STUDY OF G6PD DEFICIENCY WITH CLINICAL AND HEMATOLOGICAL CORRELATION IN
NEWBORN INFANTS

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การศึกษาภาวะพร่องเอนไซม์ G6PD ที่มีความสัมพันธ์ทางคลินิกและโลหิตวิทยาในทารกแรกเกิด

น.ส.ธนพร พิมพกรรณ์

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ธนพร พิมพกรรณ์ : การศึกษาภาวะพร่องเอนไซม์ G6PD ที่มีความสัมพันธ์ทางคลินิกและโลหิตวิทยาในทารกแรกเกิด. (STUDY OF G6PD DEFICIENCY WITH CLINICAL AND HEMATOLOGICAL CORRELATION IN NEWBORN INFANTS) อ.ที่ปรึกษาหลัก : ผศ. ดร.ชาลิสา หลุยเจริญ ชีพสุนทร, อ.ที่ปรึกษาร่วม : รศ. ดร.พูลลาภ ชีพสุนทร

ภาวะพร่องเอนไซม์จีซิกพีดี (G6PD) เป็นความผิดปกติของเอนไซม์ที่พบได้บ่อยที่สุด โดยพบได้ในประชากรหลายร้อยล้านคนทั่วโลก หากทารกแรกเกิดไม่ได้รับการวินิจฉัยและรักษาภาวะพร่องเอนไซม์จีซิกพีดีได้ทัน จะนำไปสู่ภาวะดีซ่าน (jaundice) และภาวะบิลิรูบินในเลือดสูง (hyperbilirubinemia) ซึ่งภาวะบิลิรูบินในเลือดสูงเกิดจากการเสียความสมดุลระหว่างการผลิตบิลิรูบินที่เพิ่มขึ้นและการกำจัดบิลิรูบินที่น้อยลงเนื่องจากตับของทารกยังทำหน้าที่ได้ไม่สมบูรณ์ ด้วยเหตุนี้จึงอาจทำให้ทารกมีอาการผิดปกติทางสมอง เรียกว่า kernicterus ส่งผลให้พัฒนาการล่าช้ากว่าปกติและอาจมีระดับสติปัญญาลดลงด้วย นอกจากนี้มีรายงานว่าการการเพิ่มขึ้นของเรทติคิวโลไซท์ (reticulocytes) จำนวนมากในทารกแรกเกิดอาจขัดขวางต่อการวินิจฉัยภาวะพร่องเอนไซม์ G6PD ได้ เนื่องจาก reticulocytes มีเอนไซม์ G6PD ในปริมาณที่สูงกว่าเม็ดเลือดแดงที่โตเต็มวัย ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินผลของ reticulocytosis ในการกำหนดค่ากัมมันตภาพของเอนไซม์ G6PD ในเลือดของทารกแรกเกิดไทยด้วยวิธี automated UV enzymatic เพื่อศึกษาความสัมพันธ์ระหว่างระดับของ G6PD activity และระดับของบิลิรูบิน และเพื่อตรวจสอบประสิทธิภาพของการทดสอบนี้สำหรับการตรวจหาภาวะพร่องเอนไซม์ G6PD ในตัวอย่างเลือดของทารกแรกเกิด จากการศึกษาพบความชุกของภาวะพร่องเอนไซม์ G6PD ในทารกแรกเกิดชายไทยเท่ากับ 10.0% และ 2.3% ในทารกเพศหญิง รูปแบบการกลายพันธุ์ของยีน *G6PD* ที่แพร่หลายที่สุดในกลุ่มประชากรที่ศึกษาคือ *G6PD Viangchan*^{G871A} (MAF = 0.066) จำนวน reticulocyte ในทารกแรกเกิดที่มีภาวะพร่องเอนไซม์ G6PD สูงกว่าทารกแรกเกิดเพศชายที่มีเอนไซม์ G6PD ปกติอย่างมีนัยสำคัญ ($p<0.001$) แต่ไม่พบความสัมพันธ์ระหว่างค่าการทำงานของเอนไซม์ G6PD และระดับของ reticulocyte ในทารกแรกเกิดที่มีภาวะพร่องเอนไซม์ G6PD ($r=-0.019$, $p=0.881$) นอกจากนี้ยังพบว่าระดับของ total serum bilirubin และ indirect bilirubin ในทารกแรกเกิดที่มีภาวะพร่องเอนไซม์ G6PD ปานกลาง และมีภาวะพร่องเอนไซม์ G6PD นั้นสูงกว่าทารกแรกเกิดที่มี G6PD ปกติอย่างมีนัยสำคัญ ระดับ agreement ในการตรวจหาภาวะพร่องเอนไซม์ G6PD มีค่าเท่ากับ 0.999 ในขณะที่ค่า AUC แสดงให้เห็นว่าวิธี automated UV enzymatic มีค่าความไว (sensitivity) 98.4% ความจำเพาะ (specificity) 99.5% ค่าการทำนายผลบวก (PPV) 92.4%, ค่าพยากรณ์เชิงลบ (NPV) 99.9% และความแม่นยำ (accuracy) 99.4% ผลการศึกษาแสดงให้เห็นว่าภาวะ reticulocytosis พบบ่อยในทารกแรกเกิดไม่มีผลกระทบอย่างมีนัยสำคัญทางสถิติต่อการตรวจวินิจฉัยภาวะพร่องเอนไซม์ G6PD ด้วยวิธี spectrophotometric และ automated UV enzymatic นอกจากนี้การศึกษาของเราพบว่าทารกแรกเกิดที่มีภาวะพร่องเอนไซม์ G6PD มีระดับของ indirect bilirubin สูง ดังนั้นหากมีการสะสมของ indirect bilirubin เป็นจำนวนมากอาจทำให้เกิดผลเสียต่อทารกแรกเกิดได้

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KEYWORD: G6PD deficiency, Reticulocytosis, Hyperbilirubinemia, Thai newborn

Thanaporn Pimpakan : STUDY OF G6PD DEFICIENCY WITH CLINICAL AND HEMATOLOGICAL CORRELATION IN NEWBORN INFANTS. Advisor: Asst. Prof. CHALISA LOUICHAROEN CHEEPSUNTHORN, Ph.D. Co-advisor: Assoc. Prof. POONLARP CHEEPSUNTHORN, Ph.D.

G6PD deficiency is the most common enzymatic aberration, affecting more than a million people worldwide. If severe cases at birth are not diagnosed and treated promptly, they can develop into cerebral jaundice and hyperbilirubinemia. Hyperbilirubinemia is caused by an imbalance between increased bilirubin production and ineffective excretion of bilirubin. This can result in the neurotoxicity of bilirubin, kernicterus, and fatal mental retardation. Besides, It has been speculated that the presence of a high number of reticulocytes in newborns interferes with the diagnosis of G6PD deficiency since reticulocytes contain higher amounts of G6PD enzyme than mature erythrocytes. Therefore, the purposes of this study were to assess the effect of reticulocytosis in the determination of blood G6PD activity in Thai newborns by using an automated UV enzymatic assay, to examine the association between the levels of G6PD activity and bilirubin, and to validate the performance of this assay for the detection of G6PD deficiency in newborn blood samples. Our data revealed that the prevalence of G6PD deficiency was 10.0% in Thai male newborns and 2.3% in females. The minor allele frequency (MAF) of common mutation in the study population; *G6PD Viangchan^{G871A}* was 0.066. Compared with normal newborns after controlling for thalassemia and hemoglobinopathies, the reticulocyte counts in newborns with G6PD deficiency were significantly higher than that of normal newborns ($p<0.001$). There was no correlation between G6PD activity and reticulocyte counts in subjects with G6PD deficiency (r=-0.019, p=0.881), whereas the levels of G6PD activity in normal newborns were positively correlated with the percentage of reticulocytes (r=0.327, $p<0.001$). Moreover, our results showed that the total serum bilirubin and indirect bilirubin in all newborns with intermediate and deficient G6PD were significantly higher than that of normal G6PD newborns. The level of agreement in the detection of G6PD deficiency was 0.999, while the area under the curve of receiver operating characteristic (AUC) demonstrated that the automated UV enzymatic assay had 98.4% sensitivity, 99.5% specificity, 92.4% positive prediction value (PPV), 99.9% negative predictive value (NPV), and 99.4% accuracy. We report that reticulocytosis, commonly observed in newborns, does not have a statistically significant effect on the diagnosis of G6PD deficiency in newborns by both qualitative and quantitative methods. G6PD-deficient newborns had high levels of indirect bilirubin, which might cause serious consequences for newborns.

Field of Study:	Medical Sciences	Student's Signature ....
Academic Year:	2021	Advisor's Signature ....
		Co-advisor's Signature ....

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°C	Degree Celsius
μl	Microliter
μM	Micromolar
5'-UTR	5' untranslated region
6PG	6-phosphoglucolonate
AHA	Acute hemolytic anemia
AMM	Adjusted male median
APS	Ammonium persulfate
BBB	Blood brain barrier
bp	Base pair
CNSHA	Chronic non-spherocytic hemolytic anemia
DB	Direct bilirubin
DNA	Deoxyribonucleic acid
FST	fluorescent spot test
g	Gram
G6P	Glucose 6-phosphate
G6PD	Glucose 6-phosphste dehydrogenase
g/dl	Gram per deci lire
GA	Gestational age
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HCT	Hematocrit
IQ	Intelligence quotient
IQR	Interquartile range

IRB	Institutional review board
Kb	Kilo base pair
kDa	Kilo dalton
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
ml	milliliter
mM	millimolar
NADP$^+$	Nicotinamide adenine dinucleotide phosphate (Oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (Reduced form)
ng	Nanogram
ng/μl	Nanogram per microliter
nm	Nanometer
NPV	Negative predictive value
PBS	Phosphate-buffered saline solution
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction restriction fragment length polymerase
pH	Positive potential of Hydrogen ions
PLT	Platelet
PPP	Pentose phosphate pathway
PPV	Positive predictive value
RBC	Red blood cell
RDW	Red blood cell distribution width
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
rpm	Revolution per minute
S.D.	Standard deviation
SNP	Single nucleotide polymorphism
TB	Total bilirubin
TBE	Tris Borate EDTA solution

TCF	Temperature correction factor
U	Unit
UV	Ultraviolet
WBC	White blood cell
WHO	The World Health Organization

CHAPTER 1

Introduction

1.1. Background and Rationale

Glucose 6-phosphate dehydrogenase (G6PD) is a substantial enzyme in the pentose phosphate pathway (PPP) to produce reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is particularly necessary for protection against reactive oxygen species (ROS) induced oxidative cellular damage (1, 2). G6PD deficiency is the most common enzymatic aberration, affecting approximately 400 million people worldwide (3). G6PD deficiency is the most common enzymatic aberration caused by mutations in the *G6PD* gene (2). G6PD deficiency is an X-linked recessive inherited disorder that typically affects hemizygous males, whereas heterozygous females can have a normal, intermediate, or severely deficient phenotype depending on lyonization mosaicism (4).

Individuals with G6PD deficiency are generally asymptomatic unless they are exposed to exogenous factors such as infections, certain foods intake, and antimalarial medication (5). Clinical syndromes frequently associated with G6PD deficiency are acute hemolytic anemia (AHA) and favism (2). If severe cases in the newborn period are not diagnosed and treated promptly, they can progress to cerebral jaundice and hyperbilirubinemia (6). Hyperbilirubinemia is caused by an imbalance between increased bilirubin production and inefficient bilirubin excretion, which can result in severe and fatal bilirubin neurotoxicity, kernicterus, and mental retardation (7). Therefore, early detection of G6PD deficiency in newborns can help to prevent congenital disorders caused by bilirubin poisoning. In Thailand, the prevalence of G6PD deficiency in male and female newborns are around 11.1-17.0% and 2.0-5.8%, respectively (8, 9). Around 65% of severe neonatal jaundice (8) and 19.7%-21.2% of hyperbilirubinemia were coincidently observed in G6PD deficient Thai newborns (8, 10). The World Health Organization (WHO) has recommends routine screening for G6PD deficiency in newborns, living in areas where the prevalence of G6PD deficiency is as high as 3–5% in males to avoid potentially serious complications (11).

Various techniques are currently available to detect G6PD deficiency. The fluorescent spot test (FST) is a simple and widely used technique for the qualitative screening of G6PD deficiency (4), but is limited in detecting intermediate cases. The spectrophotometric assay is the reference method for the quantitative determination of G6PD activity, which can discriminate between intermediate and normal activity levels. However, performing this technique is manual, time-consuming, and requires experienced professionals to analyze the results (12). Recently, an automated UV enzymatic assay has been developed to measure G6PD activity levels in 50 samples simultaneously. This might solve the weaknesses of these two common techniques. It has been reported that the detection of G6PD deficiency in newborn's blood can be disturbed by reticulocytosis. Reticulocytes, which typically have higher levels of enzyme activity than mature RBCs, may be an important interfering factor in the detection of G6PD deficiency in newborns by false-negative errors (13). Therefore, our aims in this study were to investigate the prevalence of G6PD deficiency and the effect of reticulocytosis, in the determination of newborn blood G6PD activity measured by the reference spectrophotometric assays and automated UV enzymatic assay. The performance of the automated UV enzymatic assay for detecting G6PD deficiency and intermediate was compared with the reference spectrophotometric assay.

1.2. Research Questions

1.2.1. Does reticulocytosis affect the determination of blood G6PD activity measured by the reference spectrophotometric assays and automated UV enzymatic assay in Thai newborns?

1.2.2. Do the levels of G6PD activity associate with the levels of bilirubin in Thai newborn infants?

1.2.3. How is the performance of automated UV enzymatic assay in the detection of G6PD deficiency and intermediate by comparing with the reference spectrophotometric assay?

1.3. Objectives

1.3.1. To determine the effect of reticulocytosis in the determination of blood G6PD activity measured by the reference spectrophotometric assays and automated UV enzymatic assay in Thai newborns

1.3.2. To examine the association between the levels of G6PD activity and bilirubin in Thai newborn infants

1.3.3. To evaluate the performance of automated UV enzymatic assay in the detection of G6PD deficiency and intermediate by comparing with the reference spectrophotometric assay

1.4. Hypotheses

1.4.1. Reticulocytosis affects the determination of blood G6PD activity measured by the reference spectrophotometric assays and automated UV enzymatic assay in Thai newborns by increase G6PD activity levels.

1.4.2. The levels of G6PD activity associates with the levels of bilirubin in Thai newborn infants.

1.4.3. The performance of automated UV enzymatic assay in detection of G6PD deficiency and intermediate is comparable to spectrophotometric assay.

1.5. Keywords

G6PD deficiency

Reticulocytosis

Hyperbilirubinemia

Thai newborn

1.6. Research Design

Descriptive research

1.7. Conceptual Framework

G6PD deficiency is the most common enzymatic abnormality, affecting approximately million people worldwide.

↓

Clinical syndromes frequently associated with G6PD deficiency are acute hemolytic anemia (AHA) and favism. →

If severe cases in the newborn period are not diagnosed and treated promptly, they can progress to life-threatening bilirubin neurotoxicity.

↓

In Thailand, the prevalence of G6PD deficiency in male and female newborns was 11.1% to 17.0% and 2% to 5.8% respectively.

Around 65% of severe neonatal jaundice and 19.7%-21.2% of hyperbilirubinemia were coincidently observed in G6PD deficient Thai newborns.

↓

WHO has recommended routine screening for G6PD deficiency in newborns, living in areas where the prevalence of G6PD deficiency is as high as 3–5% in males in order to prevent poor consequences outcomes.

↓

G6PD deficiency is an X-linked recessive inherited disorder therefore the heterozygous female with partial G6PD activity may be misdiagnosed.

↓

The reference spectrophotometric assay is used for the assessment of G6PD enzyme activity. →

Lately, an automated UV enzymatic assay has been developed to simultaneously measure the level of G6PD activity in 50 samples with an automated system.

↓

High-enzyme activity of reticulocytes, known as reticulocytosis, commonly observed in newborn.

↓

Reticulocytosis may affect the interpretation of G6PD deficiency.

↓

Objectives

1. To determine the effect of reticulocytosis in the determination of blood G6PD activity measured by the reference spectrophotometric assays and automated UV enzymatic assay in Thai newborns

2. To examine the association between the levels of G6PD activity and bilirubin in Thai newborn infants

3. To evaluate the performance of automated UV enzymatic assay in the detection of G6PD deficiency and intermediate by comparing with the reference spectrophotometric assay?

1.8.   Benefits of Study

The expected benefit of this study is to provide evidence on the effect of reticulocytosis in the determination of newborn blood G6PD activity, the association between the levels of G6PD activity and bilirubin in Thai newborn infants, and the performance of automated UV enzymatic assay in determination of G6PD deficiency.

1.9.   Definition

Reticulocytosis is a condition in which there is an increase in the amount of immature red blood cells. It occurs to compensate for the loss of mature red blood cells called hemolytic anemia. Reticulocytosis in newborns is defined as a reticulocyte count greater than 4% on day 3 of life (14).

Hyperbilirubinemia is a condition in which elevated levels of bilirubin in the newborn's blood result in yellowish staining of the skin and whites of the eyes by the bilirubin (pigment of bile). According to the Bhutani nomogram, neonates with hyperbilirubinemia (NH) define as having total serum bilirubin (TSB) levels above the 95th percentile for their age in hours (15).

CHAPTER 2

Literature review

2.1.   Function of G6PD

Glucose 6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) is a housekeeping enzyme; however, its concentration varies with tissue. G6PD catalyzes the first step in the pentose phosphate pathway (PPP) (2) in producing reduced nicotinamide adenine dinucleotide phosphate (NADPH) by converting glucose 6-phosphate (G6P) to 6-phosphogluconate (6PG). NADPH functions as a proton donor for the generation of reduced glutathione (GSH) from glutathione (GSSG) by glutathione reductase (GR) (2). Then, GSH works with glutathione peroxidase (GPX) to detoxify hydrogen peroxide (H_2O_2), which is produced by oxygen radicals to water (H_2O) (16). Thus, the enzymatic activity of G6PD is required for reducing ROS. Besides, NADPH is involved in several metabolic processes including glycolysis, oxidative phosphorylation, biosynthesis of lipids as well as in the formation of ribose 5-phosphate required for DNA synthesis (5, 17) (**Figure 1**).
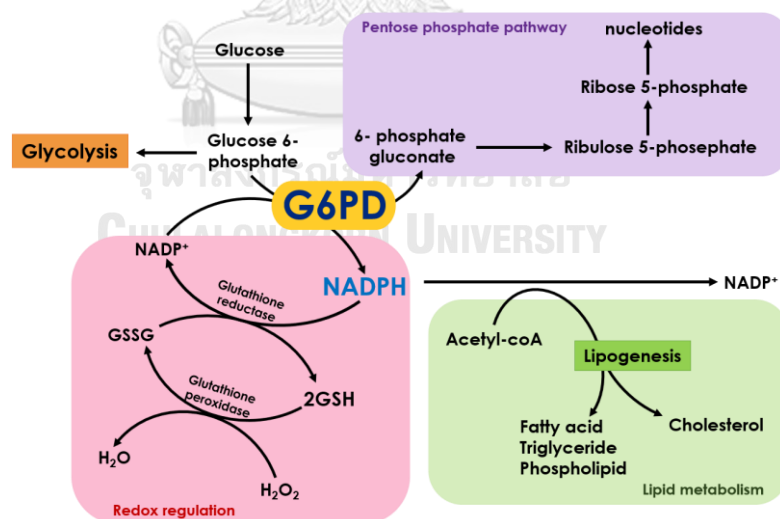
Figure 1 The role of glucose 6-phosphate dehydrogenase (G6PD). G6PD = glucose 6-phosphate dehydrogenase; $NADP^+$ = nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH = nicotinamide adenine dinucleotide phosphate (reduced form); GSH = reduced glutathione; GSSG = oxidized glutathione; H_2O_2 = peroxide.

2.2.   Structure of G6PD

The human G6PD enzyme is active in dimer (**Figure 2**) or tetramer (**Figure 3**) equilibrium, which relied on ionic strength and pH (18). The high pH (approximately above pH8) and the ionic strength conditions shift the equilibrium state to the dimer forms, while the low pH condition (approximately below pH6) shifts the equilibrium state to the tetramer. In conditions where the pH is very high, G6PD might be able to change reversibly from an active dimer to an inactive monomer form (19, 20). The dimer form has two monomers symmetrically located across a complex interface of β-sheets and each subunit binds to a NADP$^+$ molecule which provides its structural stability (**Figure 2.2**). The monomer of G6PD is composed of 515 amino acids with 59 kDa molecular weight (21). Each monomer consists of 2 domains;

1. N-terminal domain (amino acid residues 27-200) adopts a classic **β**–**α**–**β** dinucleotide-binding fold with the fingerprint sequence GASGDLA (residues 38–44), which is coenzyme-binding site (NAPD$^+$) (19).

