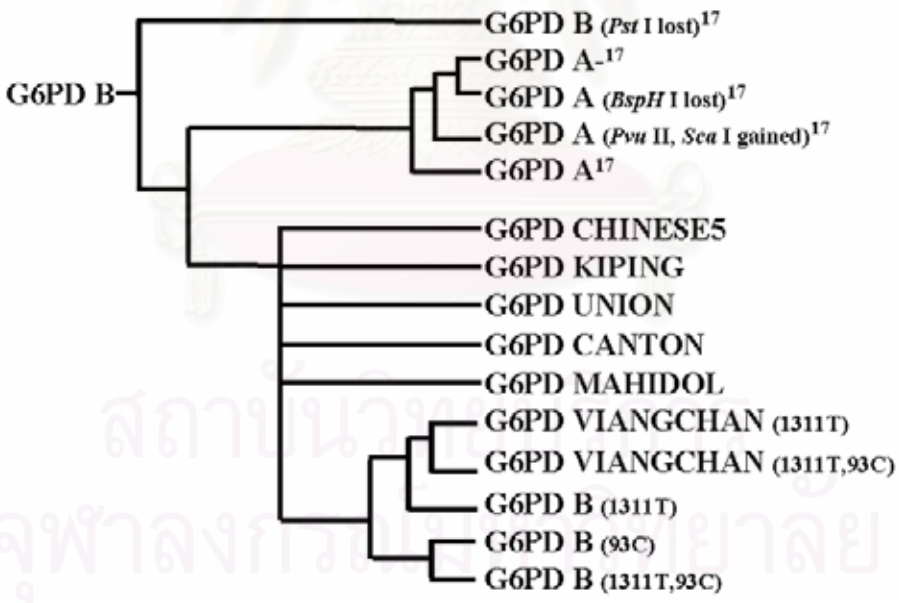


Figure 36 Phylogenetic tree of G6PD mutations.

CHAPTER V

CONCLUSION AND DISCUSSION

The objective of this study is to analyze mutation of *G6PD* in Cambodian who have G6PD deficiency and analyze RFLP haplotypes of *G6PD* in Thai, Laotian, Myanmars and Cambodian. Previous reports[1,5,12] suggested that G6PD Viangchan is the most common mutation in Southeast Asian. However, there were no information on mutation of *G6PD* in Cambodians and RFLP haplotypes of *G6PD* in Southeast Asian population.

1. G6PD Deficiency Highly Prevalent in Southeast Asians

Through a population study for G6PD deficiency using a quantitative G6PD assay, we found prevalence of G6PD deficiency in Thai, Cambodian, Laotian and Myanmars male is 11.1, 18.5, 17.4, 9.7% respectively and 5.8, 4.2, 8.6, 1.7% in female respectively. Among as prevalence of G6PD deficient jaundiced newborns in Cambodian, Laotian and Thai males is 51.9, 53.4, 16.3% respectively and 0.0, 5.0, 7.9% in female respectively.

There were many papers reported the prevalence of G6PD deficiency in Thai male ranges from 11-21.7%[5,10,67] and prevalence of G6PD deficiency in neonatal jaundice Thai male is 22.1%[5]. In this study, the sample size of Thai was too small to represent Thai population. Thus we used the prevalence of G6PD deficient Thai in previous report was 0.11[5] represent Thai population. From the result, prevalence of G6PD deficient neonatal jaundice is higher than prevalence of G6PD deficient neonate supporting the hypothesis that G6PD deficiency contribute to neonatal jaundice.

In previous study, the prevalence of G6PD deficiency in Laotian male were reported to be 7.2%[1] and 20.3%[72]. For neonatal jaundice, our study is the only study, which report high prevalence of G6PD deficiency in Laotians.

The prevalence of G6PD deficient Myanmars that comes from Burma were similar to previous study, reported prevalence of G6PD deficient Myanmars in ranges from 0-10.8% depending upon the ethnic group, consists of Akha, Lisu, Chinese, Kachin, Mon, Danu, Burma, Indian and Shan respectively[1].

As, prevalence of G6PD deficient Cambodian never been reported, our study is the only study that report prevalence of Cambodian.

The prevalence in female is higher than predicted from Hardy Weinberg principle due to unequal X-inactivation (Lyon) in some heterozygotes cause of the excess of G6PD deficient female.

The high prevalence of G6PD deficiency in Southeast Asian may be due to natural selection by malaria, as malaria is hyperendemic in Southeast Asia[68]. The mechanism of resistances malaria infection is abnormality of hemoglobin and glutathione in red blood cell from G6PD deficiency. G6PD deficiency makes an imbalance between NADP and NADPH in oxidative stress. The lacking of NADPH affects growth of *Plasmodium*. From the previous study[69], they studied the expression of *P. falciparum* glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase (PfG6PD-6PGL) in G6PD deficient and normal erythrocyte host cells both *in vitro* and *in vivo* systems. Their result suggested a lower relative abundance of PfG6PD-6PGL, and presumably antioxidant activity, in malaria parasites from G6PD deficient hosts, thus extending the current knowledge of the mechanism of G6PD deficiency related host protection.

2. G6PD Viangchan is the Most Common G6PD Mutation in Southeast Asian

We used PCR-RFLP to identify *G6PD* mutations in G6PD deficient Cambodian. We screened 6 known mutations that were previous identified in Thai and South Chinese such as G6PD Viangchan, G6PD Mahidol, G6PD Canton, G6PD Union, G6PD Kaiping and G6PD Chinese-5. G6PD Viangchan (871G→A) was the most common mutation in Cambodians (28 in 34; 82.4%), Thais (21 in 44; 47.7%) and Laotians (12 in 26; 46.2%) but was not found in Myanmars. The gene frequency of G6PD Viangchan among Cambodian, Thai and Laotian populations was calculated

to be 0.10, 0.06 and 0.06 respectively. In contrast, G6PD Mahidol (487G→A) was the most common mutation in Myanmars (5 in 8; 62.5%), and much less prevalent in Thais (2 in 44; 4.5%) and was not found in Cambodians and Laotians. The gene frequency of G6PD Mahidol among Myanmars and Thai populations was calculated to be 0.04 and 0.006 respectively.

In Cambodian, G6PD Viangchan was the dominant mutation. We have also found G6PD Viangchan was the most mutation in Thai and Laotian similar to previous reports where G6PD Viangchan is the most common mutation in Thais[5] and Laotians[1]. Our finding that the gene frequency of G6PD Viangchan is high among Cambodian, Thai and Laotian suggested a common ancestral origin of the Cambodian, Laotian and Thai.

For Myanmars, G6PD Mahidol was found to be the most mutation in G6PD deficient Myanmars similar to previous report[1]. In contrast, G6PD Mahidol was found in 4.5% among the Thai and was not found among Cambodian and Laotian populations. Our finding suggested the different ethnic origin of Myanmars compared with the Cambodian, Laotian and Thai. G6PD Mahidol and G6PD Viangchan appear to be the important markers in the Southeast Asia.

G6PD Canton, G6PD Kaiping were found in a proportionately smaller number of Thai and Laotian. The mutations have been found to be the most two mutations in South Chinese[5,52,54]. In contrast, G6PD Viangchan was rarely found (1 in 112 G6PD deficient male neonates) among the Chinese population[52]. Our finding suggested Thai and Laotian consist of native Thai that have different ancestral origin with Chinese and assimilated Chinese because subpopulation that have G6PD Canton and G6PD Kaiping mutations could be the descendant of Chinese immigrant in to the Thai and Laotian populations[5].

3. G6PD Mutations and their Property

The existence of over 442 variants of G6PD in human provides a unique opportunity to deduce structure-function relationships. Many variants that are

of potential interest were studies many years ago, long before it was defined at the DNA level. Many variants were found to be the same by DNA studies.