2. **β** + **α** domain (amino acid residues 191–515) consists of the conserved nine-residue peptide RIDHYLGKE (residue 198-206), which is G 6-P binding side (22), and a curved nine-stranded antiparallel **β** sheet at residue 380-425 (19). These two domains are linked by the **α**-helix.
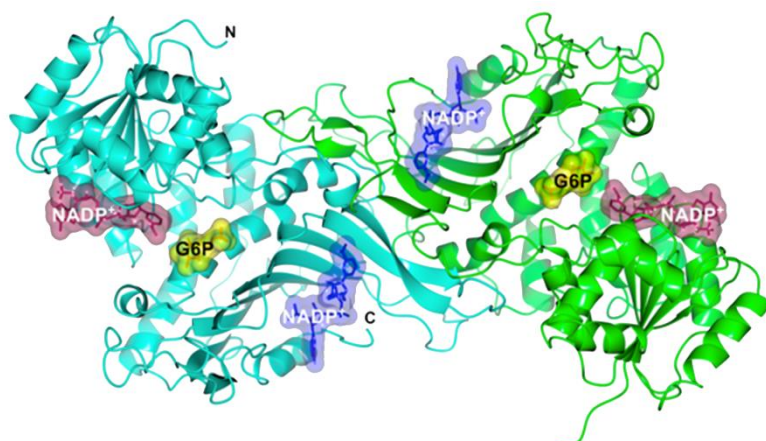
Figure 2 Three-dimensional structure of active G6PD dimer (21). The human G6PD monomers are illustrated in cyan and green. The binding sites of the G6P substrate, NADP$^+$, and structural of NADP$^+$ are shown as yellow, purple, and blue, respectively.
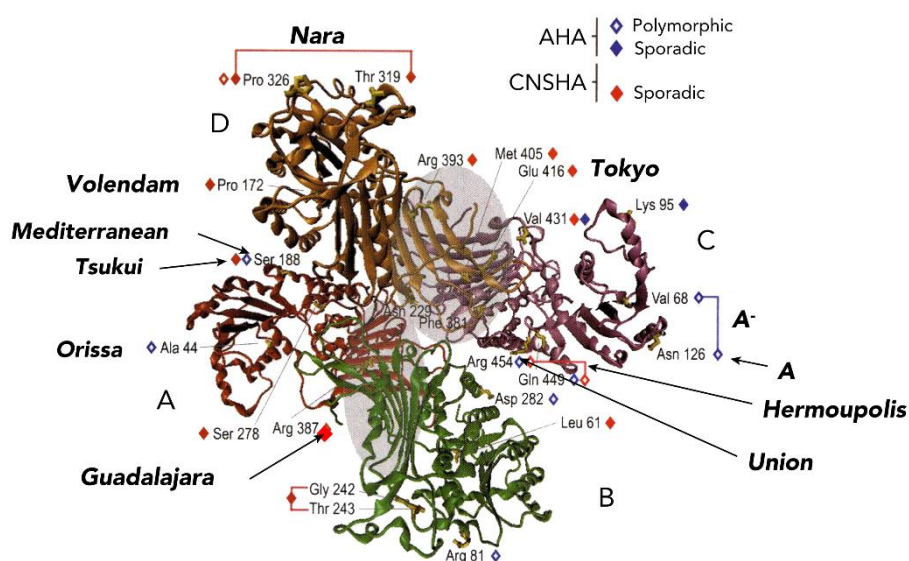
Figure 3 Three-dimensional structure of active G6PD tetramer (23). A - D exhibits four monomeric G6PD enzymes with representative locations of mutations producing a clinical phenotype such as acute hemolytic anemia (AHA) or chronic non-spherocytic hemolytic anemia (CNSHA). The grey shadowed areas from each dimer represent interfaces formed by the contact between the curved nine-stranded antiparallel β-sheet from each monomer, which in a dyad axis involving 57 amino acid residues.

2.3.   G6PD in Erythrocyte

In almost all cells except mature erythrocytes, G6PD is a source of NADPH together with other mitochondrial enzymes e.g. isocitrate dehydrogenase and malic enzyme (7). So even if the G6PD is insufficient, there may be no shortage of NADPH. The situation is considerably different in mature red blood cells. This is because their nucleus, mitochondria, and ribosomes are sacrificed during erythroid cell development. Thus, new proteins cannot be synthesized (5, 24). Consequently, G6PD is the only source of NADPH, which is necessary to protect cells and hemoglobin from oxidative stress due to their role in oxygen transport (11). The sulfhydryl groups of several enzymes and the β-chain of hemoglobin are particularly susceptible to oxidation stress, with potentially adverse consequence effects. Protection against oxidation is mediated by glutathione cycle (11). Hydrogen peroxide (H_2O_2) is normally detoxified by the NADPH-based glutathione peroxide generated from G6PD (**Figure 1**). In G6PD-deficient RBCs, it cannot produce enough NADPH, and oxidized glutathione (GSSG) cannot be reversed, resulting in oxidative damage of lipids and proteins thiol groups from H_2O_2, converting oxyhemoglobin to methemoglobin (25) (**Figure 4**). Sulfhydryl groups of membrane and cytoplasmic proteins including hemoglobin are also oxidized, resulting a precipitation and accumulation of denatured hemoglobin known as 'Heinz body' (25) (**Figure 5**). Macrophages in the spleen, which are responsible for engulfing old or abnormal RBCs, notice these Heinz bodies and try to remove them, leaving these RBCs partially devoured known as 'bite cells' (**Figure 5**). In normal RBCs, G6PD produces an abundant supply of NADPH, which can regenerate glutathione when it is oxidized by ROS (25). Besides, only 1–2% of the total G6PD enzyme capacity is used, even with severe hemolytic stress (24). The red cells aging has a progressive decrease in the activity of several enzymes, including G6PD. Thus, young red blood cells have a larger amount of G6PD enzyme, which contributes to their resistance to hemolysis (26, 27).
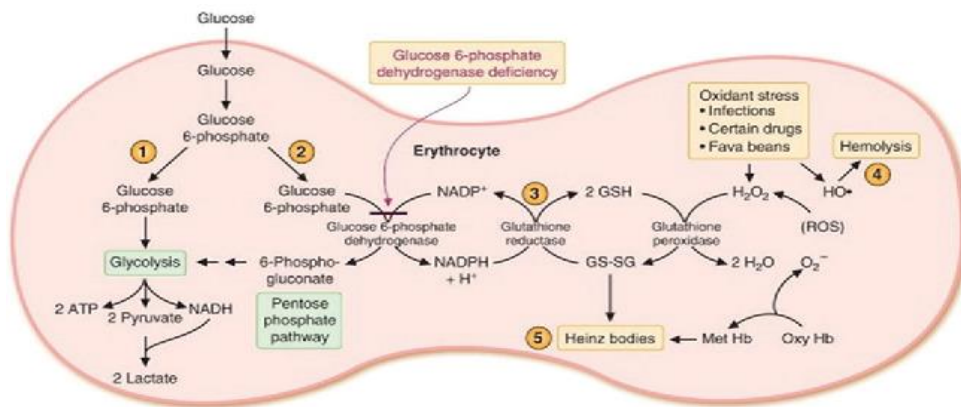
Figure 4 Role of G6PD in protection against oxidative damage (28).

Bite cells   Heinz bodies
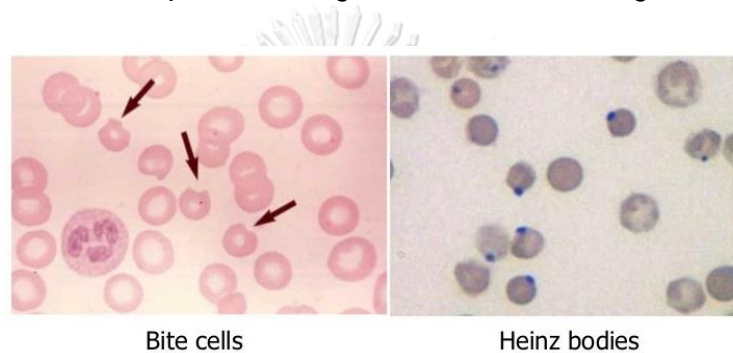
Figure 5 Heinz Bodies and Bite Cells (29).

2.4. Molecular genetic of G6PD

The *G6PD* gene is located at the telomeric region of the distal arm of the X chromosome (band Xq28), close to the genes for hemophilia A (factor VIII, *F8C*) and color blindness (*CBBM*), as illustrated in **Figure 6**. The G6PD sequence has a size of 18.5 Kb and consists of a promoter region, 13 exons, and 12 introns encoding a product of 1,545 bp (2). The 5' untranslated region (UTR) is in the first 600 bp of the *G6PD* gene, which corresponds to exon 1 and a part of exon 2. The start codon sequence (ATG) is found in amino acid residues 115–127 of exon 2. This 5' UTR sequence contains two start codons out of reading frame and CpG island (approximately 80%) which is a common characteristic among genes with constitutive expression. Several features of the G6PD promoter region have been identified, such as high GC content (over 70%), the absence of CAAT element located in the essential site for transcription factor (position -70 to -90), the TATA box with ATTAAT sequence

located at position -202 from ATG sequence and the presence of at least nine sites (CCGCCC) which involved in gene regulation (30).
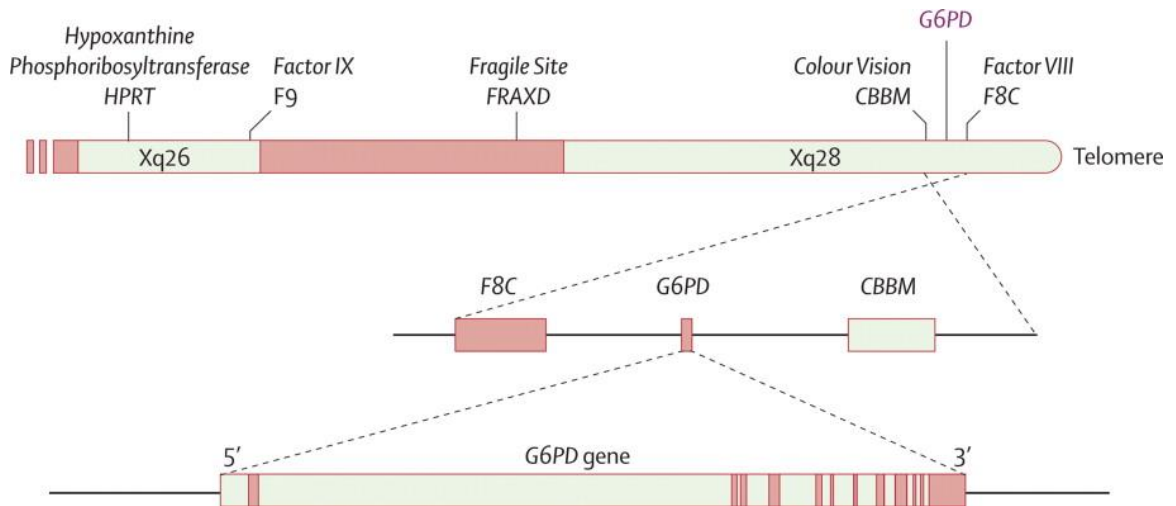
Figure 6 Location of *G6PD* gene on X chromosome (2).

2.5.   *G6PD* mutations

G6PD deficiency arises mainly from a mutation on the *G6PD* gene, which is nucleotide substitution, resulting in *G6PD* variations (23). These variants have been biochemically characterized based on the different residual enzyme activities, electrophoretic mobility patterns, and physicochemical or kinetic (k_m for G6PD or NADPH, pH dependence, and utilization of substrate analogues) properties (31). Different types of *G6PD* mutations are involved in the various clinical manifestations (32). Currently, the pattern of over 186 known mutations has been published. For example, single missense point mutations, multiple missense mutations, small in-frame deletions etc. (30). The G6PD enzyme is essential to protect cells against oxidative stress. Therefore, Large deletions, nonsense, or frameshift mutations are not found in the *G6PD* gene or its promoter because these two mutations change amino acids into stop codons and diminish the complete abolishment of G6PD functionality. Cells are unable to develop and finally die when the G6PD enzyme is in shortage (33). The mutations that underlie G6PD deficiency are spread throughout the coding region, as shown in **Figure 7**. Furthermore, the *G6PD* variants are

associated with geography and ethnicity (34) (**Figure 8**). In Thailand, the common *G6PD* mutations are *G6PD Viangchan^G871A*, and *G6PD Mahidol^G487A* (35), classified as severe and moderate deficiency, respectively. The other less common *G6PD* mutations are *G6PD Union^C1360T*, *G6PD Kaiping^G1388A*, classified as severe deficiency, and *G6PD Canton^G1376T*, classified as moderate deficiency (8, 36).
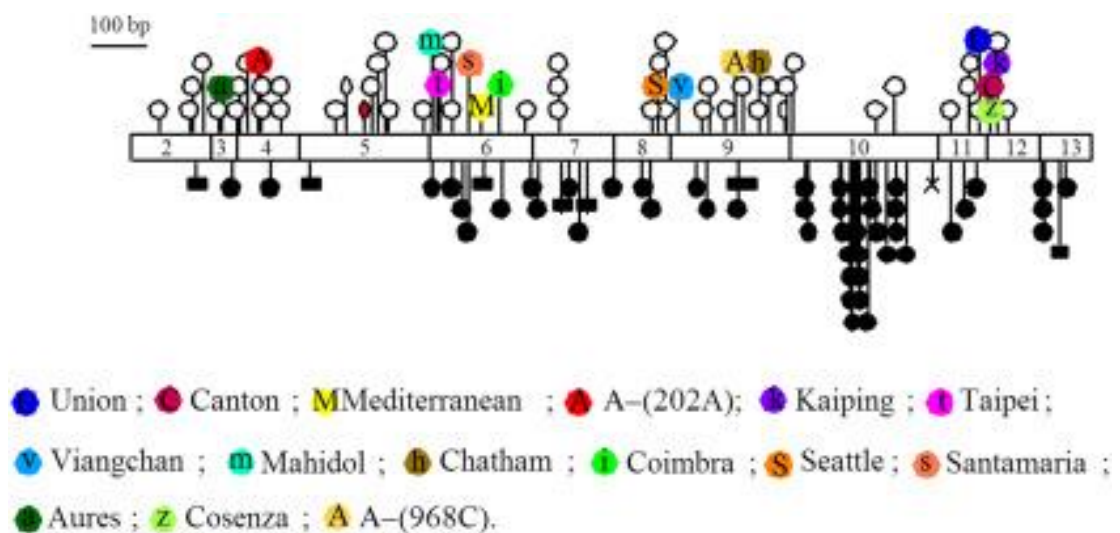
Figure 7 The common mutations along coding sequence of *G6PD* gene (5) Exons 2-13 of the *G6PD* gene are represented as a square diagram with numerals. Below the exons diagram are mutations associated with the most severe clinical phenotype (class I), and above the exon diagram are mutations associated with a milder phenotype (class II or III). The colored circles represent *G6PD* gene mutations that occur on different exons.
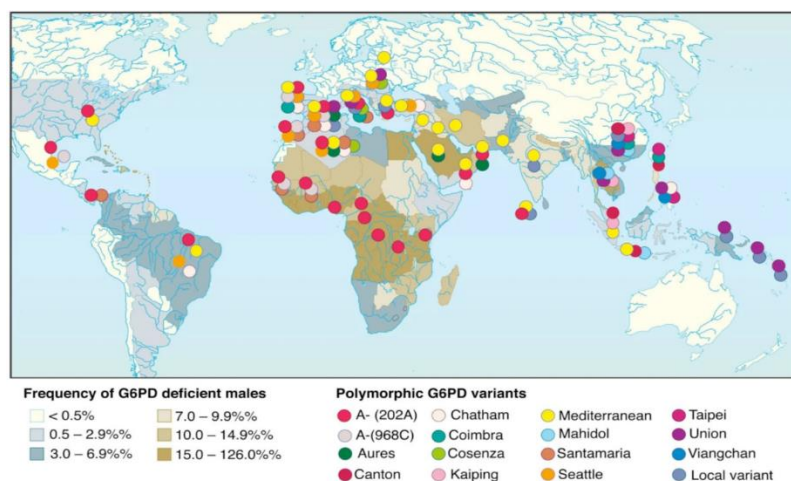
Figure 8 The global distribution of G6PD deficiency and *G6PD* mutations (37) The color shades on the map indicate the prevalence of G6PD deficiency in each region. Each colored circle illustrates the geographical distribution of *G6PD* variants in each region.

2.6.   G6PD deficiency

G6PD deficiency is the most common enzymatic abnormality, affecting approximately 400 million people worldwide or 4.9% of the global population (3, 38) (**Figure 8**). The highest frequencies have been discovered in Africa, the Mediterranean, the Middle East, Southeast Asia, and areas where malaria is endemic. In Thailand, the prevalence of G6PD deficiency in male and female newborns was 11.1% to 17.0% and 2% to 5.8% respectively (8, 9). The prevalence of G6PD deficiency in male and female adults was 8.5% to 9.2% and 3.0% to 6.3% respectively (12, 39). It is an X-linked recessive genetic disorder with a high prevalence in males more than females because of DNA methylation and mosaicism of X-chromosome inactivation (2). Males containing one X chromosome may carry normal or abnormal *G6PD* gene, resulting in either normal or deficient G6PD hemizygotes. In contrast, females containing two X chromosomes may have normal or heterozygotes or deficient homozygotes. Because of the random inactivation of one of the two X chromosomes, heterozygous deficient women have two RBC populations, resulting in intermediate G6PD activity (24, 40). Individuals with G6PD deficiency are mostly asymptomatic but they can develop jaundice and acute

hemolysis after exposure to exogenous agents such as infections, fava beans intake, and antimalarial drugs (41). However, the severity of G6PD deficiency depends on the level of enzymatic dysfunction (11). The World Health Organization (WHO) classified *G6PD* variants into five classes according to the level of enzyme activity and clinical phenotypes; Class I: severe deficiency associated with chronic non-spherocytic hemolytic anemia (CNSHA), Class II: severe deficiency (enzyme activity lower than 10%), class III: moderate to mild deficiency (enzyme activity 10-60%), Class IV: normal (enzyme activity 60-100%), and Class V: increased enzyme activity (11). Class II and III are the most frequent pathological variations.

2.7.   Neonatal hyperbilirubinemia and kernicterus in G6PD deficiency

About 80% of term infants worldwide develop sign of hyperbilirubinemia (7). The occurrence of neonatal hyperbilirubinemia is attributed to various factors. G6PD deficiency has been identified as one of the risk factors (42). In Thai newborns, around 65% of severe neonatal jaundice (8) and 19.7%-21.2% of hyperbilirubinemia were coincidently observed in G6PD deficient (8, 10). The cause of hyperbilirubinemia is elevated bilirubin production with inefficient excretion of bilirubin (7). After hemolysis of RBCs, heme is broken down into bilirubin. In the bloodstream, unconjugated bilirubin binds to albumin to facilitate its transport to the liver and then conjugated with diglucuronide by glucuronyl transferase in the liver for excretion. In pre-term infants, glucuronyl transferase is insufficient to eliminate bilirubin, resulting in an increase of unconjugated bilirubin in blood flow. In G6PD deficient newborns with low erythrocytic NADPH and glutathione (GSH) levels, there is elevating hemolysis and unconjugated bilirubin in the blood circulation, then it can be disseminated into the skin and other bodily tissues. The diffusion threshold exceeds, and the unconjugated bilirubin penetrates brain cells through the blood-brain barrier (BBB), resulting in neuroinflammation, cell death, and kernicterus (7) (**Figure 9**). Likewise, bilirubin also targets the mitochondrial membrane due to its lipophilic property, which inhibits mitochondrial activities, resulting in mitochondrial swelling, membrane permeability, depolarization, cytochrome c release, and cell death by apoptosis and necrosis (7). WHO recommends routine G6PD deficiency

screening in newborns if the prevalence of G6PD deficiency reaches 3–5% in males in order to detect, and thereby ensure timely prevent poor outcome (11). According to the clinical practice guideline for the management of neonatal jaundice in Thailand, qualify of G6PD deficiency should be carried out only when newborns developed signs of jaundice. This condition may misdiagnose asymptomatic G6PD intermediate.
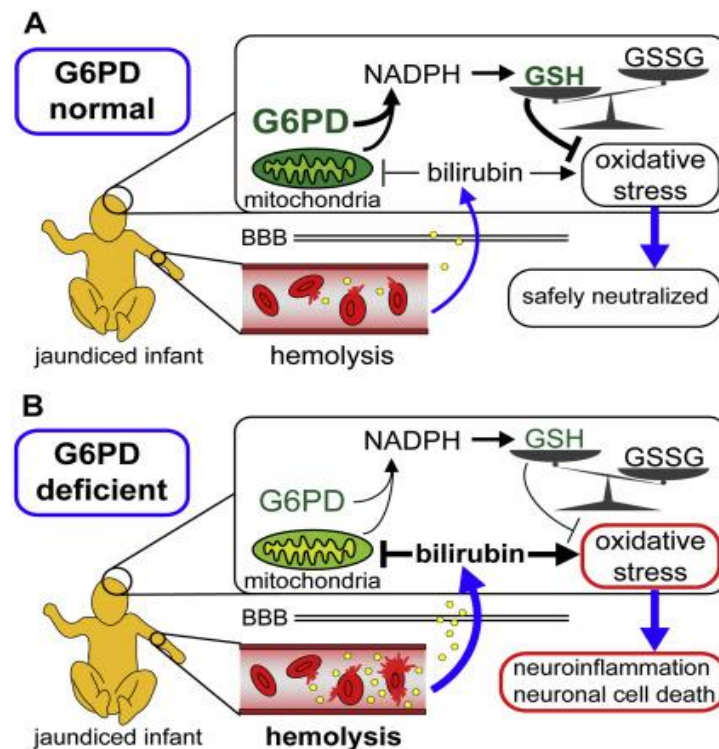
Figure 9 Mechanism of G6PD deficiency causing neurotoxicity induced by bilirubin in newborns (7).

2.8.   Detection of G6PD deficiency

The biochemical assessment of G6PD status is usually determined by either a qualitative screening method or a quantitative technique for enzyme activity. The biological basis of the biochemical tests involves the production of NADPH from NADP, the rate of NADPH production reflecting G6PD activity (40).

2.8.1.   Qualitative technique

The fluorescent spot test (FST) has been used as a qualitative test for G6PD deficiency screening. The principle of this method is to detect fluorescence of

NADPH production, which is proportional to the concentration of G6PD activity. All specimens are characterized in terms of a qualitative outcomes for G6PD activity under UV light. NADPH absorbs UV light at a wavelength of 340 nm and emits light at a wavelength of 460 nm with a fluorescence intensity proportional to the enzyme activity (43). It is widely used for the qualitative detection of G6PD deficiency due to its reasonable price and simple technique. This method is useful in field research, which requires rapid screening of large numbers of cases (43, 44). Although FST can identify severe deficient cases, discrimination intermediate levels among heterozygous females is more difficult (44).