G6PD Viangchan was found by chemical technique in 1988 from G6PD deficient Laotian immigrated to Canada[55]. This mutation was classified as WHO Class 2; severe enzyme deficiency (activity<10% of normal or <0.74 I.U./g Hb in male and <0.69 I.U./g Hb in female)[3]. In our sample, the mean value of G6PD activity was 0.42 in forth nine males and 0.51 I.U./g Hb in twelve females, consistent with the previous reports. G6PD Viangchan was identified by molecular technique to be a nucleotide substitution at nt 871 from G to A (871G→A) makes a substitution of amino acid 291 from valine to methionine (V 291 M)[2]. The change from nonpolar group to polar uncharged group affects structure of polypeptide chain. However, the structural analysis of the usual enzyme has not been report. The activity of this G6PD variant was lost at 10 uM $NADP^+$ but are reactivated by 200 uM $NADP^{+30}$. Thier finding suggested the region in which these mutations occur defines the binding domain for $NADP^+$. Thus, G6PD Viangchan mutation might results in altered NAPD binding site or G6P binding site affecting the activity of enzyme.

G6PD Mahidol was identified biochemically in 1972 from G6PD deficient Thais[11]. This mutation was classified in WHO Class 3; mild enzyme deficiency (activity 10-60% of normal or 0.74 - 4.44 I.U./g Hb in male and 0.69 - 4.14 I.U./g Hb in female)[3]. In our study, the mean value of G6PD activity was 0.62 in six males and 1.10 I.U./g Hb in one female, which is mostly below the previous report. This could be due to a biological variation in small sample size. G6PD Mahidol was identified by molecular technique to be a nucleotide substitution at nt 487 from G to A (487G→A) makes a substitution of amino acid 163 from glycine to serine (G 163 S)[56] or from nonpolar group to polar uncharged group similar to G6PD Viangchan. From the previous study[9] this position contains the G6P binding site. Thus, changing this mutation could affects activity of enzyme in producing 6-phosphoconolactone.

G6PD Canton was dominant variant found in Chinese[52]. It is classified as WHO Class 2; severe enzyme deficiency[3]. Our result meant amount of G6PD activity was 0.56 in five males and 0.62 I.U./g Hb in one female consisted with the previous report. G6PD Canton was identified by molecular technique to be a

nucleotide substitution at nt 1376 from G to T (1376G→T) makes a substitution of amino acid 459 from arginine to leucine (R 459 L)[2]. This position contains the putative NADP binding site[20]. Thus, changing from basic charged group to be nonpolar group associate with poor binding to NADP, resulting in failure to produce NADPH.

G6PD Union was assessed as a severe (WHO Class 2) variant[3]. Our result meant amount of G6PD activity in two males was 0.72 I.U./g Hb consisted with the previous report. G6PD Union was identified the point mutation at nucleotide 1360 with substitution of C with T (1360C→T), which changed translation of amino acid 454 from arginine to cysteine (R 454 C)[2]. There is previous report supports this position contains the putative NADP binding site[20]. Thus, changing from basic charged group to polar uncharged group effects activity of enzyme that decreases stability of cell.

G6PD Kaiping was prevalent in Singaporean Chinese[54]. This mutation was classified in WHO Class 2; severe enzyme deficiency[3]. Our result meant amount of G6PD activity was 0.22 I.U./g Hb in eight males and no detectable activity in one female that were consistent with the previous report. G6PD Kaiping was identified by molecular technique to be a nucleotide substitution at nt 1388 from G to A (1388G→A) makes a substitution of amino acid 463 from ariginine to histidine (R 463 H)[2] both arginine and histidine come from basic charged group. The effect of this mutation on function in protein structure has not been reported.

G6PD Chinese-5 is G6PD variant that was not been assigned WHO Class. Our result meant amount of G6PD activity was 0.95 I.U./g Hb in one male. G6PD Chinese-5 was identified the point mutation at nucleotide 1024 with substitution of C with T (1024C→T), which changed translation of amino acid 342 from leucine to phenylalanine (L 342 F)[2] or from nonpolar group to aromatic group. From the previous report, this position contains the putative NADP binding site[20]. Thus, changing this mutation could affects activity of enzyme in producing NADPH.

G6PD mutations and their effects in protein structure were shown in figure 17.

Table 17 G6PD mutations and their function in polypeptide chain.

Mutation	Nucleotide Substitution	WHO Class	Amino acid substitution	Function in polypeptide chain
Viangchan	871G→A	2	Val 291 Met	?
Mahidol	487G→A	3	Gly 163 Ser	G6P binding site
Canton	1376G→T	2	Arg 459 Leu	NADP binding site
Union	1360C→T	2	Arg 454 Cys	NADP binding site
Kaiping	1388G→A	2	Arg 463 His	?
Chinese-5	1024C→T	?	Leu 342 Phe	NADP binding site

4. Haplotypes Analysis of the *G6PD* Locus in Southeast Asian Population

The G6PD locus has many polymorphisms distributed among the gene (figure 16). These polymorphisms could be associated with ethnic groups and done evolution of ethnic groups. In line with this observation, in the present work only 4 of the 64 possible haplotypes were observed. Two restriction fragment length polymorphisms (*Bcl* I and *Nla* III) are polymorphic in the Southeast Asian population. The others RFLPs were not found because they are polymorphic only in Africans and Europeans.

We found that among Southeast Asian population G6PD B (G6PD wild type G6PD) has 4 haplotypes designated type haplotype I *Pvu* II, *Sca* I, *Bsp*H I, *Pst* I, *Bcl* I, *Nla* III (-,-,+,+,+,+), haplotype II (-,-,+,+,+,-), haplotype III (-,-,+,+,-,+) and haplotype IV (-,-,+,+,-,-). Haplotype IV was the most frequent haplotype (69.8%) of G6PD normal chromosomes (G6PD B) and haplotype I was found 21.3%. As haplotype II and III was present in only 2.5 and 6.4% respectively. This ratio is similar in all of ethnic groups. Our finding confirm previous reports[13,23,62] that G6PD B (haplotype IV) is an ancestral (figure 37) to other G6PD variants. The 2 polymorphisms (*Bcl* I and *Nla* III) arose independently in 2 individuals (haplotype II and III) so that crossing over has occurred between these 2 nucleotides (haplotype I). Alternatively, mutations of 1311T and 93C may have occurred in one individual but in different point of time (figure 38).

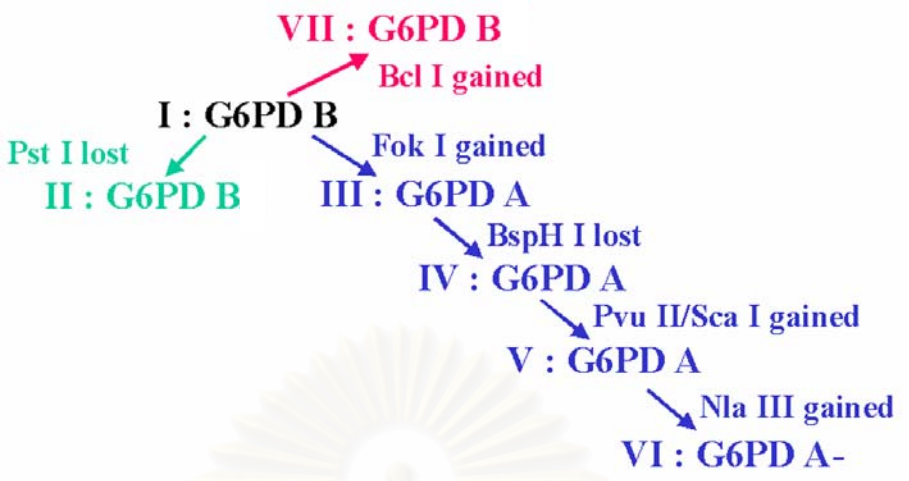


Figure 37 Postulated evolution sequence of the different polymorphisms of the *G6PD* locus based on the most simple progression from haplotype to haplotype (indicated by the Roman numerals), starting from the most common G6PD B haplotype[17].

G6PD Viangchan is always associated significantly with 1311T (*Bcl* I +) regardless of ethnic origin of the subjects with this mutation, as only haplotype I (-,-,+,+,+,+) and haplotype II (-,-,+,+,+,-) is found. Haplotype I was the most common (93.9%) in G6PD Viangchan chromosomes, while haplotype II was present in only 6.1%. For 93C (*Nla* III +), it was previously reported study that all of G6PD Viangchan chromosomes of South Chinese were found to be 93C (*Nla* III +)(N=2) [18]. In our study, both 93C (*Nla* III +) and 93T (*Nla* III -) were found in G6PD Viangchan chromosomes but G6PD Viangchan chromosome was also associated significantly with 93C (*Nla* III +). G6PD Viangchan chromosomes with 93T (*Nla* III -) were found only in Cambodians.