2.8.2. Quantitative technique

The spectrophotometer assay is a gold standard method that measures the absorbance of NADPH at the wavelength of 340 nm for 5 min. One International Unit (U) is the amount of G6PD activity converting one micromole of $NADP^+$ per minute under predefined substrate and reaction conditions. This method can accurately measure the activity of G6PD for all individuals by normalizing the enzymatic activity by hemoglobin concentration and is calculated in the following formula (45). Thus, this method is more sensitive and reliable than FST.

$$\text{G6PD (U/g Hb)} = \Delta A \text{ per } min \times \frac{4839}{Hb \left(\frac{g}{dl}\right)} \times TCF$$

Where: $\Delta A \text{ per } \min = \frac{Final A - Initial A}{5}$

Hb (g/dl) = Hemoglobin concentration determined for each sample

TCF = Temperature correction factor (1 at 37℃)

Both qualitative and quantitative assays for measuring G6PD activity are available in the market. Nonetheless, operating these methods is time-consuming, needs to be done manually, and hard to distinguish between G6PD intermediate and normal (12). Recently, an automated UV enzymatic assay has been developed to simultaneously measure the level of G6PD activity in 50 samples with an automated system. The enzyme activity is determined by measurement of the rate of

absorbance change at 340 nm due to the reduction of NADP. This method can be tested in chemistry analyzers without manual operation and provide an accurate result rapidly, which may solve the weaknesses of two common techniques.

2.8.3. Genotyping assays

Since individuals with G6PD deficiency generally asymptomatic. Females, in particular, are more difficult to classify since they may be heterozygotes, with phenotypic overlap between normal homozygous, heterozygous, and deficient homozygous. Therefore, DNA-based genotyping is the most reliable method to confirm the diagnosis of G6PD status because it can determine if any mutation is present in the *G6PD* gene (46). Several methods for the detection of *G6PD* mutations have been reported including polymerase chain reaction (PCR)-restriction fragment length polymorphism RFLP) analysis, PCR TaqMan assay, and DNA sequencing (47). PCR-RFLP has been utilized to determine common *G6PD* mutations in Thai populations. The direct sequencing is more comprehensive and reliable for detecting sequence variations within the *G6PD* genes (36).

2.9.   Hemolytic anemia

Hemolytic anemia is a condition in which red blood cells are destroyed before a normal lifespan of 120 days. It can manifest as a primary disorder or as a result of another disease process. It can be divided into immune and non-immune etiologies (48).

Immune hemolytic anemia is caused by antibodies directed against antigens on the surface of red blood cells, such as ABO-incompatible (48). Mothers with blood group O have naturally occurred antibodies against blood group A or B. If the mother's fetus has blood group A, B, or AB, the antigen of this fetus's foreign blood group will be attacked by mother's anti-A or/and anti-B antibodies. Anti-A and anti-B antibodies are IgG antibodies that can cross the placenta and break down the baby's RBCs after birth, causing hemolytic anemia (49).

Non-immune hemolytic anemias are caused by intracorpuscular defects within the red blood cells or extracorpuscular by environmental factors. For example, thalassemia is a diverse group of inherited multifactorial anemias, with defects in the synthesis of the alpha or beta subunits of the hemoglobin tetramer. The loss of the globin chain leads to a decrease in overall hemoglobin and excess intracellular precipitation of the chain, which damages cell membranes and leads to clinically apparent hemolysis in severe alpha-thalassemia (hemoglobin H disease) and beta-thalassemia (48).

2.10. Interfering substances in detection of G6PD deficiency

The performance of the G6PD test and the enzyme capacity can be disturbed in a number of situations, including reticulocytosis, leukocytosis, and thrombocytosis (50, 51).

Reticulocytosis is a condition where there is an increase in reticulocytes (immature red blood cells). They are produced from the bone marrow to compensate for the loss of mature red blood cells. The reticulocyte has a higher enzyme activity than that of mature erythrocyte (2, 52). Since the reticulocytes contain mitochondria, alternative pathways for NADPH production have been created. They also have nucleus, making it possible to synthesize new proteins. Testing for G6PD deficiency might be falsely negative because the mature erythrocytes has been hemolyzed, the reticulocytes with typically higher levels of enzyme activity than normal enzyme levels were able to sustain the oxidative damage (53). Problems can also be encountered in evaluating neonates with large amounts of young red-blood-cell populations (2). This is supported by several studies that revealed that the neonatal erythrocyte enzyme activity was significantly higher than that of adults (52, 54).

Leukocytosis is a condition in which white blood cells build up, typically in response to inflammation or infection. Thrombocytosis is a condition in which there is an excessive number of platelets in the blood. These two situations might lead to a falsely negative for testing for G6PD deficiency because leukocytes contain nucleus

and mitochondria and thrombocytes contain mitochondria so, they can synthesize new proteins and have alternative pathways to produce NADPH that might interfere with the test.

Hence, factors such as increased number of reticulocytes, leukocytes, or thrombocytes might be interfering substances for G6PD deficiency detection (13)

CHAPTER 3
Research Methodology

3.1.   Study population

A total of 1,015 leftover blood samples collected from the research project "Incidence of G6PD deficiency and clinical correlation of G6PD conjugated and unconjugated hyperbilirubinemia, reticulocytosis, and leukocytosis in newborn infants delivered in King Chulalongkorn Memorial Hospital" approved by the Institution Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 954/2021, IRB No. 254/62) was enrolled in the study.

Inclusion criteria: Healthy term-newborn infants with weight ≥ 2,000 g
Exclusion criteria: Subjects with infection, congenital anemia (Hct≤30%), or congenital disorder

3.2.   Hematological parameters

Hematological parameters including hemoglobin (Hb), red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT), hematocrit (HCT), reticulocyte counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were performed in all blood samples using a fully automated hematology analyzer BC-6200 (Mindray Medical International, PRC).

3.3.   Assessment of bilirubin level

All serum were tested for total bilirubin (TB) and direct bilirubin (DB) level by using a clinical chemistry analyzer (BS- 360E, Mindray Medical International, PRC). Total bilirubin (unconjugated + conjugated) and direct (conjugated) bilirubin concentrations were determined in the presence of a catalyst, where bilirubin reacts with a diazo- salt to form a red product of azobilirubin, which absorbs at 546 nm. The increase in absorbance is directly proportional to the concentration of bilirubin, which is calculated by the equation below.

$$\Delta A = [\Delta A \text{ sample}] - [(\Delta A \text{ blank}]$$

3.4. G6PD analytical method

3.4.1. Fluorescent spot test (FST)

This assay utilizes G6PD, which in the presence of NADP, catalyzes the oxidation of glucose 6-phosphate to 6-phosphogluconate. The NADPH produced is directly proportional to the concentration of G6PD in the sample. When activated by UV radiation (340 nm), NADPH emits blue light with a wavelength of 460 nm, with the intensity of fluorescence proportional to the G6PD enzyme activity.

All blood samples were screened for G6PD deficiency using the fluorescent spot test kits (Trinity Biotech, Bray, Co. Wicklow, Ireland, and R&D Diagnostic, Athens, Greece) according to the manufacturer's instructions. Briefly, 1.25 μl of the whole blood sample was added to 25 μl of the reagent mixture and spotted on the filter paper at time 0. The mixture of samples was then incubated at 37°C for 15 minutes before being spotted on filter papers again and allowed to air-dry in the dark at room temperature. Afterward, spotted samples were exposed under UV light to visualize the emitted fluorescence of the reaction. The fluorescence intensity was assessed with the naked eye to classify specimens into two groups: normal (moderate to strong fluorescence) and deficient (very faint or non-fluorescence).

3.4.2. Quantitative spectrophotometric assay

The G6PD activity was determined using a quantitative spectrophotometric assay (Cat. No. G7583; Pointe Scientific INC., Canton, Michigan, USA) and was carried out according to the manufacturer's instruction as the reference assay for all tests. Normal and deficient controls (Cat. No. G6888, G5888; Trinity Biotech PLC, Bray, Ireland) were performed parallel with samples in each assay run. Concisely, 5 μl of whole blood sample was added to 500 μl reagent and incubated at room temperature for 10 minutes. Then one ml of the substrate was added to the solution and mixed. The enzyme activity was determined at 37°C using a temperature-regulated spectrophotometer (Shimadzu UV- 1800; Shimadzu, Kanagawa, Japan) by measuring changes in the absorbance rate at 340 nm over 5 minutes. G6PD activities were then divided by the relative Hb value from individual subjects giving the

absolute activities presented in U/g Hb. Hemoglobin concentration was determined using an automated hematology analyzer BC-6200 (Mindray Medical International, PRC).

3.4.3. Automated UV enzymatic assay

All samples were measured G6PD activity using an automated UV enzymatic method, according to the manufacturer's instruction. In brief, hemolysate from 20 μl of packed red blood cells was added to 1 ml of distilled water, mixed for 5 minutes, and then measured G6PD activity using a clinical chemistry analyzer (BS 360E, Mindray Medical International, PRC) at 340 nm for 2 minutes of a time interval. The absorbance change per minute of the reaction (ΔA/min) was calculated by the following equation,

$$\Delta A/min = [\Delta A/min \ sample] - [(\Delta A/min \ blank]$$

3.5. DNA extraction

Genomic DNA samples were extracted from leftover blood samples using Nucleospin® Blood kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations. Briefly, 100 μl of whole blood was mixed with 100 μl of PBS in a 1.5 ml Eppendorf tube to adjust the volume to 200 μl. The samples were then mixed with 25 μl of proteinase K and 200 μl of buffer B3, and the mixture was vortexed and incubated at 70°C for 15 minutes to ensure complete digestion of the aged samples. After incubation, the color of lysates became brownish. Two hundred and ten μl of absolute ethanol was added to each sample and vortexed again. The sample were loaded on Nucleospin® Blood column and centrifuged for 3 minutes at 14,000 rpm. Silica membrane was washed with two washing steps. First step was performed by adding 500 μl of buffer BW to the column and centrifuge for 3 minutes at 14,000 rpm. The second step consist of adding 600 μl buffer B5 into the column and centrifuge for 3 minutes at 14,000 rpm. After washing steps, the column was centrifuged for 3 minutes at 14,000 rpm to dry silica membrane. Pre-warmed Buffer BE (70°C) was added to the column and incubated for 30 minutes at room temperature. Then, DNA was recovered by centrifugation at

14,000 rpm. DNA was eluted in 50 μl of elution buffer and stored at -20°C until use. The DNA concentration was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA).

3.6.   Mutation analysis of the *G6PD* Gene

3.6.1. TaqMan SNP Genotyping Assay

All DNA samples were molecularly analyzed for common *G6PD* mutations found in Thai population, *G6PD Viangchan*[G871A] and *G6PD Mahidol*[G487A] using TaqMan® SNP genotyping assay (Applied Biosystem, Foster City, CA, USA). Reactions were set up in 96-well microplates. The total volume of the PCR reaction was 5 μl and consisted of 2.5 μl of 2X TaqMan Universal PCR master mix, 0.125 μl of 40x of TaqMan SNP genotyping assay mix (**Table 1**), and 2.375 μl of nuclease-free water. One μl of DNA (about 10-50 ng) was dried on 96-well microplate before loading master mix. Thermocycling was performed with an initial 60°C incubation for 30 seconds, followed by 95°C denaturation for 10 minutes, then 45 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute. The samples were run together with the negative control in order to check the reaction efficiency and to exclude any possible contamination. Amplification and real-time detection were performed on StepOnePlus Real-time PCR (Applied Biosystem, Foster City, CA, USA) and the results were analyzed according to the instrument's software.

Table 1 Primers and probes for Taqman® SNP genotyping assay

G6PD Mutation	Assay ID	Sequence of primer (5'–3')	Sequence of probe (5'–3')
Mahidol	C__27862231_10 rs137852314	5'-TGATCCTCACTCCCCGAAGAG-3' 5'-AAGGGCTTCTCCACGATGATG-3'	VIC-CAGCAGA**G**GCTGGAA-NFQ FAM-CCAGCAGA**A**GCTGGAA-NFQ
Viangchan	C___2228724_10 rs137852327	5'-CCATTCTCTCCCTTGGCTTTCTC-3' 5'-GGCCTGCACCTCTGAGATG-3'	VIC-CAGGTCAAG**G**TGTTGAA-NFQ FAM-TCAGGTCAAG**A**TGTTGAA-NFQ

3.6.2. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The G6PD intermediate and deficiency samples that did not exhibit any of *G6PD Viangchan*[G871A] or *G6PD Mahidol*[G487A] were subjected to screen for the other less common *G6PD* variants previously reported in Thai population (8) using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP). These included *G6PD Canton*[G1376T], *G6PD Union*[C1360T], and *G6PD Kaiping*[G1388A]. PCR amplification was carried out in a total volume of 20 μl with DNase free water, 1X PCR buffer, 0.5 U Taq DNA Polymerase (Thermo Scientific, CA, USA), 200 μM dNTPs (Invitrogen), 1.5 mM MgCl$_2$ (Invitrogen), 0.2 μM of the appropriate primers (**Table 2**) (Bio Basic Inc.), and approximate 50 ng/μl DNA template. The PCR condition was initial denaturing at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 30 seconds of the annealing temperature for each primer pair (**Table 2**), 72°C for 30 seconds, and final extension at 72°C for 7 minutes using Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystem, Foster City, CA, USA). The PCR products were digested using the appropriate enzymes (**Table 3**). The reaction was performed in a total of 20 μl containing DNase-free water, 1U restriction enzyme (Fermentas), 1X reaction buffer (Fermentas), and 10 μl PCR products, which were then incubated overnight at the proper temperature (**Table 3**). PCR-RFLP products were analyzed using gel electrophoresis with 8% polyacrylamide gel (29% acrylamide + 1% N, N' methylenebisacrylamide (Bio-RAD), (5X) TBE buffer (Bio-RAD), 10% ammonium persulfate (APS) (Pharmacia Biotech), TEMED (Bio-RAD) and water. Five μl of PCR-RFLP product were mixed with 1 μl of novel juice (Bio-Helix). The mixture was then loaded

onto 8% polyacrylamide gel and run at 75 volts for 75 minutes. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad).

Table 2 G6PD primer sequences and annealing temperature

G6PD Mutations	Primer Name	Primer sequence (5'–3')	Annealing Temperature (°C)
Union	1360F	5'-ACGTGAAGCTCCCTGACGC-3'	65
	1360R	5'-GTGAAAATACGCCAGGCCTTA-3'	
Canton	1360F	*G6PD*Union primer	65
	1360R	*G6PD*Union primer	
Kaiping	1360F	5'-ACGTGAAGCTCCCTGACGC-3'	65
	1388R	5'-GTGCAGCAGTGGGGTGAACATA-3'	

Table 3 Sequence of G6PD restriction enzymes and product size

G6PD Mutation	Restriction Enzyme	Buffer Mutation	Restriction Size	Product Size (bp)		Temperature (°C)
				Wild type	Mutant	
Union	*Hha*I	Tango	5'-GCG**^**C-3' 3'-C**^**GCG-5'	142+45+27	187+27	37
Canton	*Afl*II	O	5'-C**^**TTAAG-3' 3'-GAATT**^**C-5'	214	194+20	37
Kaiping	*Nde*I	O	5'-CA**^**TATG-3' 3'-GTAT**^**AC-5'	227	206+21	37

3.6.3. Direct sequencing

For all unknown mutation samples, PCR direct sequencing was conducted to determine the *G6PD* gene mutations of exon3 - 12. The primer sequences shown in **Table 4** were used in the study. Final 50 µl PCR reaction mixture contained 1X PCR buffer, 0.20-0.25 mM dNTPs, 1.0-2.5 mM MgCl$_2$, 0.40 µM of each primer, 0.50-1.50 U Taq DNA polymerase (Thermo Scientific, CA, USA), and 50 ng/µl DNA template. The final concentration of the master mix for each exon and the optimum conditions are shown in **Tables 5 and 6**. Three µl of PCR product was mixed with 1 µl of novel juice (Bio-Helix). The mixture was then loaded onto 2% agarose gel and run at 100 volts for 60 minutes. Results were visualized under Molecular Imager® Gel Doc™ XR+ with Image Lab ™ Software (BioRad). The PCR products with exactly one size band were purified using NucleoSpin Gel and PCR Cleanup (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The PCR product containing a non-specific band was loaded onto 2% agarose gel, run at 100 volts for 60 minutes again, gel purified, and sequenced in forward direction. All sequences were aligned to the NCBI reference sequence (NC 000023.11) to ensure the specificity of the PCR product.

Table 4 PCR primer sequences and amplicon size of *G6PD* gene

PCR pair	Primer name	Primer sequence (5'–3')	Exon coverage	PCR product (bp)
1	G6PD_Ex3-4F	5'-TGTCCCCAGCCACTTCTAA-3'	Exon 3-4	400
	G6PD_Ex3-4R	5'-GGAGAGGAGGAGAGCATCC-3'		
2	Ex5F	5'-GTGTGTCTGTCTGTCCGTGTC-3'	Exon 5	320
	Ex5R	5'-CACGCTCATAGAGTGGTGGG-3'		
3	G6PD_Ex6-7F	5'-ACACAAGGCACGGGAGGT-3'	Exon 6-7	697
	G6PD_Ex6-7R	5'-GAGGAGCTCCCCCAAGATAG-3'		
4	Ex8F	5'-CATGCCCTTGAACCAGGTGA-3'	Exon 8	241
	Ex8R	5'-GCATGCACACCCCAGCTC-3'		
5	871F	5'-TGGCTTTCTCTCAGGTCTAG-3	Exon9 -11	993

	1388R	5'-GTGCAGCAGTGGGGTGAACATA-3'		
6	1360F	5'-ACGTGAAGCTCCCTGACGC-3'	Exon 12	469
	G6P13R	5'-CCAGGGCTCAGAGCTTGTG-3'		

Table 5 The final concentration of the master mix for each exon

Reagent	Final concentration of the master mix					
	Exon 3-4	Exon 5	Exon 6-7	Exon 8	Exon 9-11	Exon 12
10X PCR Buffer	1X					
10 mM dNTPs	0.25 mM	0.20 mM	0.25 mM	0.20 mM		
50 mM MgCl$_2$	1.0 mM	1.0 mM	2.5 mM	1.5 mM		1.0 mM
5 U/µl Taq polymerase	1.5 U	0.5 U	1.5 U	0.5 U		
Forward primer	0.4 µM					
Reverse primer	0.4 µM					

Table 6 The optimum conditions for each exon

Steps	Exon 3-4	Exon 5	Exon 6-7	Exon 8	Exon 9-11	Exon 12
Pre - denaturation	94 °C, 5 min					
Denaturation	94 °C, 30 sec	94 °C, 45 sec			94 °C, 30 sec	94 °C, 45 sec
Annealing	62 °C, 30 sec	65 °C, 45 sec	67 °C, 45 sec	65 °C, 45 sec	60 °C, 30 sec	65 °C, 45 sec
Extension	72 °C, 1 min	72 °C, 45 sec			72 °C, 30 sec	72 °C, 45 sec
Cycles	40	35				
Post - extension	72 °C, 10 min	72 °C, 15 min			72 °C, 7 min	72 °C, 15 min

3.7.   Data Analysis

Statistical analyses were carried out using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). The distribution of data was determined using Kolmogorov-Smirnov/Shapiro-Wilk tests. The G6PD activity values of male and female newborns were expressed as median ± interquartile range (IQR). The G6PD reference value in newborn infants was calculated from the median G6PD activity of male subjects. The cut-off points of deficiency and intermediate were determined as less than 30%, and 30 to 80% G6PD activity of the normal median G6PD activity of male subjects (46). Patients with G6PD activity more than 80% of the AMM was classified as normal (46). The frequency of G6PD deficiency and G6PD variants were present as percentages. Multivariate linear regression was used to test for the correlation between G6PD activity from two G6PD enzymatic assays and between G6PD activity and hematological parameters. Cohen's Kappa statistic was performed to evaluate the degree of agreement between two G6PD enzymatic assays. The area under Receiver operating characteristic (ROC) curve (AUC) was calculated to test the efficacy of automated UV enzymatic assay in detection of G6PD deficiency and intermediate. The sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) of fluorescent spot test and automated UV enzymatic assay were evaluated in comparison with the gold standard method. The two-tailed Student's t-test was used to assess the differences between data groups. P -value less than 0.05 was considered as statistically significant.

CHAPTER 4
Results

4.1. Demographic data of population

A total of 1,015 Thai newborns consisted of 502 (49.5%) males and 513 (50.5%) females. Demographic data of the newborns are summarized in **Table 7**. Mean gestational age (GA) and average age of all subjects were 39.2±18.0 weeks and 2.7±0.8 days, respectively. There were no significant differences in gestation age or average age between gender (p=0.152 and p=0.191, respectively). Mean birth weight of male newborns was 3,179.5±414.1 g, which significantly higher than that of female newborns (3,061.4±389.4 g; p<0.001) (**Table 7**).

Hemolytic disease of the fetus and newborn (HDFN) is a blood disorder that causes the baby's red blood cells to break down rapidly. The causes of HDFN can be immune or non-immune mediated HDFN. Blood group incompatibility between mother and the fetus is the main cause of immune-mediated HDFN. The non-immune mediated HDFN are enzyme abnormalities, RBC membrane problems, thalassemia, and hemoglobinopathies. In the study population, there were 8 newborns with ABO incompatibility, 43 newborns with thalassemia/hemoglobinopathies, and 1 coincidence of ABO incompatibility and thalassemia/hemoglobinopathies. The prevalence of G6PD deficiency in male and female populations of the study based on three different diagnostic techniques were shown in **Table 7**. The gold standard spectrophotometric assay was used to verify the results of the other two techniques.

Table 7 Demographic data of newborn subjects enrolled in the study.

	Total (n=1,015)	Male (n=502)	Female (n=513)	*p*-value[*]
Gestational age (GA) (week)	39.2±18.0	38.3±1.0	40.1±25.3	0.152
Average age (day)	2.7±0.8	2.8±0.8	2.7±0.8	0.191
Birth weight (g)	3,119.8±405.9	3,179.5±414.1	3,061.4±389.4	<0.001
ABO incompatibility (n) (%)	9 (0.89)	3 (0.60)	6 (1.17)	
Thalassemia/hemoglobinopathy (n) (%)	44 (4.33)	15 (2.99)	29 (5.65)	
G6PD deficiency by FST (n) (%)	54 (5.3)	46 (9.2)	8 (1.6)	<0.001
G6PD deficiency by spectrophotometric assay (n) (%)	62 (6.1)	50 (10.0)	12 (2.3)	<0.001
G6PD deficiency by automated UV enzymatic assay (n) (%)	66 (6.5)	52 (10.4)	14 (2.7)	<0.001

Values are expressed as mean ± SD; [*] Compared between male and female.