At this stage, we can envisage two models to account for the origin of the two Viangchan alleles. It is possible that the G6PD Viangchan mutation (871G→A) has occurred on two independent occasions, once on a chromosome carrying a haplotype like haplotype II leading to Viangchan (*Nla* III -) and once on a haplotype I leading to Viangchan (*Nla* III +). Alternatively, the 871G→A mutation may have occurred only once as a final step in the production of allele Viangchan on haplotype II and subsequently a recombination event took place somewhere between exon 11 (where nucleotide 1311 is located) and intron 11 (where nucleotide 93 is

located) of a Viangchan (*Nla* III -) chromosome and a G6PD normal chromosome having 93C (*Nla* III +). The last possibility, the 871G→A mutation may have occurred in the generation of allele Viangchan on haplotype I and led to a recombination somewhere between exon 11 and intron 11 of a Viangchan (*Nla* III +) chromosome and a G6PD normal chromosome having 93T (*Nla* III -).

G6PD Mahidol, G6PD Canton, G6PD Union, G6PD Kaiping and G6PD Chinese-5 have only haplotye IV (-,-,+,+,-,-)(N = 22). From the result, we concluded that these mutations come from G6PD B haplotype IV (-,-,+,+,-,-)(N = 141) and then led to each mutation. The exact phylogeny of each mutation could not be draw at this time due to small sample size.

When polymorphic sites were found to be closely associated, it was possible to work out a likely pattern of evolution of haplotype based on the principle of the most economical pathway in terms of successive genetic events. We have attempted to build an evolutionary diagram that links all the haplotypes described above. In the schemes depicted in figure 38 there is a stepwise progression of single base mutations from a haplotype IV.

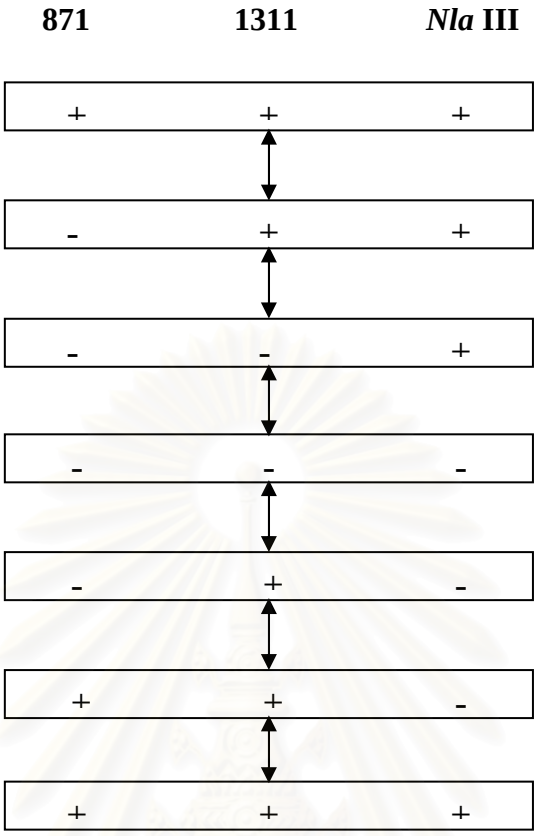


Figure 38 Hypothetical schemes of stepwise progression of single basepair mutations in the *G6PD*.

5. Phylogenetic Tree of G6PD Mutations

When we used the phylogenetic program (phylip)[65] to analyze the evolution of G6PD mutations in many ethnic groups from G6PD mutations and polymorphisms data, G6PD B was taken as the starting point for this evolutionary tree (figure 36) because it is by far the most common and also because the G6PD of the chimpanzee is G6PD B-like[69] and G6PD mutations were classified in 3 major groups that relates with the result in previous study[17]. Group one was G6PD B (*Pst* I lost) its haplotype did not similar to other groups, so it was separated from other groups first. Group two was G6PD A, G6PD A (*Bsp*H I lost), G6PD A (*Pvu* II, *Sca* I gained) and G6PD A- were specific mutations in African. Group three was classified in 2 minor groups. Minor group one consisted of G6PD Mahidol, G6PD Canton, G6PD Union, G6PD Kaiping and G6PD Chinese-5 are not found 1311T and 93C polymorphisms in their *G6PD*. In this group, we do not know the sequence of occurrence of mutation in