4.2. Cut-off value determination of G6PD activity and prevalence of G6PD deficiency calculated based on the enzyme activity among study subjects

The normality of the data was assessed using the Kolmogorov-Smirnov/Shapiro-Wilk tests. Our finding revealed that neonatal male G6PD activity as measured by spectrophotometric (**Figure 10a)** and automated UV enzymatic assays **(Figure 11a)** showed a bimodal distribution, while presenting a normal distribution in female subjects (**Figure 10b, 11b**). The median ± interquartile range (IQR) of G6PD activity of males (10.09±2.47 U/g Hb, ranging from 0.00 to 16.42 U/g Hb) and females (10.00±2.66 U/g Hb, ranging from 0.47 to 17.78 U/g Hb) from spectrophotometric assay were not significantly different (*p*=0.848 by Mann-Whitney U-test) (**Table 8**). Results from an automated UV enzymatic assay demonstrated that there was no significant difference in median G6PD activity between males (25.94±7.76 U/g Hb, ranging from 0.3 to 64.01 U/g Hb) and females (25.56±8.95 U/g Hb, ranging from 1.15 to 54.45 U/g Hb) (*p*=0.375 by Mann-Whitney U-test) (**Table 8**). The reference values of G6PD activity of newborn subjects determined by spectrophotometric and automated UV enzymatic assays were shown in **Table 9**. Since G6PD deficiency is an

X-linked recessive disorder, G6PD level can be normal, deficient, or an intermediate among females, whereas the G6PD level can be either normal or deficient in males. WHO defined G6PD activity of less than 30% the normal median as deficient, while G6PD activity between 30% and 80% the normal median as intermediate. Subjects with G6PD activity of more than 80% the normal median were classified as normal (46). In spectrophotometric assay, the reference values for deficiency in this study was less than 3.03 U/g Hb; G6PD activity between 3.03 and 8.07 U/g Hb were G6PD intermediate, and G6PD activity more than 8.07 U/g Hb were considered G6PD normal. Based on the enzyme activity cut-off values, the prevalence of G6PD activity in this study was found to be 62 (6.1%) of G6PD deficient, 116 (11.4%) of G6PD intermediate, and 837 (82.5%) of G6PD normal (**Table 9**). In automated UV enzymatic assay, the reference values for deficiency were less than 7.78 U/g Hb; G6PD activity between 7.78 and 20.75 U/g Hb were considered G6PD intermediate, and G6PD activity more than 20.75 U/g Hb were G6PD normal. The prevalence of G6PD activity was found to be 66 (6.5%) of G6PD deficient, 158 (15.6%) of G6PD intermediate, and 791 (77.9%) of G6PD normal (**Table 9**). The median values of G6PD activity in males and females as measured by spectrophotometric and automated UV enzymatic assays categorized by G6PD status were shown in **Table 10**. In the spectrophotometric assay, the median of G6PD normal was 10.48±2.18 U/g Hb, comprising 10.52±2.15 U/g Hb of male and 10.45±2.23 U/g Hb of female newborns. The median of G6PD intermediate was 6.34±2.40 U/g Hb, comprising 7.63±1.01 U/g Hb of male and 6.04±1.92 U/g Hb of female newborns. The median of G6PD deficiency was 1.33±0.75 U/g Hb, comprising 1.21±0.76 U/g Hb and 1.56±1.67 U/g Hb of female newborns (**Table 10**). In automated UV enzymatic assay, the median of G6PD normal was 27.45±6.63 U/g Hb, comprising 27.26±6.68 U/g Hb of male and 27.50±6.63 U/g Hb of female newborns. The median of G6PD intermediate was 16.49±6.37 U/g Hb, comprising 18.26±2.81 U/g Hb of male and 14.75±6.22 U/g Hb of female newborns. The median of G6PD deficiency was 1.81±0.90 U/g Hb, comprising 1.76±0.70 U/g Hb of male and 2.11±5.44 U/g Hb of female newborns (**Table 10**).

Spectrophotometric assay
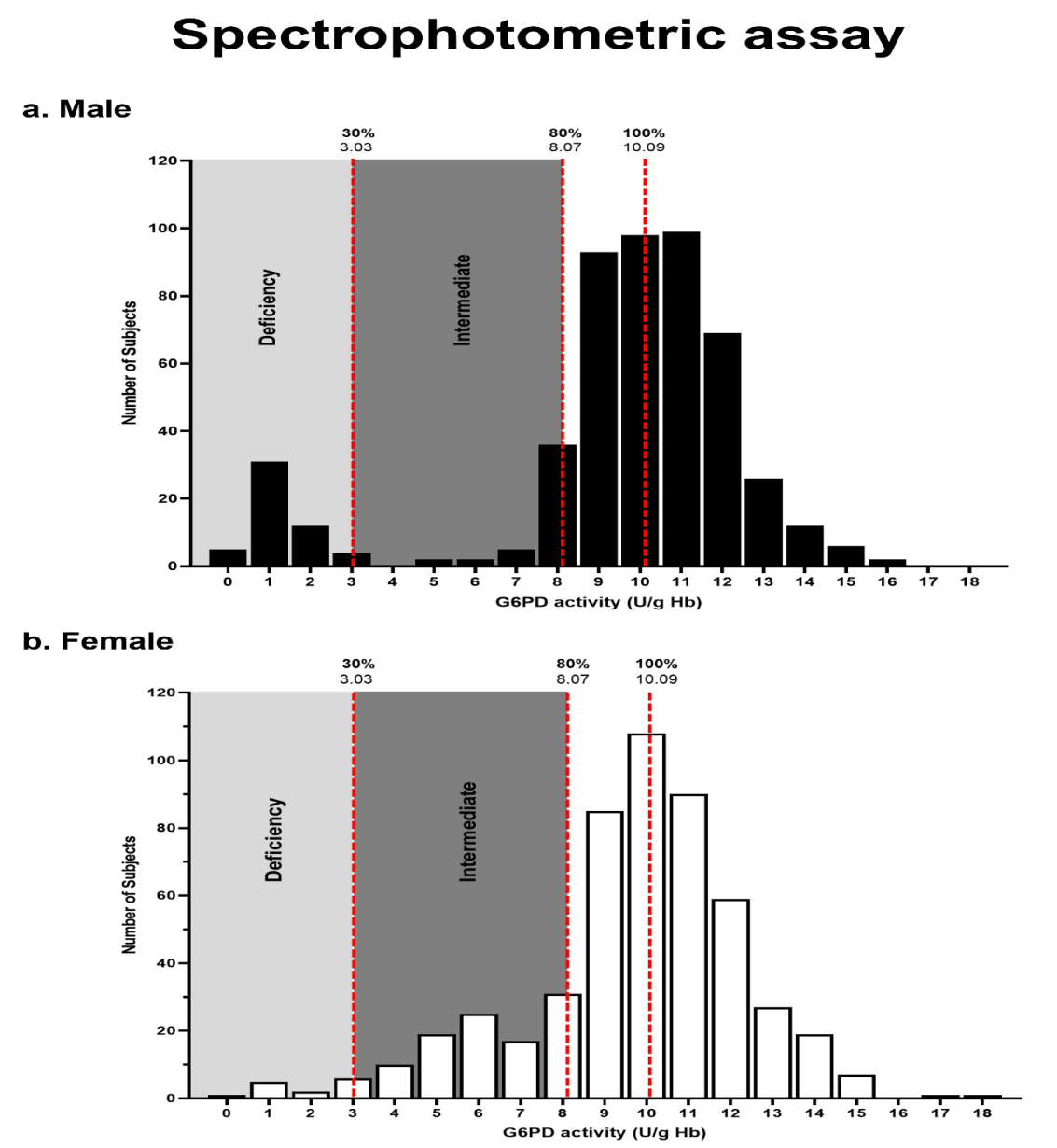
a. Male

b. Female

Figure 10 The histogram represents G6PD activities in (a) male and (b) female newborns measured by the spectrophotometric assay. The numbers on the top of each dotted line on the graph represent cut-off values as percentages of the male activity for the study population.

Automated UV enzymatic assay

a. Male
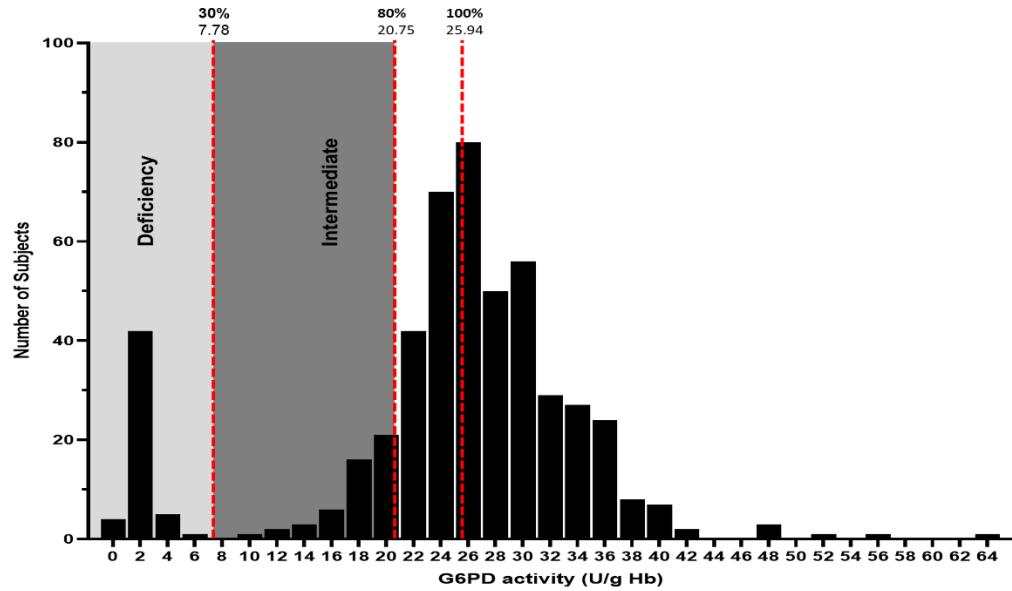
b. Female
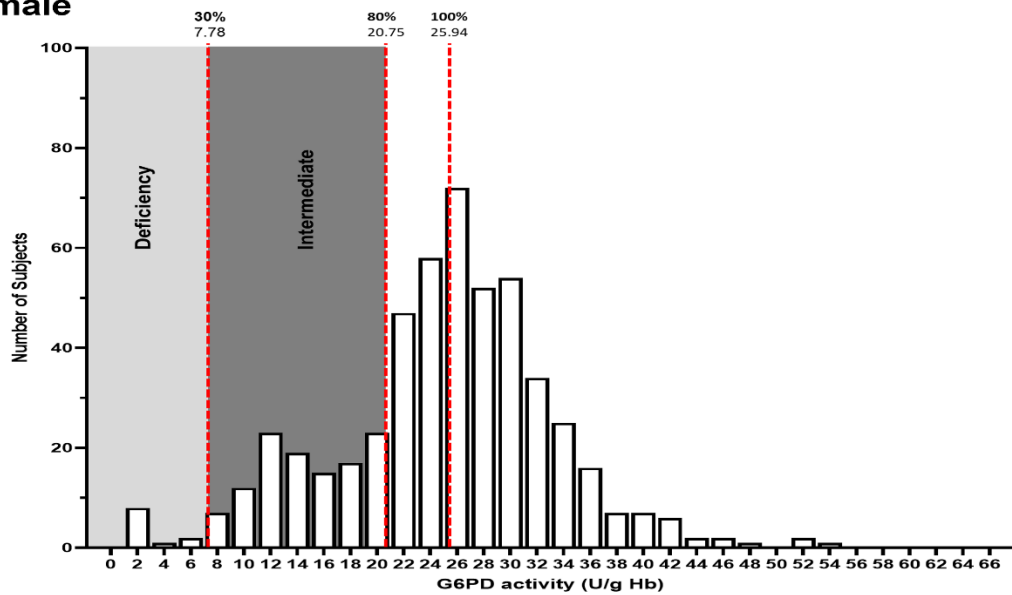
Figure 11 The histogram represents G6PD activities in (a) male and (b) female newborns measured by the automated UV enzymatic assay. The numbers on the top of each dotted line on the graph represent different cut-off values as percentages of the male activity for the study population.

Table 8 The median± interquartile range (IQR) of G6PD activity of males and females in the study population measured by spectrophotometric and automated UV enzymatic methods.

Spectrophotometric assay				
Reference values (U/g Hb)	Total (n=1015)	Female (n=513)	Male (n=502)	*p*–value*
Median	10.01	10.00	10.09	0.848
IQR	2.56	2.66	2.47	
Range	0.00 - 17.78	0.47 - 17.78	0.00 - 16.42	
Automated UV enzymatic assay				
Reference values (U/g Hb)	Total (n=1015)	Female (n=513)	Male (n=502)	*p*–value*
Median	25.81	25.56	25.94	0.375
IQR	8.35	8.95	7.76	
Range	0.3 - 64.01	1.15 - 54.45	0.3 - 64.01	

* Compared between male and female by Mann-Whitney U-test.

Table 9 Proportion of newborn subjects with G6PD status determined by the spectrophotometric and automated UV enzymatic methods.

		Spectrophotometric assay (n)			Total
		Deficiency (< 3.03 U/g Hb)	Intermediate (3.03 - 8.07 U/g Hb)	Normal (> 8.07 U/g Hb)	
FST					
Male	Deficiency	46	-	-	46
	Normal	4	29	423	456
	Total	50	29	423	502
Female	Deficiency	8	-	-	8
	Normal	4	87	414	505
	Total	12	87	414	513
Automated UV enzymatic assay					
Male	Deficiency (< 7.78 U/g Hb)	50	2	0	52
	Intermediate	0	11	36	47

	(7.78 - 20.75 U/g Hb)				
	Normal (> 20.75 U/g Hb)	0	16	387	403
	Total	50	29	423	502
Female	Deficiency (< 7.78 U/g Hb)	11	3	0	14
	Intermediate (7.78 - 20.75 U/g Hb)	1	69	41	111
	Normal (> 20.75 U/g Hb)	0	15	373	388
	Total	12	87	414	513

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Table 10 G6PD activity in group of G6PD normal, intermediate, and deficiency newborn subjects in the study.

	G6PD Normal			G6PD Intermediate			G6PD Deficiency		
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Spectrophotometric assay (n)	837	423	414	116	29	87	62	50	12
G6PD activity (U/g Hb)	10.48 ± 2.18	10.52±2.15	10.45±2.23	6.34 ± 2.40	7.63±1.01	6.04±1.92	1.33 ± 0.75	1.21 ± 0.76	1.56 ± 1.67
Automated UV enzymatic assay (n)	791	403	388	158	47	111	66	52	14
G6PD activity (U/g Hb)	27.45 ± 6.63	27.26 ± 6.68	27.50 ± 6.63	16.49 ± 6.37	18.26 ± 2.81	14.75 ± 6.22	1.81 ± 0.90	1.76 ± 0.70	2.11 ± 5.44

Values are expressed as median ± IQR.

4.3. *G6PD* mutations and their enzyme activity

Of all 62 patients with G6PD deficiency and 116 patients with G6PD intermediate detected by spectrophotometric assay, 81 patients carried *G6PD Viangchan^{G871A}* (30 hemizygous deficient males, 1 hemizygous intermediate male, 5 homozygous deficient females, 4 heterozygous deficient females, 41 heterozygous intermediate females), 5 patients carried *G6PD Mahidol^{G487A}* (2 hemizygous deficient males, 1 hemizygous intermediate male, 2 heterozygous intermediate females), 7 patients carried *G6PD Union^{C1360T}* (1 hemizygous deficient males and 6 heterozygous intermediate females), 5 patients carried *G6PD Kaiping^{G1388A}* (4 hemizygous deficient males and 1 homozygous deficient females), 4 hemizygous deficient males carried *G6PD Canton^{G1376T}*, 2 patients carried *G6PD Chinese-5^{C1024T}* (1 hemizygous intermediate males and 1 homozygous deficient females), 2 hemizygous deficient males carried *G6PD Songklanaklarind^{T196A}*, 2 hemizygous deficient males carried *G6PD Chinese-4^{G392T}*, 1 hemizygous deficient males carried *G6PD Valladolid^{C406T}*, 1 hemizygous deficient male carried *G6PD Aures^{T143C}* (**Figures 12-19, Table 11**). We were unable to identify mutations in the *G6PD* gene by a direct sequencing method in the remaining 4 deficient patients. In the study population, the minor allele frequency (MAF) of *G6PD Viangchan^{G871A}, G6PD Mahidol^{G487A},* and *G6PD Union^{C1360T}* were 0.066, 0.005, and 0.005, respectively. MAF of other mutations including *G6PD Kaiping^{G1388A}, G6PD Canton^{G1376T}, G6PD Songklanagarind^{T196A}, G6PD Chinese-4^{G392T}, G6PD Chinese-5^{C1024T}, G6PD Valladolid^{C406T},* and *G6PD Aures^{T143C}* were 0.004, 0.003, 0.001, 0.001, 0.002, 0.001, and 0.001, respectively. The G6PD activities in each genotype of *G6PD* mutations found in male and female newborns were presented in **Table 12**.
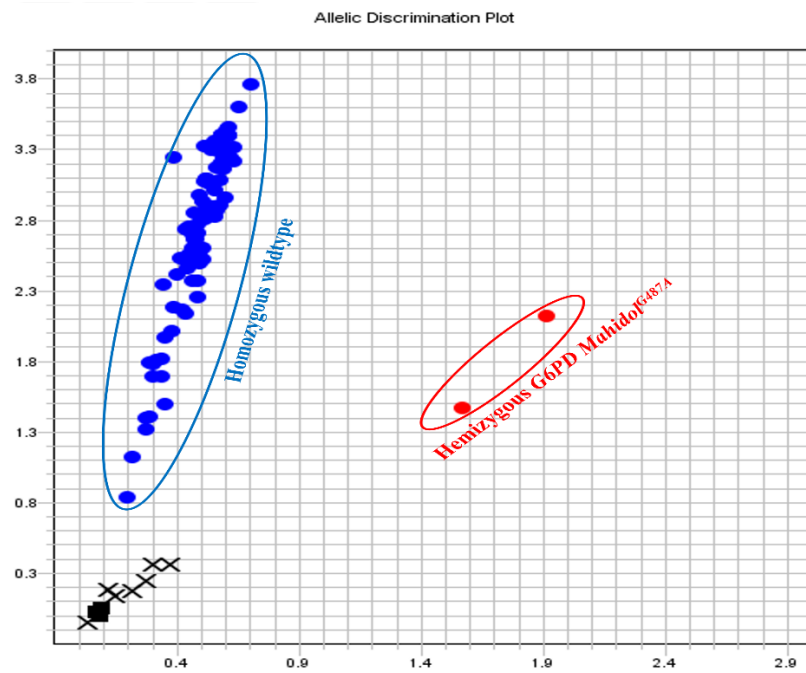
Figure 12 Allelic discrimination plots with the result of the genotyping for *G6PD Mahidol^{G487A}*, forming two clusters that represent the genotypes GG and AA, classified as homozygous wildtype and hemi/homozygous mutant, respectively.
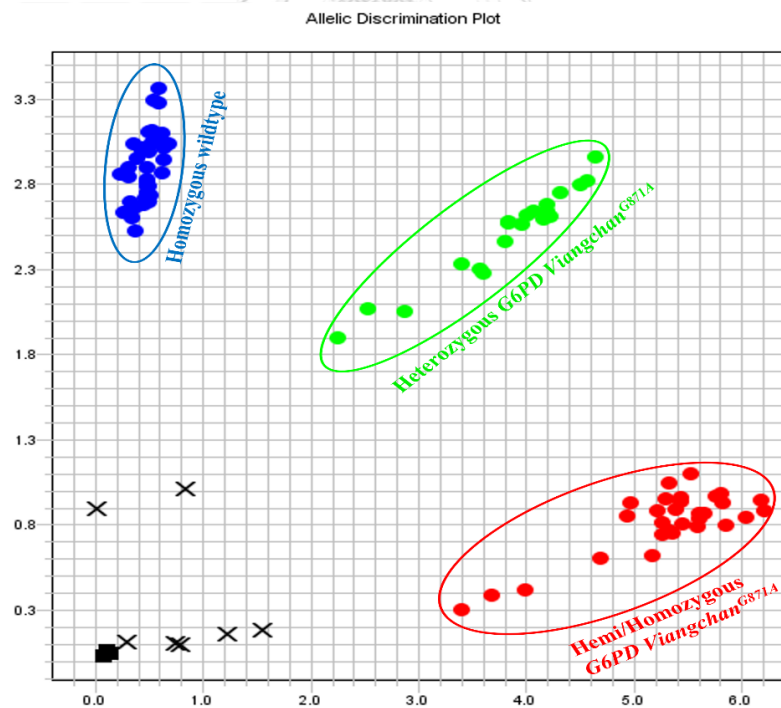
Figure 13 Allelic discrimination plots with the result of the genotyping for *G6PD Viangchan^{G871A}*, forming three clusters that represent the genotypes GG, GA, and AA, classified as homozygous wildtype, heterozygous, and hemi/homozygous mutant, respectively.
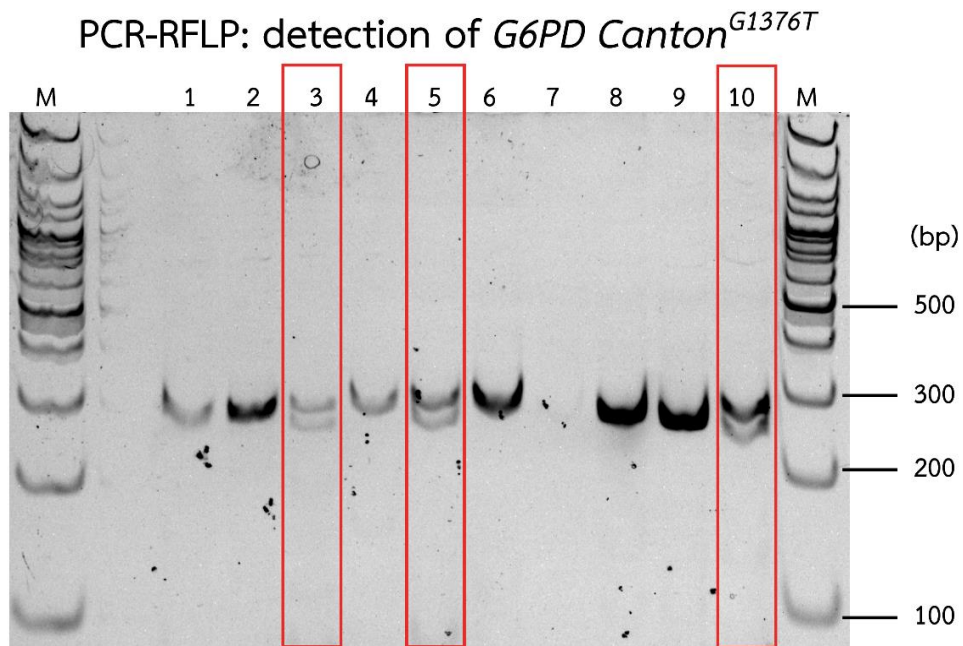
Figure 14 PCR-RFLP for *G6PD Canton*^{G1376T}. Lane M 100 bp ladder, lane 1 undigested *G6PD Canton*^{G1376T}, lane 2, 4, 6-9 digested normally showed a 214 bp band, lane 3, 5, 10 digested male hemizygous showed a 194 bp band.
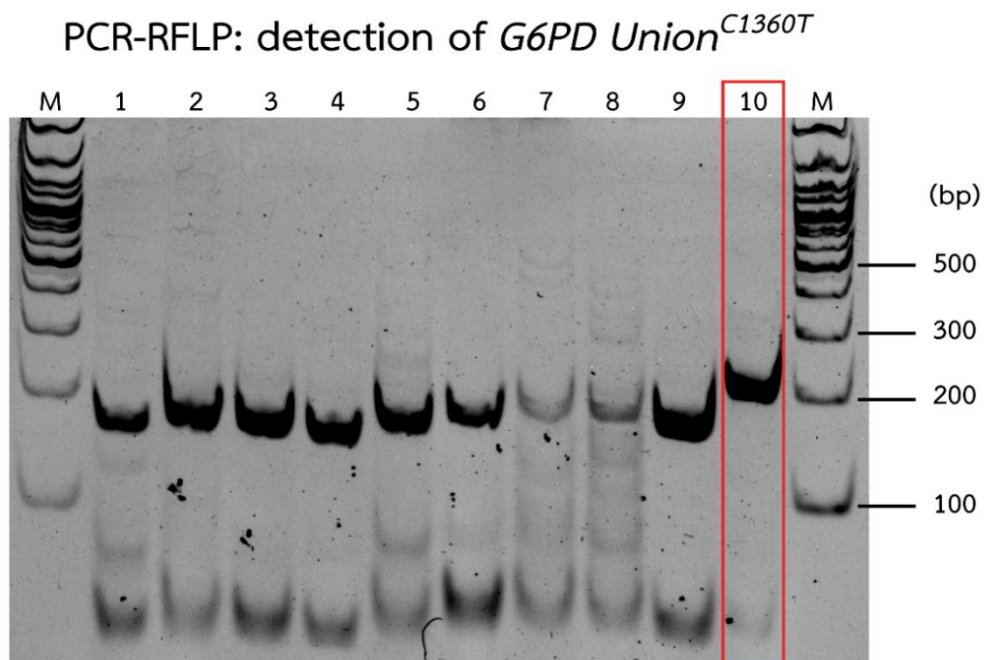
Figure 15 PCR-RFLP for *G6PD Union*^{C1360T}. Lane M 100 bp ladder, lane 1-9 digested normally showed a 142 bp band, lane ten digested male hemizygous showed a 187 bp band.
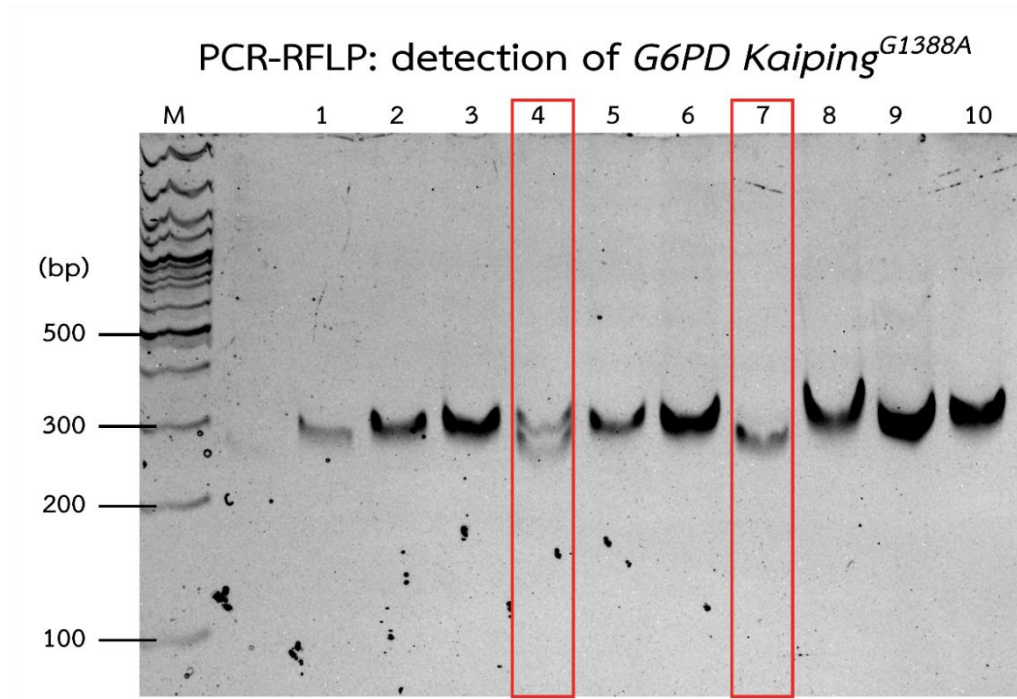
Figure 16 PCR-RFLP for *G6PD Kaiping*^{G1388A}. Lane M 100 bp ladder, lane 1 undigested *G6PD Kaiping*^{G1388A}, lane 2-3, 5-6, and 8-10 digested normal showed a 227 bp band, lane 4 and 7 digested male hemizygous showed a 206 bp band.
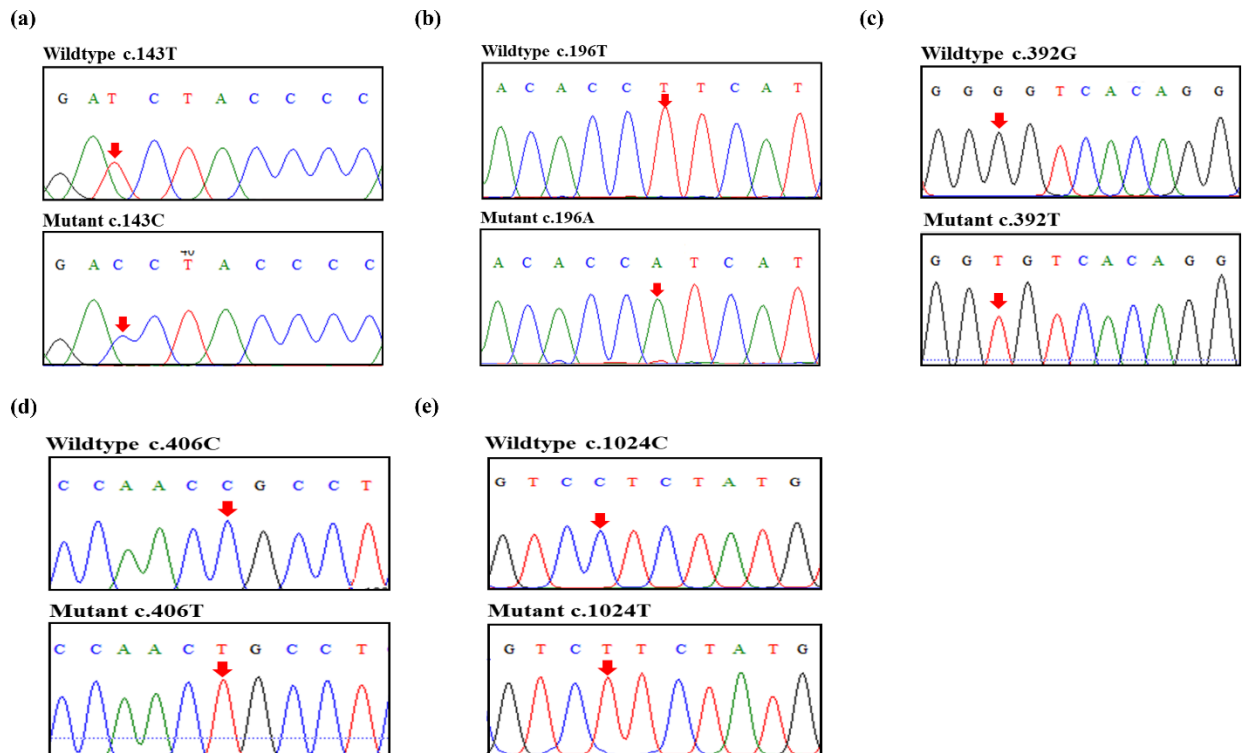
Figure 17 Sequencing results of *G6PD* mutations found in the study population.
(a) Hemizygous *G6PD Aures^{T143C}*, **(b)** Hemizygous *G6PD Songklanagarind^{T196A}*, **(c)** Hemizygous *G6PD Chinese-4^{G392T}*, **(d)** Hemizygous *G6PD Valladolid^{C406T}*, **(e)** Hemizygous *G6PD Chinese-5^{C1024T}*. The red arrows showed nucleotide substitution site.
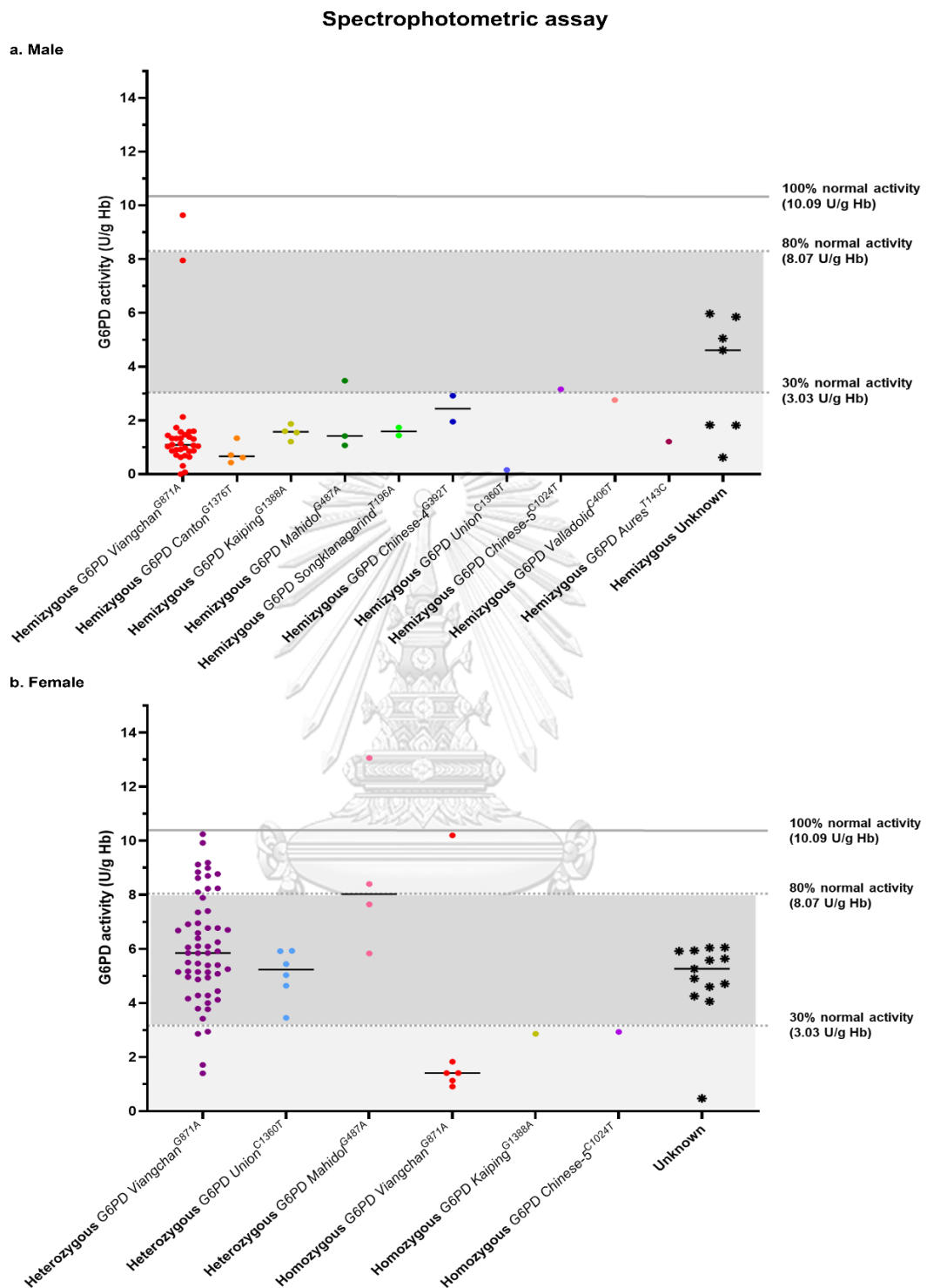
Figure 18 Distribution of *G6PD* mutations activities determined by spectrophotometric assay in **(a)** male and **(b)** female patients.

Figure 19 Distribution of *G6PD* mutations activities determined by automated UV enzymatic assay in **(a)** male and **(b)** female patients.
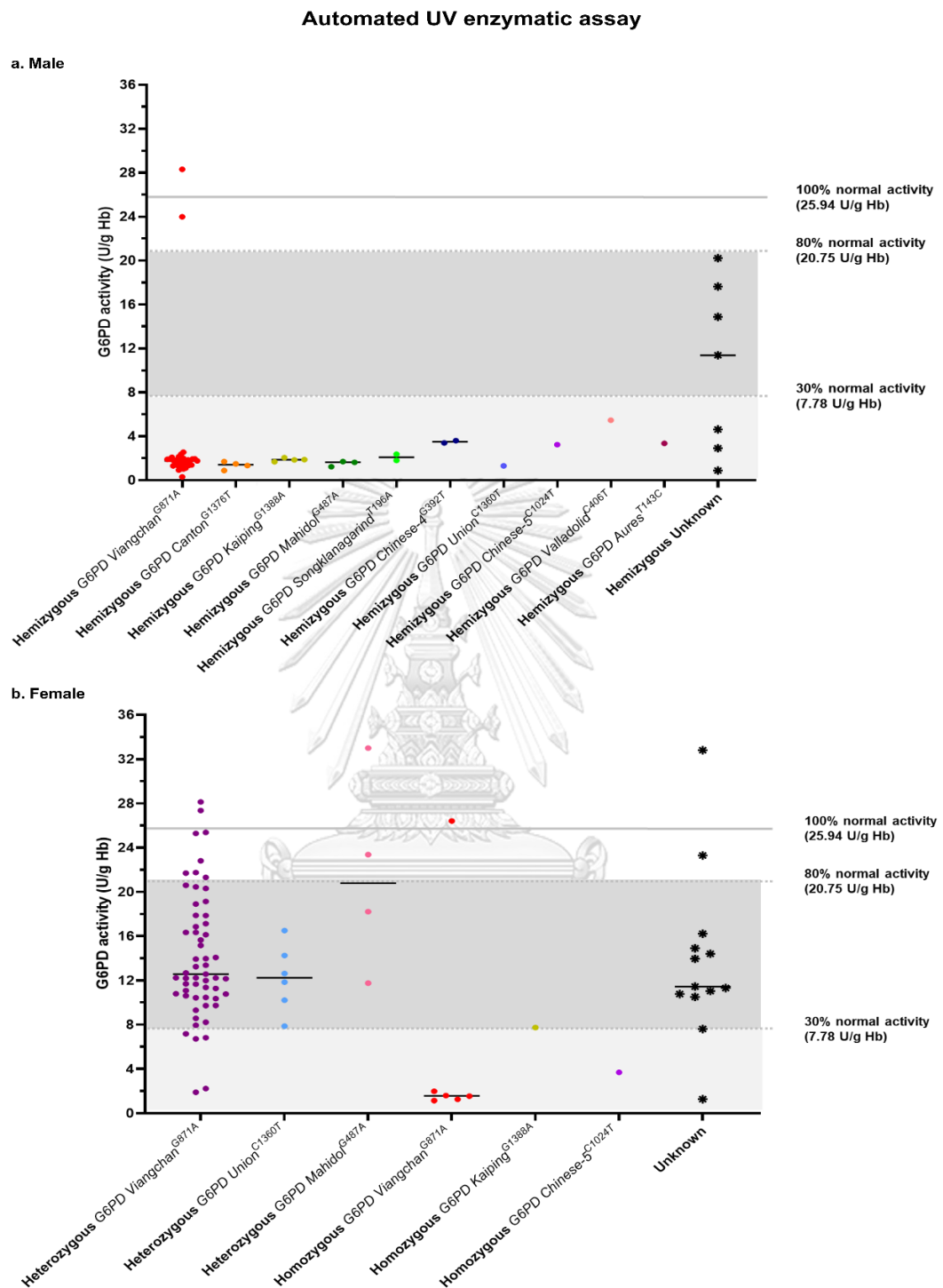
Table 11 Prevalence and frequency of *G6PD* mutations characterize based on G6PD activity determined by spectrophotometric assay.

Mutation	Normal (n)			Intermediate (n)			Deficiency (n)		
	Male	Female		Male	Female		Male	Female	
	Hemizygous	Homozygous	Heterozygous	Hemizygous	Homozygous	heterozygous	Hemizygous	homozygous	heterozygous
G6PD Viangchan^{G871A}	1	1	12	1	0	41	30	5	4
G6PD Canton^{G1376T}	0	0	0	0	0	0	4	0	0
G6PD Kaiping^{G1388A}	0	0	0	0	0	0	4	1	0
G6PD Mahidol^{G487A}	0	0	2	1	0	2	2	0	0
G6PD Songklanagarind^{T196A}	0	0	0	0	0	0	2	0	0
G6PD Chinese-4^{G392T}	0	0	0	0	0	0	2	0	0
G6PD Union^{C1360T}	0	0	0	0	0	6	1	0	0
G6PD Chinese-5^{C1024T}	0	0	0	1	0	0	0	1	0
G6PD Valladolid^{C406T}	0	0	0	0	0	0	1	0	0
G6PD Aures^{T143C}	0	0	0	0	0	0	1	0	0
Unknown	0	0	0	4	0	12	3	0	1

Table 12 G6PD activity and all genotypes of subjects enrolled in the study.