each mutation. Minor group two was classified in 2 subgroups. Subgroup one consisted of G6PD B (1311T, 93T) and G6PD B (1311C, 93C) and Subgroup two consisted of G6PD (1311T, 93C), Viangchan (1311T, 93C) and G6PD Viangchan (1311T, 93T). G6PD B (1311T) might be an origin of G6PD Viangchan but not other mutations.

Since the same haplotype is found in a G6PD Viangchan from Thais, Laotians and Cambodians, it seems reasonable to suggest that they share the same ancestral origin. Although G6PD Viangchan (haplotype II) found in Cambodians differs from G6PD Viangchan (haplotype I), it did not differ significantly. The culture and linguistics between Thai, Cambodian and Laotian are quite same[70]. There is evidence of strong connection among the Thai, Laotian and Cambodian, which is supported by the fact that all most of Thais, Laotians and Cambodians speak Daic and Austroasiatic, which is a linguistic family spoken by people of the Southern Indochinese mainland. An ancestral of Thai, Laos and Cambodian differ from ancestor of Myanmars and South Chinese because of difference of their haplotype and linguistics. Although Chinese G6PD mutations in some Thais may reflect recent Southeast Chinese immigrant assimilated to the Thai population. Intermarriage between Chinese and Thais contributed to a minority of Chinese variants of G6PD among Thais. In summary, our finding suggests that aboriginal population of Thai, Laotian and Cambodian movement from the Indochina Peninsula rather than from South China, which G6PD Viangchan is a marker of Thai ethnicity. The distribution of G6PD Viangchan is similar to hemoglobin E allele in this population[71]. The high prevalence of G6PD Viangchan and hemoglobin E alleles could both be a result of malaria selection pressure. **6. G6PD and the History of Southeast Asian Ethnic Group**

To understand the evolution of Southeast Asian population, we compared the historians' study of early and current history of Southeast Asia with the genetic evidence.

Prehistoric archeological research suggests that an advanced civilization may have previously occupied the peninsula of Southeast Asia, where lowered sea levels. In the later years of rising ocean levels, when the Northern and

Southern ice sheets melted due to climate catastrophe situations, refugees of such flooding low-land sea-side cultures would have migrated to higher land areas of such locations as Northern Thailand, Laos, Western China. There is evidence supports, the lands today known as Laos were occupied 10,000 years ago, according to village sites of stone tools, implements and human bones discovered in Huaphan, Savannakhet and Luang Prabang provinces of Laos[73].

We hypothesize that Thais, Cambodians and Laotians come from the same society, who lived in peninsula of Southeast Asia within the past 10,000 years. It possible that the ancestor of Thais, Laotians and Cambodians were these indigenous people, as malarial selection pressure may be at work in people started to do agriculture[13]. After that, mutation of *G6PD* was induced to provide reduced risk from malarial infection. Thus, Thais, Cambodians and Laotians have had the same mutation before they separated to construct their kingdom.

In 3rd-7th centuries, Funan society flourished cover Southern Myanmar, Thailand, Cambodia and Southern Vietnam. The origin of these people was unclear. In the end of 7th century it was attacked by two other early societies in the Khmer area-"water Chenla" and "land Chenla"[74]. The Khmer may the first ethnic group with G6PD Viangchan who moved to this region.

In the 13th century, several small kingdoms emerged across the regions known today as Northeast Myanmar, Central and Northern Thailand and Laos were called "Tai". We proposed that the Tai also carry G6PD Viangchan. The evidence suggests long, slow Tai migration over many centuries, beginning in Western China, or even further north, and spreading southwards from the seven century[74].

As in Myanmar, there is the first kingdom emerged in the lower Irrawaddy valley from the fifth century but they were non-Myanmars kingdoms. The first major kingdom was founded around 1044, on the banks of the Irrawaddy river at Pagon, north of the present day capital of Yangon with people who carry G6PD Mahidol. Until the end of the 13th century, Pagon was attacked by the Mongols from China[74].