Gender	Genotype (n)	Spectrophotometric assay (U/g Hb) (Range)	Automated UV enzymatic assay (U/g Hb) (Range)
Male	Hemizygous *G6PD Viangchan*^{G871A} (32)	1.08±0.61 (0.00-9.64)	1.76±0.60 (0.30-28.31)
	Hemizygous *G6PD Canton*^{G1376T} (4)	0.67±0.70 (0.43-1.34)	1.42±0.65 (0.89-1.70)
	Hemizygous *G6PD Kaiping*^{G1388A} (4)	1.58±0.51 (1.21-1.87)	1.87±0.29 (1.69-2.06)
	Hemizygous *G6PD Mahidol*^{G487A} (3)	1.42 (1.07-3.48)	1.64 (1.24-1.70)
	Hemizygous *G6PD Songklanagarind*^{T196A} (2)	1.59 (1.44-1.74)	2.09 (1.80-2.38)
	Hemizygous *G6PD Chinese-4*^{G392T} (2)	2.44 (1.95-2.92)	3.50 (3.40-3.61)
	Hemizygous *G6PD Union*^{C1360T} (1)	0.16	1.31
	Hemizygous *G6PD Chinese-5*^{C1024T} (1)	3.16	3.24
	Hemizygous *G6PD Valladolid*^{C406T} (1)	2.76	5.47
	Hemizygous *G6PD Aures*^{T143C} (1)	1.21	3.36
	Hemizygous Unknown (7)	4.61±4.03 (0.62-5.97)	11.38±14.71 (0.89-20.22)
Female	Heterozygous *G6PD Viangchan*^{G871A} (57)	5.85±2.47 (1.40-10.24)	12.57±7.33 (1.89-28.13)
	Heterozygous *G6PD Union*^{C1360T} (6)	5.23±1.58 (3.45-5.93)	12.23±5.19 (7.88-16.51)
	Heterozygous *G6PD Mahidol*^{G487A} (4)	8.03±5.61 (5.83-13.06)	20.78±17.21 (11.76-32.99)
	Homozygous *G6PD Viangchan*^{G871A} (6)	1.41±2.85 (0.91-10.20)	1.58±6.86 (1.15-26.41)
	Homozygous *G6PD Kaiping*^{G1388A} (1)	2.86	7.75
	Homozygous *G6PD Chinese-5*^{C1024T} (1)	2.93	3.7
	Unknown (13)	5.26±1.50 (0.47-6.05)	11.45±4.92 (1.27-32.80)

4.4. The hematological characteristics and G6PD activity

The clinical data of newborn infants were presented in **Table 13.** The mean red blood cell (RBC) counts in G6PD normal, intermediate and deficiency groups were 4.93±0.64, 5.12±0.62, and 4.62±0.52 $\times 10^6$/μl, respectively, with a statistically significant difference in groups of G6PD intermediate and G6PD deficiency in comparing with G6PD normal group ($p<0.05$ and $p<0.001$, respectively). Male patients with G6PD deficiency had lower mean RBC counts than male patients with normal G6PD (4.63±0.53 and 4.93±0.63 $\times 10^6$/μl, respectively, $p<0.05$), whereas mean RBC counts in male patients with G6PD intermediate were higher than in those with normal G6PD (5.38±0.77 and 4.93±0.63 $\times 10^6$/μl, respectively, $p<0.05$). The mean hemoglobin (Hb) levels in G6PD normal, intermediate and deficiency groups were 17.48±2.03, 18.47±2.20, and 16.78±1.84 g/dl, respectively, with a statistically significant difference in groups of G6PD intermediate and G6PD deficiency in comparing with G6PD normal ($p<0.001$ and $p<0.05$, respectively). Male patients with G6PD deficiency had lower mean Hb levels (16.85±1.89 g/dl) than patients with normal G6PD (17.51±2.08 g/dl, $p<0.05$). In the other hand, male patients with G6PD intermediate had higher mean Hb levels (19.57±2.57 g/dl) than patients with normal G6PD (17.51±2.08 g/dl, $p<0.05$). The mean hematocrit in G6PD normal, intermediate, and deficiency groups were 49.98±5.89, 52.92±6.45, and 48.06±5.19%, respectively, with a statistically significant difference in G6PD intermediate and G6PD deficiency in comparing with G6PD normal ($p<0.001$ and $p<0.05$, respectively). Male patient with G6PD intermediate had higher mean hematocrit levels than male patients with G6PD normal (55.88±7.57 and 49.92±6.06%, respectively, $p<0.05$). Moreover, mean hematocrit in female patients with G6PD intermediate were higher than in those with normal G6PD (51.94±5.75 and 50.04±5.71%, respectively, $p<0.05$). The mean corpuscular volume (MCV) in G6PD normal, intermediate and deficiency groups were 101.80±6.11, 103.49±5.13 and 104.15±5.88 fl, respectively, with a statistically significant difference in groups of G6PD intermediate and G6PD deficiency in comparing with G6PD normal ($p<0.05$). Male patients with G6PD intermediate and G6PD deficiency, compared to male patients with G6PD normal, had a higher mean MCV (104.17±3.70, 104.63±5.68, and 101.39±5.41 fl, respectively, $p<0.05$). The mean

corpuscular hemoglobin (MCH) in G6PD normal, intermediate and deficiency groups were 35.61±2.42, 36.10±1.92 and 36.38±2.39 pg, respectively, with a statistically significant difference between G6PD intermediate and G6PD deficiency when compared to G6PD normal ($p<0.05$). Male patients with G6PD intermediate and G6PD deficiency, compared to G6PD normal, had a higher mean MCH (36.23±1.34, 36.50±2.34 and 35.58±2.17 pg, respectively, $p<0.05$). Besides, the mean of mean corpuscular hemoglobin concentration (MCHC) in G6PD normal, intermediate and deficiency groups were 34.97±0.73, 34.88±0.73 and 34.92±0.65 g/dl, respectively, with no significant differences were observed between G6PD status. But in male patients with G6PD intermediate and G6PD deficiency, compared to male patients with G6PD normal, had a lower mean MCHC (34.79±1.00 and 34.87±0.67 g/dl, respectively, $p<0.05$).
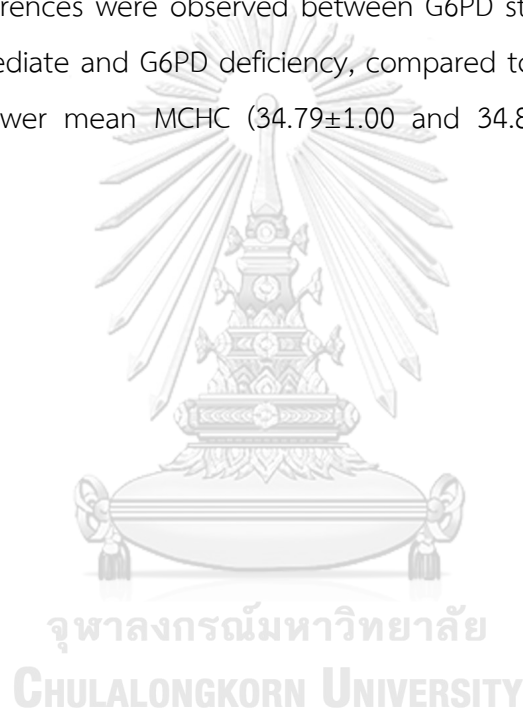
Table 13 Hematological characteristics and G6PD activity of G6PD normal, intermediate and deficiency newborns in the study.

Clinical Parameter	G6PD Normal			G6PD Intermediate			G6PD Deficiency		
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Number	837	423	414	116	29	87	62	50	12
RBC counts (X10^6/µl)	4.93±0.64	4.93±0.63	4.92±0.64	5.12±0.62[b]	5.38±0.77[c]	5.04±0.54	4.62±0.52[a]	4.63±0.53[c]	4.62±0.47
Hb (g/dl)	17.48±2.03	17.51±2.08	17.44±1.97	18.47±2.20[a]	19.57±2.57[c]	18.10±1.94[d]	16.78±1.84[b]	16.85±1.89[c]	16.51±1.68
WBC counts (X10^3/µl)	11.18±3.28	10.59±2.95	11.80±3.48	11.89±3.50	10.57±3.85	12.28±3.32	11.57±3.52	11.46±3.56	11.99±3.50
Platelet (X10^3/µl)	282.38±83.76	274.22±83.50	290.72±83.31	275.79±91.56	243.79±100.93	286.46±86.21	293.52±82.56	289.62±83.14	309.75±81.57
Hematocrit (%)	49.98±5.89	49.92±6.06	50.04±5.71	52.92±6.45[b]	55.88±7.57[c]	51.94±5.75[d]	48.06±5.19[b]	48.30±5.30	47.04±4.78
MCV (fl)	101.80±6.11	101.39±5.41	102.21±6.72	103.49±5.13[b]	104.17±3.70[c]	103.26±5.52	104.15±5.88[b]	104.63±5.68[c]	102.17±6.53
MCH (pg)	35.61±2.42	35.58±2.17	35.64±2.65	36.10±1.92[b]	36.23±1.34[c]	36.06±2.08	36.38±2.39[b]	36.50±2.34[c]	35.87±2.62
MCHC (g/dl)	34.97±0.73	35.08±0.65	34.87±0.78	34.88±0.73	34.79±1.00[c]	34.92±0.62	34.92±0.65	34.87±0.67[c]	35.08±0.59

Values are expressed as mean ± SD.

[a] significant difference from normal group ($p<0.001$) (*t*-test).

[b] significant difference from normal group ($p<0.05$) (*t*-test).

[c] significant difference from male with G6PD normal ($p<0.05$) (*t*-test).

[d] significant difference from female with G6PD normal ($p<0.05$) (*t*-test).

4.5. The correlation between G6PD activity and reticulocyte count in the study population

Reticulocytosis (reticulocyte count greater than 4%) was observed in the study population. In spectrophotometric assay, the mean value of reticulocyte counts in the G6PD deficiency group (5.82±1.73%) was significantly higher than that of G6PD normal group (4.78±1.29%, $p<0.001$). Male and female newborns with G6PD deficiency had significantly higher mean reticulocyte counts than normal male and female newborns ($p<0.001$ and $p<0.05$, respectively) (**Table 14**). In automated UV enzymatic assay, the mean value of reticulocyte counts in the G6PD deficiency group (5.83±1.71%) was significantly higher than that of G6PD normal group (4.82±1.29%, $p<0.001$). The reticulocyte counts in male and female newborns with G6PD deficiency was significantly higher than of normal male ($p<0.001$) and female ($p<0.05$) newborns, respectively (**Table 14**). To predict the effect on reticulocyte counts, we used univariate and multivariate linear regression analysis. The univariate analysis revealed that the levels of G6PD deficiency (from both assays) ($p<0.001$), *G6PD Viangchan^{G871A}* ($p<0.001$), gender ($p<0.001$), neonatal anemia (Hb<15 g/dl) ($p<0.001$), ABO incompatibility ($p<0.001$), and postpartum age ($p<0.001$) were all significantly positively associated with reticulocyte counts (**Table 15**). After monitoring for thalassemia and hemoglobinopathies frequently observed in Thailand, the multivariate analysis demonstrated gender, G6PD deficiency, *G6PD Viangchan^{G871A}*, neonatal anemia, postpartum age, and ABO incompatibility were independently associated with increasing levels of reticulocyte counts (**Table 15**). The effect of reticulocytes on spectrophotometric assay and automated UV enzymatic assay was examined. G6PD activity by both assays was found to have a significant negative correlation with the percentage of reticulocytes in newborns with intermediate G6PD status (spectrophotometric assay; $r=-0.218$, $p=0.019$, automated UV enzymatic assay; $r=-0.296$, $p<0.001$) and a significant positive correlation in newborns with normal G6PD status (spectrophotometric assay; $r=0.239$, $p<0.001$, automated UV enzymatic assay; $r=0.327$, $p<0.001$) but not in G6PD deficient newborns (**Figure 20a-f**).

Table 14 Reticulocyte counts (%) in group of G6PD normal, intermediate, and deficiency newborn subjects in the study.

Parameter	G6PD Normal			G6PD Intermediate			G6PD Deficiency		
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Spectrophotometric assay (n)	837	423	414	116	29	87	62	50	12
Reticulocyte count (%)	4.78±1.29	4.56±1.20	5.01±1.33	5.03±1.43	4.31±1.69	5.27±1.25	5.82±1.73[a]	5.86±1.90[b]	5.65±0.69[c]
Automated UV enzymatic assay (n)	791	403	388	158	47	111	66	52	14
Reticulocyte count (%)	4.82±1.29	4.60±1.20	5.04±1.34	4.76±1.37	3.94±1.25[b]	5.10±1.28	5.83±1.71[a]	5.91±1.88[b]	5.52±0.75[c]

Values are expressed as mean ± SD.

[a] significant difference from normal group ($p<0.001$) (t-test).

[b] significant difference from male with G6PD normal ($p<0.001$) (t-test).

[c] significant difference from female with G6PD normal ($p<0.05$) (t-test).

Table 15 The univariate and multivariate linear regression analyses of G6PD status (deficiency/intermediate/normal), *G6PD Viangchan^G871A/G6PD* Wildtype, and reticulocyte counts adjusted by gender, hemoglobin levels, age (day), ABO incompatibility, thalassemia and hemoglobinopathies.

Analysis type	Variable	Reticulocyte counts (%)			
		B*	SE	Beta	*p*-value
Univariate					
	G6PD status (Spectrophotometric assay) (Deficiency/Intermediate/Normal)	-0.443	0.076	-0.18	<0.001
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.319	0.073	-0.136	<0.001
	G6PD Genotype (Wildtype/Mutations)	0.738	0.119	0.191	<0.001
	G6PD Genotype (Wildtype/*G6PD Viangchan^G871A*/Others)	0.537	0.082	0.201	<0.001
	Gender (Male/Female)	0.398	0.084	0.147	<0.001
	Hemoglobin (≥15/<15g/dl)	1.286	0.132	0.292	<0.001
	Thalassemia and hemoglobinopathies	-0.158	0.209	-0.024	0.450
	ABO incompatibility	1.956	0.498	0.162	<0.001
	Age (day)	-0.314	0.053	-0.184	<0.001
Multivariate					
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.316	0.093	-0.129	0.001
	Gender (Male/Female)	0.470	0.114	0.157	<0.001
	Hemoglobin (≥15/<15g/dl)	1.303	0.171	0.292	<0.001
	ABO incompatibility	1.225	0.466	0.102	0.009
	Age (day)	-0.339	0.064	-0.203	<0.001
	Constant	5.884	0.350		<0.001
Multivariate					
	***G6PD* Genotype (Wildtype/Mutations)**	0.593	0.157	0.144	<0.001
	Gender (Male/Female)	0.424	0.114	0.141	<0.001
	Hemoglobin (≥15/<15g/dl)	1.269	0.171	0.284	<0.001
	ABO incompatibility	1.275	0.465	0.106	0.006

Age (day)	-0.334	0.064	-0.200	<0.001
Constant	4.410	0.307		<0.001
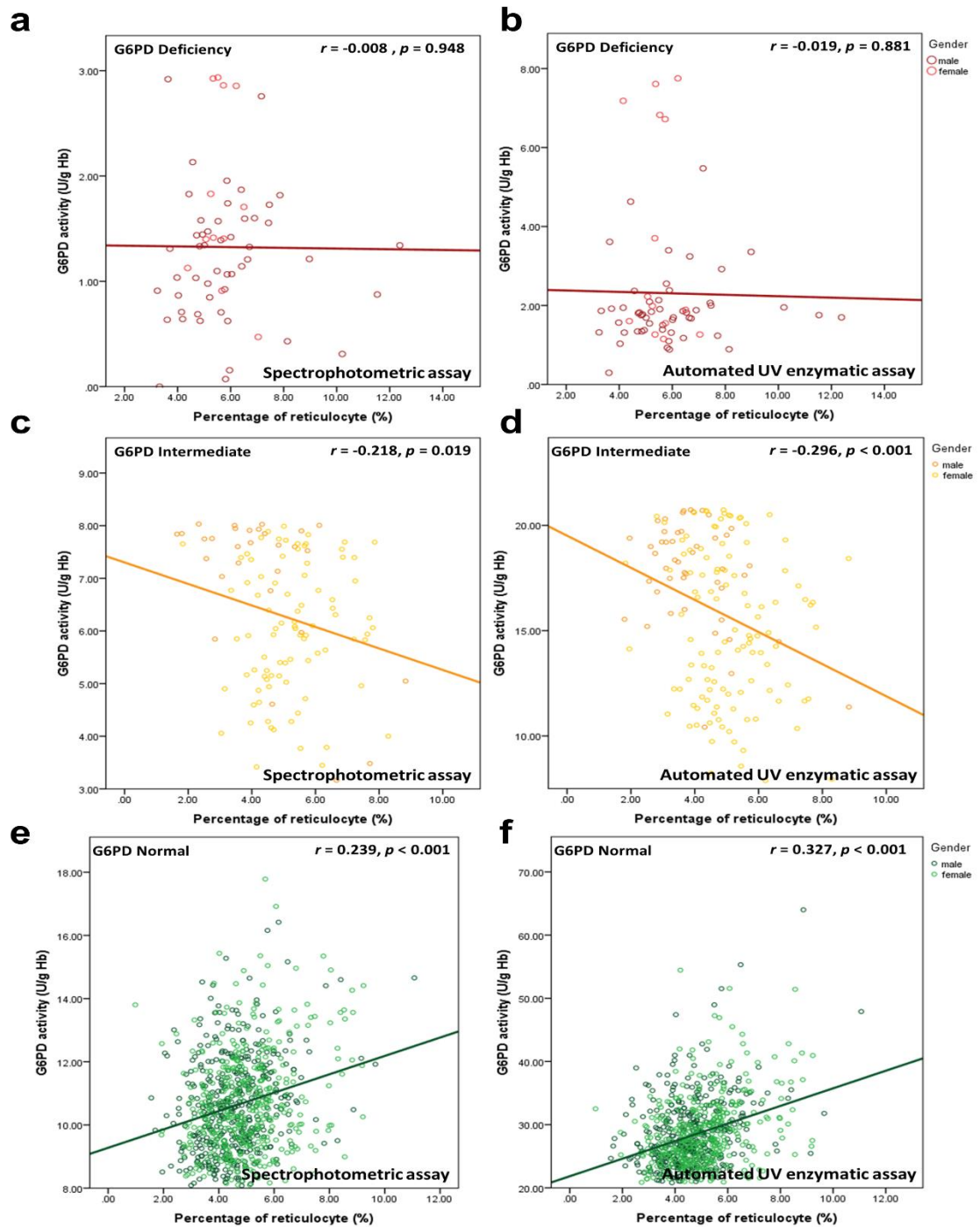
Figure 20 Correlation between G6PD activity and the percentage of reticulocytes in newborns with various G6PD status. G6PD activity and reticulocyte counts in newborns with **(a, b)** G6PD deficient, **(c, d)** G6PD intermediate, and **(e, f)** G6PD normal.

4.6. The correlation between G6PD activity and hyperbilirubinemia

The mean value of total serum bilirubin in all newborns with intermediate and deficient G6PD was significantly higher than that of normal G6PD newborns by both assays ($p<0.05$) (**Table 16**). The mean value of direct bilirubin in all newborns with deficient G6PD was significantly higher than that of normal G6PD newborns by both assays ($p<0.05$) (**Table 16**). The mean value of indirect bilirubin in all newborns with intermediate and deficient G6PD was significantly higher than that of normal G6PD newborns by both assays ($p<0.05$) (**Table 16**). The univariate analysis revealed that the level of total serum bilirubin was significantly positively associated with G6PD deficiency (from both assays) ($p<0.001$), *G6PD Vinagchan*[G871A] ($p<0.001$), neonatal anemia (Hb<15 g/dl) ($p=0.006$), and postpartum age ($p<0.001$) (**Table 17**). The multivariate analysis included all variables with $p<0.05$ observed in the univariate analysis and demonstrated that G6PD deficiency, *G6PD Vinagchan*[G871A], neonatal anemia, and postpartum age were independently associated with increasing levels of total serum bilirubin (**Table 17**). The univariate analysis revealed that the level of indirect bilirubin was significantly positively associated with G6PD deficiency (from both assays) ($p<0.001$), *G6PD Vinagchan*[G871A] ($p<0.001$), neonatal anemia (Hb<15 g/dl) ($p=0.008$), and postpartum age ($p<0.001$) (**Table 18**). The multivariate analysis included all variables with $p<0.05$ observed in the univariate analysis and demonstrated that G6PD deficiency, *G6PD Vinagchan*[G871A], neonatal anemia, and postpartum age were independently associated with increasing levels of total serum bilirubin (**Table 18**). **Table 19** was shown the number and percentages of the cases of hyperbilirubinemia in male and female newborns. The prevalence of G6PD deficiency in newborns with hyperbilirubinemia was 12.0% in males and 16.7% in females (**Table 19**).

Table 16 Total serum bilirubin, direct bilirubin, and indirect bilirubin in group of G6PD normal, intermediate, and deficiency newborn subjects in the study.

Parameter	G6PD Normal			G6PD Intermediate			G6PD Deficiency		
	Total	Male	Female	Total	Male	Female	Total	Male	Female
Spectrophotometric assay	837	423	414	116	29	87	62	50	12
Total serum bilirubin (mg/dl)	11.63±2.56	11.76±2.44	11.50±2.68	12.16±2.71[b]	11.86±3.09	12.26±2.58[d]	12.58±2.75[b]	12.53±2.62[c]	12.79±3.39
Direct bilirubin (mg/dl)	0.48±0.11	0.49±0.12	0.47±0.10	0.48±0.08	0.48±0.07	0.48±0.09	0.52±0.12[b]	0.52±0.12	0.51±0.11
Indirect bilirubin (mg/dl)	11.15±2.53	11.27±2.40	11.04±2.65	11.68±2.67[b]	11.38±3.06	11.78±2.54[d]	12.06±2.73[b]	12.01±2.59[c]	12.28±3.35
Automated UV enzymatic assay	791	403	388	158	47	111	66	52	14
Total serum bilirubin (mg/dl)	11.59±2.59	11.68±2.49	11.51±2.69	12.11±2.51[b]	12.36±2.23	12.01±2.62	12.74±2.73[b]	12.64±2.63[c]	13.11±3.14[d]
Direct bilirubin (mg/dl)	0.48±0.11	0.49±0.11	0.47±0.11	0.49±0.11	0.51±0.15	0.48±0.09	0.52±0.12[b]	0.52±0.12[c]	0.51±0.10
Indirect bilirubin (mg/dl)	11.12±2.55	11.19±2.46	11.04±2.65	11.62±2.47[b]	11.84±2.21	11.53±2.58	12.23±2.70[b]	12.12±2.61[c]	12.61±3.11[d]

Values are expressed as mean ± SD.

[a] significant difference from normal group ($p<0.001$) (*t*-test).

[b] significant difference from normal group ($p<0.05$) (*t*-test).

[c] significant difference from male with G6PD normal ($p<0.05$) (*t*-test).

[d] significant difference from female with G6PD normal ($p<0.05$) (*t*-test).

Table 17 The univariate and multivariate linear regression analyses of G6PD status (deficiency/intermediate/normal), *G6PD Viangchan^G871A/G6PD* Wildtype, and total serum bilirubin adjusted by gender, hemoglobin levels, postpartum age (day), ABO incompatibility, thalassemia and hemoglobinopathies.

Analysis type	Variable	Total serum bilirubin (mg/dl)			
		B*	SE	Beta	*p*-value
Univariate					
	G6PD status (Spectrophotometric assay) (Deficiency/Intermediate/Normal)	-0.489	0.148	-0.104	0.001
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.556	0.14	-0.123	<0.001
	G6PD Genotype (Wildtype/Mutations)	0.817	0.232	0.11	<0.001
	G6PD Genotype (Wildtype/*G6PD Viangchan^G871A*/Others)	0.652	0.160	0.127	<0.001
	Gender (Male/Female)	-0.177	0.163	-0.034	0.279
	Hemoglobin (≥15/<15g/dl)	-0.729	0.264	-0.086	0.006
	Thalassemia and hemoglobinopathies	-0.713	0.401	-0.056	0.075
	ABO incompatibility	0.474	0.942	0.021	0.615
	Age (day)	0.959	0.098	0.293	<0.001
Multivariate					
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.595	0.133	-0.132	<0.001
	Hemoglobin (≥15/<15g/dl)	-0.785	0.251	-0.093	0.002
	Age (day)	0.986	0.097	0.302	<0.001
	Constant	10.775	0.447		<0.001
Multivariate					
	G6PD Genotype (Wildtype/Mutations)	0.947	0.220	0.128	<0.001
	Hemoglobin (≥15/<15g/dl)	-0.847	0.252	-0.100	0.001
	Age (day)	0.991	0.097	0.303	<0.001
	Constant	8.072	0.380		<0.001

Table 18 The univariate and multivariate linear regression analyses of G6PD status (deficiency/intermediate/normal), *G6PD Viangchan*^{G871A}/*G6PD* Wildtype, and indirect bilirubin adjusted by gender, hemoglobin levels, postpartum age (day), ABO incompatibility, thalassemia and hemoglobinopathies.