In 9th-14th centuries, the Khmer society consolidated to be Angkor kingdom. Meanwhile, in 1279-1298, the most celebrated of early Tai states was the kingdom of Sukhothai. Modern Thais regard Sukhothai as the birthplace of the Thai nation. Then Tai attacked Angkor's imperial outposts, and eventually upon Angkor itself in the 14th and 15th centuries that is a period of Ayudhya kingdom flourished, would lead to a direct transfer of human[74].

From the early and current history of Southeast Asia and result that Thai, Cambodian and Laotian have similar haplotype of *G6PD*, we conclude that Thai, Cambodian and Laotian come from the same genetic pool.

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APPENDIX

APPENDIX

CHEMICAL AGENTS AND INSTRUMENTS

A. **Research Instruments**

Automatic adjustable micropipette (Eppendorf, Germany)
Balance (Precisa, Switzerland)
Beaker (Pyrex)
Centrifuge
Chemi Doc (BIO-RAD, USA)
Combs (BIO-RAD, USA)
Cuvette 80-100 μl.
Disposable cuvette 2.5 ml (Plastibrand)
DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)
Electrophoresis Chamber set (BIO-RAD, USA)
Flask (Pyrex)
Heat block (Bockel)
Ice block (Eppendorf)
Incubator
Needle 21G x 11 ½" (Nipro)
Parafilm (American National Can, USA)
Pipette rack (Autopack,USA)
Pipette tip (Axygen, USA)
Power supply model
pH meter (Eutech Cybernataics)
Microcentrifuge (Eppendorf, USA)
Microcentrifuge tube (BIO-RAD, Elkay, USA)
Reagent bottle (Duran)
Spectrophotometer (Milton ROY spectronic 401)
Spectrophotometer (BIO-RAD, USA)
Syringe Disposable 5 ml. (Nipro)

Techicon H.3 (Bayer)

Thermometer (Precision, Germany)

Vacuum pump

Vortex (scientific Industry, USA)

Water bath

3 ml test tube

3 ml of Vacutainer™ tube (Becton Dickinson)

5 ml of Vacutainer™ tube (Becton Dickinson)

B. General Reagents

Absolute ethanol (Merck)

Acetic acid (Merck)

Acrylamide:Bisarylamide (Phamacia Amersham)

Agarose (USB)

Ammonium persulphate (Phamacia Amersham)

Bromophenol blue (USB)

Citric acid powder (The British Drug House LTD.)

Dextrose (Fluka AG. Chem)

EDTA (Merck)

Ethidium bromide (Sigma)

Glucose-6-Phosphate (Sigma)

Hydrochloric acid (Merck)

Isopropanol (Sigma)

Magnesium chloride (Eiken chemical Co;LTD)

Methanol (Merck)

Methylene blue (Merck)

Saline

Sodium chloride (Scharlau)

Sodium nitrite (Mallinckrodt)

SDS (Sigma)

Sodium hydroxide (Merck)

Sucrose (Sigma)

TEMED (Gibco)

TPN (Sigma)

Tris base (USB)

Tris HCl (Merck)

Tris sodium citrate (Fluka Garantic)

100 bp DNA ladder (Biolabs)

C. Buffer of enzymes

1. *Taq* DNA Polymerase 10X buffer (50 mM KCl, 10 mM Tris-HCl (pH9.0 at 25^{O}C), 1.5 mM $MgCl_2$ and 0.1% TritonX-100 when diluted 1:10)
2. *Pfu* DNA Polymerase 10X buffer (100 mM KCl, 200 mM Tris-HCl (pH8.8 at 25^{O}C), 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$, 1 mg/ml nuclease-free BSA and 1% TritonX-100)

BIOGRAPHY

Name	Miss Chalisa Louicharoen	**Sex**	Female
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Place of birth	Bangkok, Thailand		
Home address	296/16 Soi 14, Rama III Rd, Bangkholamp, Bangkok, 10120 Thailand		
Education	2001 : Degree of Bachelor of Science (Health Science) Thammasat University		

Publications

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