Analysis type	Variable	Indirect bilirubin (mg/dl)			
		B*	SE	Beta	*p*-value
Univariate					
	G6PD status (Spectrophotometric assay) (Deficiency/Intermediate/Normal)	-0.475	0.146	-0.102	0.001
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.540	0.138	-0.122	<0.001
	G6PD Genotype (Wildtype/Mutations)	0.802	0.228	0.110	<0.001
	G6PD Genotype (Wildtype/*G6PD Viangchan*^{G871A}/Others)	0.639	0.158	0.126	<0.001
	Gender (Male/Female)	-0.155	0.161	-0.030	0.336
	Hemoglobin (≥15/<15g/dl)	-0.694	0.261	-0.083	0.008
	Thalassemia and hemoglobinopathies	-0.708	0.395	-0.056	0.073
	ABO incompatibility	0.417	0.928	0.019	0.654
	Age (day)	0.94	0.097	0.292	<0.001
Multivariate					
	G6PD status (Automated UV enzymatic assay) (Deficiency/Intermediate/Normal)	-0.578	0.132	-0.13	<0.001
	Hemoglobin (≥15/<15g/dl)	-0.75	0.248	-0.09	0.003
	Age (day)	0.966	0.096	0.30	<0.001
	Constant	10.298	0.441		<0.001
Multivariate					
	G6PD Genotype (Wildtype/Mutations)	0.929	0.217	0.127	<0.001
	Hemoglobin (≥15/<15g/dl)	-0.811	0.248	-0.097	0.001
	Age (day)	0.971	0.096	0.301	<0.001
	Constant	7.66	0.376		<0.001

Table 19 Prevalence of hyperbilirubinemia in male and female newborns with G6PD deficiency.

	No. of Newborn Subjects	Newborn with G6PD deficiency		G6PD deficiency newborn with Hyperbilirubinemia	
		No.	%	No.	%
Male	502	50	10.0	6	12.0
Female	513	12	2.3	2	16.7

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4.7. Performance of automated UV enzymatic assay for G6PD enzyme activity detection in newborns

G6PD activity levels as assessed by the automated UV enzymatic assay was highly correlated with that of spectrophotometric assay (r= 0.835, p<0.001) (**Figure 21a-c**). The proportion of newborn subjects with different G6PD status determined by spectrophotometric and automated UV enzymatic assays was illustrated in **Table 9**. The level of agreement (Cohen's Kappa) between two tests in detecting G6PD status in newborns was 0.651 (Kappa's SE = 0.043, 95% CI: 0.566 to 0.735), indicating a high level of agreement. The areas under the receiver operating characteristic (ROC) curve (AUC) in detecting G6PD deficient and G6PD intermediate newborns between the spectrophotometric and the automated UV enzymatic assays were 0.999 (95%CI: 0.997 to 1.000) (p<0.001) (**Figure 22a**) and 0.839 (95%CI: 0.805 to 0.874) (p<0.001) (**Figure 22b**). **Table 20** shows a summary of the performance of FST and automated UV enzymatic assay against the spectrophotometric assay in detecting G6PD deficient and G6PD intermediate in newborns, such as the sensitivity, specificity, positive predictive value (PPV), negative prediction value (NPV), positive likelihood ratio, negative likelihood ratio, and accuracy with 95%CI. FST had 87.1% sensitivity and 100.0% specificity for detecting G6PD deficiency. The automated UV enzymatic assay test was 98.4% sensitive and 99.5% specific when using a cut-off number of 7.8 U/g Hb to define overall G6PD deficiency status, but when using the number of 7.8-20.8 U/g Hb to determine G6PD intermediate sensitivity dropped to 69.0%, and specificity fell to 91.3% (**Table 20**). A previous study by Domingo Gj *et al.* (2019) (55) used a 10% prevalence of G6PD deficiency in males to predict the number of cases in males and females at different G6PD deficiency cut-off thresholds. Based on 10% G6PD deficiency prevalence found in our male newborn subjects, the proportion of total deficiency in females detected by both spectrophotometric and automated UV enzymatic assay at 30%, 40%, 60% and 80% threshold G6PD activity was shown in **Table 21**.
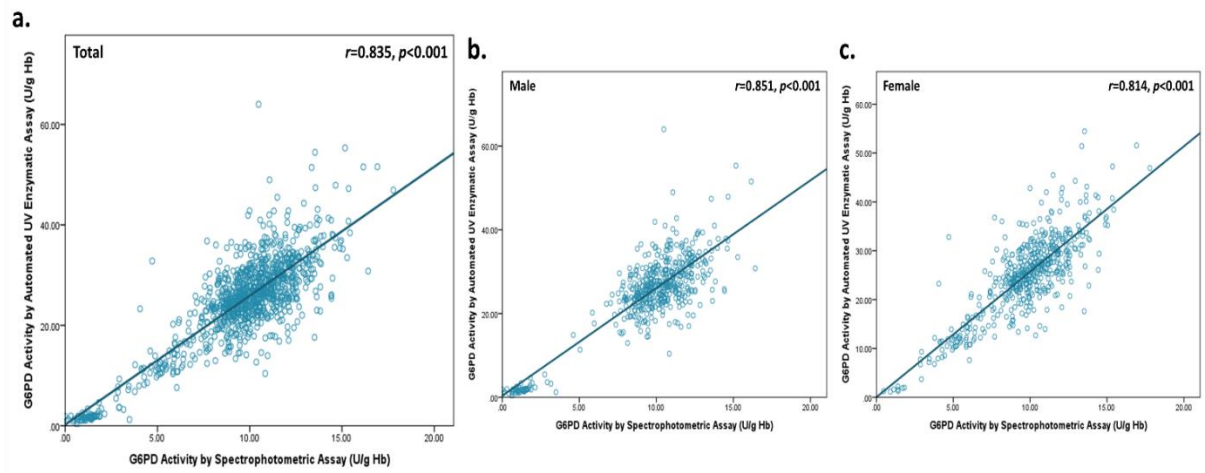
Figure 21 Correlation of G6PD activities measured by the spectrophotometric and the automated UV enzymatic assay.
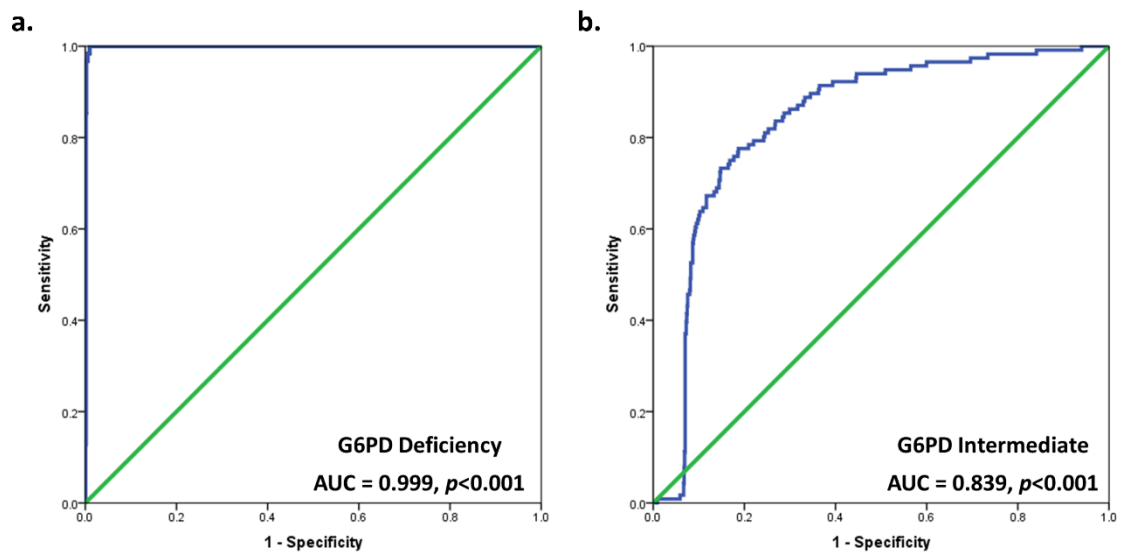
Figure 22 Receiver operating characteristic (ROC) analysis of the automated UV enzymatic method in detecting **(a)** G6PD deficient and **(b)** G6PD intermediate newborns.

Table 20 Assessing the accuracy of the two techniques, FST, and automated UV enzymatic assay, in detecting G6PD deficiency and G6PD intermediate newborns.

G6PD status	G6PD activity cut-off value (U/g Hb)	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)	Accuracy (%) (95% CI)
FST								
Deficiency	-	87.1 (76.2-94.3)	100.0 (99.6-100.0)	100.0	99.2 (98.4-99.6)	-	0.1 (0.1-0.3)	99.2 (98.5-99.7)
Automated UV enzymatic method								
Deficiency	<7.8	98.4 (91.3-100.0)	99.5 (98.8-99.8)	92.4 (83.6-96.7)	99.9 (99.3-100.0)	187.5 (78.2-449.8)	0.02 (0.0-0.1)	99.4 (98.7-99.8)
Intermediate	7.8-20.8	69.0 (59.7-77.2)	91.3 (89.3-93.1)	50.6 (44.5-56.7)	95.8 (94.6-96.8)	8.0 (6.2-10.2)	0.3 (0.3-0.5)	88.8 (86.7-90.7)

Table 21 The prevalence of G6PD deficiency in male and female newborn subjects, utilizing different deficiency thresholds.

Male G6PD deficiency prevalence		Threshold G6PD activity expressed as percent of normal													
At 10%		30%			40%			60%			70%			80%		
		M	F	T	M	F	T	M	F	T	M	F	T	M	F	T
Domingo GJ. *et al.*	No. def	500	124	624	500	237	737	517	656	1173	595	968	1563	864	1313	2177
	% Def	80	**20**	100	68	**32**	100	44	**56**	100	38	**62**	100	40	**60**	100
	% Pop	10.0	**2.5**	6.2	10.0	**4.7**	7.4	10.3	**13.1**	11.7	11.9	**19.4**	15.6	17.3	**26.3**	21.8
G6PD activity method																
Spectrophotometric assay (n)	No.	502	513	1015	502	513	1015	502	513	1015	502	513	1015	502	513	1015
	No. def	50	12	62	52	17	69	56	57	113	58	78	136	79	99	178
	% Def	80.6	**19.4**	100.0	75.4	**24.6**	100.0	49.6	**50.4**	100.0	42.6	**57.4**	100.0	44.4	**55.6**	100.0
	% Pop	10.0	**2.3**	6.1	10.4	**3.3**	6.8	11.2	**11.1**	11.1	11.6	**15.2**	13.4	15.7	**19.3**	17.5
Automated UV enzymatic assay (n)	No. def	52	14	66	52	23	75	61	74	135	75	97	172	99	125	224
	% Def	78.8	**21.2**	100.0	69.3	**30.7**	100.0	45.2	**54.8**	100.0	43.6	**56.4**	100.0	44.2	**55.8**	100.0
	% Pop	10.4	**2.7**	6.5	10.4	**4.5**	7.4	12.2	**14.4**	13.3	14.9	**18.9**	16.9	19.7	**24.4**	22.1

CHAPTER 5

Discussion

From a total of 1,015 newborn subjects in our study, there were no significant differences in mean gestational age and mean average age between male and female newborns because the inclusion criteria of the study in which all enrolled newborns were full-term and the standard operating procedure (SOP) that blood was drawn the day before going home, usually 2-3 days after deliver. However, the mean birth weight of male newborns was significantly higher than that of female newborns, which is consistent with previous research by Halileh et al., which found that males weight at birth more than female newborns (56).

Glucose 6-phosphate dehydrogenase is involved in the pentose phosphate pathway, generating NADPH, which maintains reduced glutathione to defends against oxidative damage in red blood cells (2). G6PD deficiency makes red corpuscles more vulnerable to oxidative stresses, causing hemolysis. G6PD deficiency is characterized by clinical, biochemical, and molecular heterogeneity. Prevalence varies between geographic regions and ethnic groups. For examples, the prevalence of G6PD-deficient males was 7.3% in Thailand, 8.1% in Lao PDR, 15.8% in Myanmar, 18.8% in Cambodia, and 8.9% in Vietnam (57). In this study, the frequency of G6PD deficiency was 10.0% in Thai male newborns and 2.3% in females using the WHO-recommended gold standard method. The incidence in male newborns was in agreed with the reported of Bancone et al. (57), but the incidence in female newborns was lower than that of reported by Nuchprayoon et al. (8). The WHO defines G6PD activity less than 30% as deficient, whereas G6PD activity between 30% and 80% as intermediate. Subjects with G6PD activity more than 80% is classified as normal (46). The distribution of G6PD activity values as measured by both spectrophotometric and automated UV enzymatic methods in male newborns was bimodal, clearly discriminating grouping between G6PD deficiency and G6PD normal. This might be affected by enzymatic deficient values of mutated hemizygous males. Whereas a normal distribution of female G6PD activity showing wide range of enzymatic activities resulted from lyonization in heterozygous females. G6PD activity

from an automated UV enzymatic assay was higher than that of the reference spectrophotometric assay. This might be due to sample preparation between both methods. Even though used the same Hb values to normalize G6PD activity, the spectrophotometric assay was performed on whole blood sample, but an automated UV enzymatic assay was performed on packed red cells. Because subjects of this study were neonates with reported a high reticulocyte count, the packed red cell contains a large amount of reticulocyte. Thus, we also found the G6PD activity values of the automated UV enzymatic assay was higher than in adults previously reported by Anantasomboon et al. (12). This finding supports by Riskin et al. (58) and Algur et al. (59), who reported that G6PD activity is higher in newborn blood than in adult blood.

According to the molecular analysis of the *G6PD* genotype, the most prevalent mutation in the study population was *G6PD Viangchan^{G871A}* (MAF = 0.066), which is consistent with other studies (8, 12). Additionally, *G6PD Mahidol^{G487A}, G6PD Union^{C1360T}, G6PD Kaiping^{G1388A}, G6PD Canton^{G1376T}, G6PD Chinese-5^{C1024T}, G6PD Songklanaklarind^{T196A}*, and *G6PD Chinese-4^{G392T}* frequent mutations in Southeast Asian and Chinese were found in the study population (60). Furthermore, we also detected *G6PD Valladolid^{C406T}* and *G6PD Aures^{T143C}* in our samples. *G6PD Valladolid^{C406T}* located in exon 5 and originally reported in Spain (60). This mutation has been reported to occur in Burmese and Thai subjects (12, 61). *G6PD Aures^{T143C}* located in exon 3 and originally reported in Algeria and Tunisia (60). This mutation has been proposed to be associated with G6PD deficiency in Thai subjects (12). The hemizygous males and homozygous females had G6PD enzyme activity value from severe deficiency to intermediate, whereas the heterozygous female had G6PD enzyme activity value varying from intermediate to normal. This might be explained by as the inheritance of G6PD is X-linked, homozygous females and hemizygous males fully express the deficiency phenotype, whereas heterozygous females present partial expression as a result of random X-inactivation. Our findings revealed some intermediate cases that no mutation could be identified by direct sequencing. This might possible that these cases may contain mutations in cis-acting regulatory sequences or in the noncoding region of the *G6PD* gene that may interfere with its

expression. Because many noncoding regions are involved in the regulation of gene activity, a mutation in this region can turn a gene on or off, resulting in the production or reduction of an important protein. Besides, Our findings showed that G6PD activity in each mutation was similar to G6PD activity in each genotype than in a group of G6PD activity classified by the World Health Organization.

Hematological parameters including hemoglobin (Hb), red blood cell (RBC) count, white blood cell (WBC) count, platelet (PLT), hematocrit (Hct), reticulocyte counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) are important diagnostic and prognostic clinical parameters for several human diseases. It has been reported that G6PD deficiency is associated with decreased RBC count, hemoglobin, and hematocrit (62), which was also found in our study (**Table 4.7**). Interestingly, the decrease of RBC count, hemoglobin, hematocrit and increase of MCV and MCH was found in our study which was agreed with that of reported by previous study (63). Thus, slightly increased MVC and MCH in G6PD deficient subjects could be explained by an increased number of reticulocytes, which is probable due to a slight increase in RBC elimination rate (63).

Reticulocytosis is a condition in which there is an increase in the amount of immature red blood cells. They are released into the circulation in response to compensate for the loss of mature red blood cells. It has been reported that the presence of a large number of reticulocytes may interfere with the diagnosis of G6PD deficiency since reticulocytes have more G6PD enzyme activity than mature red blood cells. Our findings showed that newborn subjects have a significantly higher reticulocyte count than adults, which was consistent with the findings of Jansen et al. (54), who discovered that the activities of several enzymes in neonatal red blood cells are significantly higher than in adults red blood cells. Moreover, the reticulocyte counts in newborns with G6PD deficiency was significantly higher than that of normal newborns both in males and females. This might be a result of the compensatory effect of hemolysis. However, our results demonstrated that the levels of G6PD activity in deficient newborns were not significantly correlated with the percentage of

reticulocytes. This might be explained by G6PD deficiency is a genetic disorder caused by a mutation in the *G6PD* gene affecting G6PD deficiency in all cell types, including reticulocytes. Although there are numerous reticulocytes in the circulation during the hemolytic crisis, their G6PD activity cannot increase the total blood G6PD activity to the normal level. The levels of G6PD activity in intermediate newborns were negatively correlated with the percentage of reticulocytes obtained from both assays. This might be explained by newborns with G6PD intermediate had a wide range of G6PD activity values. In the group with high enzyme activity, there was no production of reticulocyte to compensate hemolysis. Reticulocytes were produced to compensate for hemolysis in the low enzyme group. However, because it is a mutation in the *G6PD* gene, even if an increase in reticulocyte could not restore G6PD activity to normal levels. Nevertheless, G6PD activity was found to be positively correlated with the percentage of reticulocytes in normal newborns. This finding was consistent with a previous study by Kitcharoen et al. (13), who discovered that high reticulocytosis in neonatal blood samples did not result in a false negative diagnosis of G6PD deficiency. Hence, elevated levels of G6PD activity in reticulocytes only interfere with the detection of G6PD activity in normal blood samples. The multivariate showed that G6PD deficiency was associated with increasing levels of reticulocyte counts. This finding was in line with that of report by Gupte et al. (64). Besides, female also showed associated with increasing levels of reticulocyte. This finding was consistent with that of reported by Yamada et al. (65). Postpartum age was associated with increasing levels of reticulocyte count. This might be explained by a higher neonatal reticulocyte count may be one of the physiological responses to a more rapid environmental change during the early postnatal period (65) and the reticulocyte count value will subsequence decrease with the growing age of newborns (66).

G6PD deficiency is one of a major risk factor for the development of severe hyperbilirubinemia and increases the risk of bilirubin neurotoxicity. Our data revealed that the mean value of total serum bilirubin and indirect bilirubin in all newborns with intermediate and deficient G6PD was significantly higher than that of normal G6PD newborns. This might be indicated that higher level of total serum bilirubin

came from indirect bilirubin. Bilirubin is mainly formed by the breakdown of hemoglobin in red blood cells, which the liver enzyme glucoronyl transferase can be conjugated into a form that can be excreted from the body. The red blood cells of G6PD deficient newborns are more easily hemolysis than normal newborn, resulting in increasing bilirubin accumulation. Since the capacity of newborns to eliminate bilirubin is not well developed. Consequently, resulting in an imbalance between bilirubin production and excretion ability. It was led to hyperbilirubinemia in newborns. The multivariate showed that G6PD deficiency were associated with increases total serum bilirubin and indirect bilirubin. Our result was consistent with that of reported by Prachukthum et al. (10) that have demonstrated that the bilirubin levels in G6PD deficient newborns were significantly higher than that in G6PD normal newborns. And previous studies reported that 19.7% - 21.2% of hyperbilirubinemia were coincidently observed in G6PD deficient Thai newborns (8, 10). So, there may be a correlation between G6PD deficiency and neonatal hyperbilirubinemia. Besides, neonatal anemia also showed associated with increasing levels of total serum bilirubin and indirect bilirubin. This could be explained by increased hemolysis, which causes bilirubin levels to rise. Interestingly, our results showed that postpartum age were associated with increasing levels of total serum bilirubin and indirect bilirubin. This might be explained by newborns produce bilirubin at a rate of approximately 6 to 8 mg per kg per day. Moreover, bilirubin production will be typically declines to the adult level within 10 to 14 days after birth (67).

Our results revealed that the frequency of G6PD deficient males and females obtained using the FST and the automated UV enzymatic method were not different from those obtained using the reference spectrophotometric method, indicating the reliability of the automated method. Although the FST method is useful in field research, where large numbers of cases are required to be rapidly screened, it can identify a severe deficiency, discrimination intermediate levels in heterozygous females are more difficult. The quantitative automated UV enzymatic method is a new robotic spectrophotometric system that is capable of quantifying G6PD activity in 50 blood samples simultaneously. Nevertheless, the automated UV enzymatic methods interfered with reticulocytes-enriched packed red cells but also

demonstrated a strong positive correlation with the reference spectrophotometric method. The results from ROC analysis revealed that the optimal cut-off value of the automated UV enzymatic method for G6PD deficiency in newborns was less than 7.8 U/g Hb, and for those with intermediate levels of G6PD activity was of 7.8 to 20.8 U/g Hb. Based on a previous report Domingo GJ. *et al.* 2019 (55), the number of female newborn subjects in our study, which used a 30% cut-off to determine G6PD deficiency, was comparable to the estimation of the relative proportion of females that lie under any given threshold. The diagnostic performance of the automated UV enzymatic method was practically accurate for detecting G6PD deficiency, while the effectiveness of intermediate screening was suitable.

In conclusion, reticulocytosis, commonly observed in newborn, did not interfere with the diagnosis of G6PD deficiency and intermediate. However, G6PD activity in normal group was positively correlated with the percentage of reticulocyte count. This affects the activity of G6PD as measured by the automated UV enzymatic method. Furthermore, our results showed that G6PD deficiency were associated with increasing indirect bilirubin. Therefore, the accumulation of large amounts of indirect bilirubin can cause serious consequences effect on the newborn. However, the robotic quantitative method using reticulocytes-enriched packed red cells shows a strong diagnostic capability to identify G6PD deficiency and intermediate with high cut off value. Quantitative spectrophotometric assay is suitable to quantify newborn G6PD activity and detect G6PD deficiency in the prevention of hemolytic anemia of G6PD deficiency.

REFERENCES

1.   Beutler E. G6PD deficiency. Blood. 1994;84(11):3613-36.

2.   Cappellini MD, Fiorelli G. Glucose-6-phosphate dehydrogenase deficiency. Lancet. 2008;371(9606):64-74.

3.   Nkhoma ET, Poole C, Vannappagari V, Hall SA, Beutler E. The global prevalence of glucose-6-phosphate dehydrogenase deficiency: a systematic review and meta-analysis. Blood Cells Mol Dis. 2009;42(3):267-78.

4.   Thielemans L, Gornsawun G, Hanboonkunupakarn B, Paw MK, Porn P, Moo PK, et al. Diagnostic performances of the fluorescent spot test for G6PD deficiency in newborns along the Thailand-Myanmar border: A cohort study. Wellcome Open Res. 2018;3:1.

5.   Luzzatto L, Nannelli C, Notaro R. Glucose-6-Phosphate Dehydrogenase Deficiency. Hematol Oncol Clin North Am. 2016;30(2):373-93.

6.   Fu C, Luo S, Li Q, Xie B, Yang Q, Geng G, et al. Newborn screening of glucose-6-phosphate dehydrogenase deficiency in Guangxi, China: determination of optimal cutoff value to identify heterozygous female neonates. Sci Rep. 2018;8(1):833.

7.   Cunningham AD, Hwang S, Mochly-Rosen D. Glucose-6-Phosphate Dehydrogenase Deficiency and the Need for a Novel Treatment to Prevent Kernicterus. Clin Perinatol. 2016;43(2):341-54.

8.   Nuchprayoon I, Sanpavat S, Nuchprayoon S. Glucose-6-phosphate dehydrogenase (G6PD) mutations in Thailand: G6PD Viangchan (871G>A) is the most common deficiency variant in the Thai population. Hum Mutat. 2002;19(2):185.

9.   Charoenkwan P, Tantiprabha W, Sirichotiyakul S, Phusua A, Sanguansermsri T. Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficiency in northern Thailand. Southeast Asian J Trop Med Public Health. 2014;45(1):187-93.

10.   Prachukthum S, Nunnarumit P, Pienvichit P, Chuansumrit A, Songdej D, Kajanachumpol S, et al. Genetic polymorphisms in Thai neonates with hyperbilirubinemia. Acta Paediatr. 2009;98(7):1106-10.

11.   WHO Working Group. Glucose-6-phosphate dehydrogenase deficiency. Bull

World Health Organ. 1989;67(6):601-11.

12. Anantasomboon P, Chanda M, Jugnam-Ang W, Witoonpanich P, Cheepsunthorn P, Nuchprayoon I, et al. Evaluating the performance of automated UV enzymatic assay for screening of glucose 6-phosphate dehydrogenase deficiency. Int J Lab Hematol. 2019;41(2):192-9.

13. Kitcharoen S, Dechyotin S, Khemtonglang N, Kleesuk C. Relationship among glucose-6-phosphate dehydrogenase (G-6-PD) activity, G-6-PD variants and reticulocytosis in neonates of northeast Thailand. Clin Chim Acta. 2015;442:125-9.

14. Xavier R, V. C. M, T. M. SK, Cherian VJ. Reticulocytosis: the unevaluated parameter in neonatal jaundice requiring phototherapy. 2016. 2016;3(4):5.

15. Wisnumurti DA, Sribudiani Y, Porsch RM, Maskoen AM, Rahayuningsih SE, Asni EK, et al. G6PD genetic variations in neonatal Hyperbilirubinemia in Indonesian Deutromalay population. BMC Pediatr. 2019;19(1):506.

16. Luzzatto L. Glucose 6-phosphate dehydrogenase deficiency: from genotype to phenotype. Haematologica. 2006;91(10):1303-6.

17. Luzzatto L, Ally M, Notaro R. Glucose-6-phosphate dehydrogenase deficiency. Blood. 2020;136(11):1225-40.

18. Cohen P, Rosemeyer MA. Subunit interactions of glucose-6-phosphate dehydrogenase from human erythrocytes. Eur J Biochem. 1969;8(1):8-15.

19. Au SW, Gover S, Lam VM, Adams MJ. Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. Structure. 2000;8(3):293-303.

20. Au SW, Naylor CE, Gover S, Vandeputte-Rutten L, Scopes DA, Mason PJ, et al. Solution of the structure of tetrameric human glucose 6-phosphate dehydrogenase by molecular replacement. Acta Crystallogr D Biol Crystallogr. 1999;55(Pt 4):826-34.

21. Gómez-Manzo S, Marcial-Quino J, Ortega-Cuellar D, Serrano-Posada H, González-Valdez A, Vanoye-Carlo A, et al. Functional and Biochemical Analysis of Glucose-6-Phosphate Dehydrogenase (G6PD) Variants: Elucidating the Molecular Basis of G6PD Deficiency. Catalysts. 2017;7(5):135.

22. Kotaka M, Gover S, Vandeputte-Rutten L, Au SW, Lam VM, Adams MJ. Structural studies of glucose-6-phosphate and NADP+ binding to human glucose-6-phosphate

dehydrogenase. Acta Crystallogr D Biol Crystallogr. 2005;61(Pt 5):495-504.

23.   Mason PJ, Bautista JM, Gilsanz F. G6PD deficiency: the genotype-phenotype association. Blood Reviews. 2007;21(5):267-83.

24.   Peters AL, Van Noorden CJ. Glucose-6-phosphate dehydrogenase deficiency and malaria: cytochemical detection of heterozygous G6PD deficiency in women. J Histochem Cytochem. 2009;57(11):1003-11.

25.   Luzzatto L, Arese P. Favism and Glucose-6-Phosphate Dehydrogenase Deficiency. N Engl J Med. 2018;378(11):1068-9.

26.   Marks PA, Johnson AB, Hirschberg E. Effect of Age on the Enzyme Activity in Erythrocytes. Proc Natl Acad Sci U S A. 1958;44(6):529-36.

27.   Bonsignore A, Fornaini G, Fantoni A, Leoncini G, Segni P. RELATIONSHIP BETWEEN AGE AND ENZYMATIC ACTIVITIES IN HUMAN ERYTHROCYTES FROM NORMAL AND FAVA BEAN-SENSITIVE SUBJECTS. J Clin Invest. 1964;43(5):834-42.

28.   Ragupathi S, Priya A, Alagarsamy K, Kumar S, UmaMuralidharan, editors. Methemoglobinemia in a Child with Glucose-6- phosphate Dehydrogenase Deficiency-A Case Report2018.

29.   Humphreys C. Heinz bodies vs. Howell jolly bodies: Medicine Specifics; 2016 [Available from: https://medicinespecifics.com/heinz-bodies-vs-howell-jolly-bodies/.

30.   Gomez-Manzo S, Marcial-Quino J, Vanoye-Carlo A, Serrano-Posada H, Ortega-Cuellar D, Gonzalez-Valdez A, et al. Glucose-6-Phosphate Dehydrogenase: Update and Analysis of New Mutations around the World. Int J Mol Sci. 2016;17(12).

31.   Minucci A, Giardina B, Zuppi C, Capoluongo E. Glucose-6-phosphate dehydrogenase laboratory assay: How, when, and why? IUBMB Life. 2009;61(1):27-34.

32.   Phompradit P, Kuesap J, Chaijaroenkul W, Rueangweerayut R, Hongkaew Y, Yamnuan R, et al. Prevalence and distribution of glucose-6-phosphate dehydrogenase (G6PD) variants in Thai and Burmese populations in malaria endemic areas of Thailand. Malar J. 2011;10:368.

33.   Longo L, Vanegas OC, Patel M, Rosti V, Li H, Waka J, et al. Maternally transmitted severe glucose 6-phosphate dehydrogenase deficiency is an embryonic lethal. EMBO J. 2002;21(16):4229-39.

34.   Howes RE, Dewi M, Piel FB, Monteiro WM, Battle KE, Messina JP, et al. Spatial

distribution of G6PD deficiency variants across malaria-endemic regions. Malar J. 2013;12:418.

35.   Nantakomol D, Paul R, Palasuwan A, Day NP, White NJ, Imwong M. Evaluation of the phenotypic test and genetic analysis in the detection of glucose-6-phosphate dehydrogenase deficiency. Malar J. 2013;12:289.

36.   Boonyawat B, Phetthong T, Suksumek N, Traivaree C. Genotype-Phenotype Correlation of G6PD Mutations among Central Thai Children with G6PD Deficiency. Anemia. 2021;2021:6680925.

37.   White NJ, Qiao LG, Qi G, Luzzatto L. Rationale for recommending a lower dose of primaquine as a Plasmodium falciparum gametocytocide in populations where G6PD deficiency is common. Malaria Journal. 2012;11(1):418.

38.   Howes RE, Piel FB, Patil AP, Nyangiri OA, Gething PW, Dewi M, et al. G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. PLoS Med. 2012;9(11):e1001339.

39.   Kittisares K, Palasuwan D, Noulsri E, Palasuwan A. Thalassemia trait and G6PD deficiency in Thai blood donors. Transfus Apher Sci. 2019;58(2):201-6.

40.   Kaplan M, Hammerman C. Glucose-6-phosphate dehydrogenase deficiency and severe neonatal hyperbilirubinemia: a complexity of interactions between genes and environment. Semin Fetal Neonatal Med. 2010;15(3):148-56.

41.   Howes RE, Battle KE, Satyagraha AW, Baird JK, Hay SI. Chapter Four - G6PD Deficiency: Global Distribution, Genetic Variants and Primaquine Therapy. In: Hay SI, Price R, Baird JK, editors. Advances in Parasitology. 81: Academic Press; 2013. p. 133-201.

42.   Liu H, Liu W, Tang X, Wang T. Association between G6PD deficiency and hyperbilirubinemia in neonates: a meta-analysis. Pediatr Hematol Oncol. 2015;32(2):92-8.

43.   Keihanian F, Basirjafari S, Darbandi B, Saeidinia A, Jafroodi M, Sharafi R, et al. Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency in the neonatal period. Int J Lab Hematol. 2017;39(3):251-60.

44.   LaRue N, Kahn M, Murray M, Leader BT, Bansil P, McGray S, et al. Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency.

Am J Trop Med Hyg. 2014;91(4):854-61.

45.   Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser. 1967;366:1-53.

46.   WHO Working Group. Guide to G6PD deficiency rapid diagnostic testing to support P. vivax radical cure. Geneva: World Health Organization; 2018 2018.

47.   Zhang L, Yang Y, Liu R, Li Q, Yang F, Ma L, et al. A multiplex method for detection of glucose-6-phosphate dehydrogenase (G6PD) gene mutations. Int J Lab Hematol. 2015;37(6):739-45.

48.   Dhaliwal G, Cornett PA, Tierney LM, Jr. Hemolytic anemia. Am Fam Physician. 2004;69(11):2599-606.

49.   Hall V, Avulakunta ID. Hemolytic Diseases Of The Newborn. StatPearls. Treasure Island (FL)2022.

50.   Domingo GJ, Satyagraha AW, Anvikar A, Baird K, Bancone G, Bansil P, et al. G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests. Malar J. 2013;12:391.

51.   Bancone G, Kalnoky M, Chu CS, Chowwiwat N, Kahn M, Malleret B, et al. The G6PD flow-cytometric assay is a reliable tool for diagnosis of G6PD deficiency in women and anaemic subjects. Sci Rep. 2017;7(1):9822.

52.   Lakomek M, Schröter W, De Maeyer G, Winkler H. On the diagnosis of erythrocyte enzyme defects in the presence of high reticulocyte counts. Br J Haematol. 1989;72(3):445-51.

53.   Frank JE. Diagnosis and management of G6PD deficiency. Am Fam Physician. 2005;72(7):1277-82.

54.   Jansen G, Koenderman L, Rijksen G, Cats BP, Staal GE. Characteristics of hexokinase, pyruvate kinase, and glucose-6-phosphate dehydrogenase during adult and neonatal reticulocyte maturation. Am J Hematol. 1985;20(3):203-15.

55.   Domingo GJ, Advani N, Satyagraha AW, Sibley CH, Rowley E, Kalnoky M, et al. Addressing the gender-knowledge gap in glucose-6-phosphate dehydrogenase deficiency: challenges and opportunities. Int Health. 2019;11(1):7-14.

56.   Halileh S, Abu-Rmeileh N, Watt G, Spencer N, Gordon N. Determinants of

birthweight: gender based analysis. Matern Child Health J. 2008;12(5):606-12.

57.   Bancone G, Menard D, Khim N, Kim S, Canier L, Nguong C, et al. Molecular characterization and mapping of glucose-6-phosphate dehydrogenase (G6PD) mutations in the Greater Mekong Subregion. Malar J. 2019;18(1):20.

58.   Riskin A, Gery N, Kugelman A, Hemo M, Spevak I, Bader D. Glucose-6-Phosphate Dehydrogenase Deficiency and Borderline Deficiency: Association with Neonatal Hyperbilirubinemia. The Journal of Pediatrics. 2012;161(2):191-6.e1.

59.   Algur N, Avraham I, Hammerman C, Kaplan M. Quantitative neonatal glucose-6-phosphate dehydrogenase screening: distribution, reference values, and classification by phenotype. J Pediatr. 2012;161(2):197-200.

60.   Minucci A, Moradkhani K, Hwang MJ, Zuppi C, Giardina B, Capoluongo E. Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations. Blood Cells Mol Dis. 2012;48(3):154-65.

61.   Nuchprayoon I, Louicharoen C, Charoenvej W. Glucose-6-phosphate dehydrogenase mutations in Mon and Burmese of southern Myanmar. J Hum Genet. 2008;53(1):48-54.

62.   Lo KS, Wilson JG, Lange LA, Folsom AR, Galarneau G, Ganesh SK, et al. Genetic association analysis highlights new loci that modulate hematological trait variation in Caucasians and African Americans. Hum Genet. 2011;129(3):307-17.

63.   Motshoge T, Ababio G, Aleksenko L, Souda S, Muthoga CW, Mutukwa N, et al. Prevalence of G6PD deficiency and associated haematological parameters in children from Botswana. Infect Genet Evol. 2018;63:73-8.

64.   Gupte SC, Shaw A, Shah K. Hematological findings and severity of G6PD deficiency in Vataliya Prajapati subjects. JAPI. 2005;53.

65.   Yamada M, Chishiki M, Kanai Y, Goto A, Imamura T. Neonatal reticulocyte count during the early postnatal period. Pediatr Neonatol. 2020;61(5):490-7.

66.   Bukhari KT, Zafar H. Reference values of reticulocyte counts in five age groups of healthy infants at Rawalpindi, Pakistan. J Pak Med Assoc. 2013;63(9):1108-11.

67.   Porter ML, Dennis BL. Hyperbilirubinemia in the term newborn. Am Fam Physician. 2002;65(4):599-606.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Appendix

COA No. 1579/2021

IRB No. 744/64

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama 4 Road, Pathumwan, Bangkok 10330, Thailand, Tel 662-256-4493

Certificate of Expedited Review Approval

(COA No. 1579/2021)

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title	: Study of G6PD deficiency with clinical and hematological correlation in newborn infants
Study Code	: -
Principal Investigator	: Miss Thanaporn Pimpakan
Affiliation of PI	: M.Sc. Medical Sciences Program, Faculty of Medicine, Chulalongkorn University.
Review Method	: Expedited
Continuing Report	: At least once annually or submit the final report if finished.

Document Reviewed :

1. Research Proposal Version 2.0 Date 27/10/2021
2. Protocol Synopsis Version 2.0 Date 27 October 2021
3. Case Report Form Version 1.0 Date 27 October 2021
4. Budget Version 2.0 Date 27/10/2021
5. Poster Version 1.0 Date 21/07/2021

Approval granted is subject to the following conditions: (see back of this Certificate)

6. Curriculum Vitae and GCP Training

- Miss Thanaporn Pimpakan
- Asst.Prof. Chalisa Loucharoe Cheepsunthorn, M.D.

Signature ....   Signature ....

(Emeritus Professor Tada Sueblinwong MD)   (Associate Professor Supeecha Wittayalertpanya)

Chairperson   Member and Assistant Secretary, Acting Secretary

The Institutional Review Board   The Institutional Review Board

Date of Approval   : November 8, 2021

Approval Expire Date   : November 7, 2022

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;

2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;

3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;

4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;

5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.

6. If the research project is completed, the researcher must send closing/final report using the closing/final report form of the Faculty of Medicine, Chulalongkorn University.

* A list of the Institutional Review Board members (names, positions and expertises) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

COA No. 1579/2021

IRB No. 744/64

คณะกรรมการพิจารณาจริยธรรมการวิจัย

คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

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เอกสารรับรองการพิจารณาจริยธรรมแบบเร่งด่วน

(COA No. 1579/2021)

คณะกรรมการจริยธรรมการวิจัยในคน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ	: การศึกษาภาวะพร่องเอนไซม์ G6PD ที่มีความสัมพันธ์ทางคลินิกและไซต์ไทควิทยาใน ทารกแรกเกิด
เลขที่โครงการวิจัย	: -
ผู้วิจัยหลัก	: นางสาวธนพา พิมพการณ์
สังกัดหน่วยงาน	: ภ.บ.สหเวชวิทยาศาสตร์การแพทย์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
วิธีทบทวน	: แบบเร่งด่วน
รายงานความก้าวหน้า	: ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หาก ดำเนินโครงการเสร็จสิ้นก่อน 1 ปี

เอกสารรับรอง   :

1. โครงร่างการวิจัย Version 2.0 Date 27/10/2564

2. โครงการวิจัยฉบับย่อ Version 2.0 Date 27 October 2021

3. แบบฟอร์มเก็บบันทึกข้อมูล Version 1.0 Date 27 October 2021

4. งบประมาณ Version 2.0 Date 27/10/2564

5. Poster Version 1.0 Date 21/07/2021

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

6. Curriculum Vitae and GCP Training
   - Miss Thanaporn Pimpakan
   - Asst.Prof. Chalisa Louicharoe Cheepsunthorn, M.D.

ลงนาม ....
(ศาสตราจารย์โกติคุณแพทย์หญิงชาดา สืบหลินวงศ์)
ประธาน
คณะกรรมการพิจารณาจริยธรรมการวิจัย

ลงนาม ....
(รองศาสตราจารย์อุทิศา วิทยสิทธิญญา)
กรรมการและผู้ช่วยเลขานุการปฏิบัติหน้าที่แทนเลขานุการ
คณะกรรมการพิจารณาจริยธรรมการวิจัย

วันที่รับรอง   : 8 พฤศจิกายน 2564
วันหมดอายุ   : 7 พฤศจิกายน 2565

ทั้งนี้ การรับรองนี้เปลี่ยนไปตั้งที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารการรับรองโครงการวิจัย)

นักวิจัยทุกท่านที่ผ่านการรับรองจริยธรรมการวิจัยต้องปฏิบัติดังต่อไปนี้

1. ดำเนินการวิจัยตามที่ระบุไว้ในโครงร่างการวิจัยอย่างเคร่งครัด
2. ใช้เอกสารแนะนำอาสาสมัคร ใบยินยอม (และเอกสารเชิญเข้าร่วมวิจัยหรือใบโฆษณาถ้ามี) แบบสัมภาษณ์ และหรือ แบบสอบถาม เฉพาะที่มีตราประทับของคณะกรรมการพิจารณาจริยธรรมเท่านั้น และส่งสำเนา เอกสารดังกล่าวที่ใช้กับผู้เข้าร่วมวิจัยจริงรายงานมาที่ฝ่ายวิจัย คณะแพทยศาสตร์ เพื่อเก็บไว้เป็นหลักฐาน
3. รายงานเหตุการณ์ไม่พึงประสงค์ร้ายแรงที่เกิดขึ้นหรือการเปลี่ยนแปลงกิจกรรมวิจัยใดๆ ต่อคณะกรรมการ พิจารณาจริยธรรมการวิจัย ภายใน 5 วันทำการ
4. ส่งรายงานความก้าวหน้าต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ตามเวลาที่กำหนดหรือเมื่อได้รับการ ร้องขอ
5. หากการวิจัยไม่สามารถดำเนินการเสร็จสิ้นภายในกำหนด ผู้วิจัยต้องยื่นขออนุมัติใหม่ก่อน อย่างน้อย 1 เดือน
6. หากการวิจัยเสร็จสมบูรณ์ ผู้วิจัยต้องแจ้งปิดโครงการตามแบบฟอร์มของคณะแพทยศาสตร์ จุฬาลงกรณ์ มหาวิทยาลัย

* รายชื่อของคณะกรรมการจริยธรรมการวิจัยในคน (ชื่อและตำแหน่ง) ที่อยู่ในที่ประชุมวันที่รับรองโครงการวิจัย ได้แนบมาด้วย เอกสารที่รับรองทั้งหมดจะถูกส่งไปยังผู้วิจัยหลัก

VITA

NAME	Miss Thanaporn Pimpakan
DATE OF BIRTH	27 February 1995
PLACE OF BIRTH	Nakhon ratchasima province
INSTITUTIONS ATTENDED	2017 graduated with a Bachelor's degree in Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.
HOME ADDRESS	213 Moo 3 Pho Klang sub-district, Mueang Nakhon Ratchasima District, Nakhon Ratchasima 30000
PUBLICATION	Sankhom, R., Warrit, N., Pimpakan, T. and Wiwegweaw, A. Genetic Diversity of Captive Eastern Sarus Crane in Thailand Inferred from Mitochondrial Control Region Sequence and Microsatellite DNA Markers. Trop. Nat. His. 2018, 18, 84-96.
AWARD RECEIVED	1st round of Astrophysics camp 9th The Thai Astronomy Olympaid (TAO), Faculty of Science, Khon Kaen University, Khon Kean province, Thailand.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY