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Original Article

Prevalence and Molecular Characterization of Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency in Females from Previously Malaria Endemic Regions in Northeastern Thailand and Identification of a Novel G6PD Variant

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Abstract. ***Introduction*: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common X-linked enzymopathy, highly prevalent in the areas where malaria is or has been endemic. Prevalence of G6PD deficiency and characterization of G6PD variants in females from previously malaria-endemic areas of northeastern Thailand remain unstudied.**
***Methods*: Prevalence of G6PD deficiency was determined by a fluorescent spot test (FST), quantitative G6PD activity assay, and multiplex allele-specific (AS)- and restriction fragment length polymorphic (RFLP)-PCR developed for detection of common G6PD variants in the Thai population.**
***Results*: Prevalence of G6PD deficiency in female samples (n = 355) was 18% by FST, 29.6% by quantitation of G6PD activity, and 28.1% by PCR-based genotyping. The most common variant was G6PD Viangchan (54%), followed by G6PD Canton (11%) and G6PD Union (11%); in addition, a novel heterozygous variant, G6PD Khon Kaen (c.305T>C, p.F102S), was identified. The majority of heterozygotes expressed G6PD activity within the intermediate deficiency range (30-70% median of normal enzyme activity).**
***Conclusion*: High prevalence of G6PD deficiency was present in females from northeastern Thailand, the majority being due to heterozygosity of G6PD variants. The findings will have a bearing on the inclusion of primaquine in antimalarial-based policies for malaria elimination in populations with a high prevalence of G6PD deficiency.**

Keywords: Female, G6PD deficiency, Northeastern Thailand, Novel G6PD variant.

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Introduction. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common X-linked genetic enzyme disorder. It is recently estimated to affect over 500 million individuals worldwide.[1] G6PD deficiency

arises from mutations in *G6PD* resulting in low levels of enzyme activity, giving rise to a spectrum of disorders, ranging from neonatal hyperbilirubinemia to chronic non-spherocytic hemolytic anemia and acute hemolysis. However, most people with this condition are asymptomatic but can undergo hemolytic crisis when exposed to infection, certain types of food (e.g., fava beans), or oxidant drugs (e.g., 8-aminoquinoline antimalarials, primaquine, and tafenoquine).[1] The World Health Organization (WHO) recommends neonatal screening for G6PD deficiency in populations with a 3-5% prevalence of G6PD deficiency.[2] In malaria-endemic countries, including Thailand, the G6PD test is required before administering 8-aminoquinolines for the radical cure of *Plasmodium vivax* malaria to eliminate latent stages in the liver (hypnozoites).[3,4]

G6PD deficiency can be identified either by red blood cell (RBC) enzyme activity assay (qualitative or quantitative) or genotyping of variants.[5] Quantitative G6PD activity measurement (<30% normal activity) is sufficient to identify male G6PD deficient hemizygotes and female homozygotes;[3,5] however, for heterozygous females, a recent meta-analysis showed inter-study variations in enzyme assay at the 70% level precludes a universal diagnostic cutoff for G6PD deficiency.[6]

Female heterozygotes are also at risk of neonatal hyperbilirubinemia[7,8] and vulnerable to hemolysis triggered by 8-aminoquinoline antimalarials.[9,10] However, the severity of hemolysis depends on the dose of drugs and phenotypes stemming from G6PD variants as the diversity of G6PD variants leads to variations in residual enzyme activity and clinical severity.[11,12] In heterozygous females, there is substantial variability in the ratio of G6PD normal to G6PD-deficient RBCs generated by Lyonization, and thus quantitation of G6PD activity is superior to genotyping in predicting drug-induced hemolysis,[1,9,11]

Northeastern Thailand shares borders with PDR Laos and Cambodia. Although these regions were previously endemic for malaria, there is a looming risk of reemergence of this infection, particularly now that *P. falciparum* strains with reduced sensitivity to artemisinin, a first-line antimalarial, are prevalent in the Greater Mekong Subregion (GMS).[13,14] Moreover, the predominant malaria parasite in the GMS tends to shift from *P. falciparum* to *P. vivax*,[15] leading to an increased requirement for 8-aminoquinolines radical treatment. The prevalence of G6PD deficiency in northeastern Thailand has been reported to range from 8-24%.[16] However, there are a limited number of reports on the prevalence of G6PD deficiency in females in this region of the country; the majority of studies are focused on the prevalence of G6PD deficiency in hemizygotes.[16–20] A survey conducted in Burirum Provincial Hospital on G6PD deficiency prevalence using a quantitative G6PD activity assay of cord blood samples from male and female newborns reported prevalence of G6PD deficiency of 21.7 and 8%, respectively.[21]

Here, the prevalence of G6PD deficiency in women of reproductive age residing in northeastern Thailand was carried out by a fluorescent spot test (FST), measurement of G6PD activity, and genotyping of G6PD variants. The findings should contribute to determining the relationship between G6PD variants and enzyme deficiencies in this region of the country.

Materials and Methods.

Study population. Women of reproductive age (n = 355), 18-45 years of age, from northeastern Thailand who initially enrolled in a screening program for anemia between January and May 2017 were recruited.[22] All participants appeared physically healthy.

The research protocol was approved by the Institutional Review Board, Khon Kaen University, Thailand (HE602158). Every participant provided prior written consent.

Detection of G6PD deficiency. EDTA blood samples were transported in a cold box to the laboratory within two hours of collection, stored at 4-8°C and analyzed within one week of collection. Samples were initially screened for G6PD activity by FST. The FST reagent was prepared according to recommendations of the International Council (formerly called Committee) for Standardization in Haematology (ICSH)[23] and stored at -20°C until used. FST was performed by adding 5 μL aliquot of whole blood to 100 μL of test reagent and 10 μL aliquot of the mixture was immediately spotted onto Whatman No.1 filter paper while the remaining solution was incubated at room temperature. The second and third aliquot of the mixture was then placed onto the same filter paper next to previous spot of the same sample after 5 and 15 minutes of incubation, respectively. Filter papers were dried and examined visually under UV light (365 nm). A sample showing a moderate to strong fluorescence at 5 minutes and strong fluorescence at 15 minutes was classified as G6PD normal, showing weak fluorescence at 5 minutes and moderate fluorescence at 15 minutes as G6PD intermediate deficient and showing no or very faint fluorescence after 15 minutes as G6PD deficient. FST assays were conducted alongside in-house G6PD normal and intermediate G6PD deficient controls.

G6PD activity was quantified using a Trinity Biotech G6PD kit (Trinity Biotech, Bray, Ireland). In brief, 10 μL aliquot of blood sample was added to 90 μL of lysing reagent and mixed using a vortex mixer, and hemolysate then was placed into a clinical chemistry analyzer (BS-400; Mindray, Shenzhen, PR China) for measurement of G6PD activity and hemoglobin (Hb) concentration. G6PD activity assay was based on NADP reduction rate to NADPH catalyzed by G6PD as monitored at 37°C by an increase in $A_{340\ nm}$ measured at regular intervals from

3 to 8 minutes post-addition of reagent mixture. G6PD activity was calculated by multiplying the rate of increase in absorbance ($\Delta A_{340\ nm}$/min) by 4,754 and expressed as U/g Hb. Each assay was accompanied by parallel measurements of G6PD normal (11.3 ± 0.7 U/g Hb) and intermediate (6.0 ± 0.2 U/g Hb) controls, with an inter-assay coefficient of variation of 5.6 and 3.8%, respectively. Median G6PD activity of all individuals carrying wild-type *G6PD* is defined as normal G6PD activity, and G6PD status is classified as deficient, intermediate deficient, and normal if the measured activity is <30, 30-70, and >70% normal activity, respectively.

Genotyping of G6PD variants.

Multiplex allele-specific (MAS)-PCR. DNA was extracted from peripheral blood leukocytes using a DNAzol reagent kit (Invitrogen, Carlsbad, CA, USA); MAS-PCR was performed using primer sets specific for seven common G6PD variants in Southeast Asia; namely, primer set 1 for G6PD Canton (c.1376G>T, p.R459L), Union (c.1360C>T, p.R454C) and Viangchan (c.871G>A, p.V291M); primer set 2 for G6PD Kaiping (c.1388G>A, p.R463H) and Mahidol (c.487G>A, p.G163S); primer set 3 for G6PD Chinese-4 (c.392G>T, p.G131V); and primer set 4 for G6PD Chinese-5 (c.1024C>T, p.L342F) (**Table 1**). Locations of primers on the *G6PD* gene are shown in **Supplementary Figure S1**. Reaction mixture (50 μL) contained reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, and 0.001% gelatin), 200 mM dNTPs, 0.8 U *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA), primer set (**Table 1**), and 50 ng of extracted DNA. In each reaction mixture, primers GF1 and GR1 or GER4 (**Table 1**) generating an internal control amplicon (339 or 825 bp respectively) were also added. Thermocycling was carried out in a Biometra's T-personal Thermal Cycler (Biocompare, San Francisco, CA, USA) as follows: 94°C for 3 minutes; followed by 30 cycles of 94°C for 60 s and 65°C (primer set 1), 70°C (primer sets 2 and 4) or 71°C (primer set 3) for 90 s; and a final step of 16°C and maintained at this temperature until analyzed by 2.0% agarose gel-electrophoresis, staining with ethidium bromide and visualizing under UV light (**Supplementary Figure S2**). MAS-PCR set 1 was carried out on all samples, and negative samples were subsequently subjected to MAS-PCR assay using primer sets 2, 3 and 4. All samples positive for G6PD variants were confirmed by RFLP-PCR as previously described (data not shown).[24]

DNA sequencing. Samples classified by both FST and G6PD activity assay as G6PD intermediate deficient but negative for any of the seven variants by MAS-PCR as described above were then subjected to DNA sequencing. Touchdown PCR reaction was performed to amplify the 13 exons of *G6PD* as previously described.[25] Amplicons were purified using a GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., Taipei, Taiwan), directly

Table 1 Primers used for detection of *G-6-PD* normal and mutant alleles.

G-6-PD allele	Forward primer (5′→3′)	Amount (ng)	Reverse primer (5′→3′)	Amount (ng)	Amplicon size (bp)
Internal control	GF1: GGTGGTCCTGGAGGGTCCT	16	GR1: CATAGAGGACGACGGCTGCA	24	339
			GER4: GTGTCTTGCTGATGCCACTG	12	825
MAS-PCR set 1					
Viangchan	GF2M: CTTGGCTTTCTCTCAGGTCAAGA	24	GR1: CATAGAGGACGACGGCTGCA	24	182
	GF2N: TTGGCTTTCTCTCAGGTCAAGG	24			181
Union	GF3: ACGTGAAGCTCCCTGACGC	16	GR2M: AGCTGGGCCTCACCTGCA	20	89
			GR2N: AGCTGGGCCTCACCTGCG	8	89
Canton	GF3: ACGTGAAGCTCCCTGACGC	16	GR3M: GAAAATACGCCAGGCCTCAA	20	212
			GR3N: GAAAATACGCCAGGCCTCAC	8	212
MAS-PCR set 2					
Kaiping	GF3: ACGTGAAGCTCCCTGACGC	18	GR4M: TGCAGCAGTGGGGTGAAAATAT	18	226
			GR4N: GCAGCAGTGGGGTGAAAATAC	16	225
Mahidol	GF4: GCGTCTGAATGATGCAGCTCTGAT	12	GR5M: CACGATGATGCGGTTCCAGCT	12	101
			GR5N: ACGATGATGCGGTTCCAGCC	16	100
AS-PCR set 3					
Chinese-4	C4F: CACGGACTCAAAGAGAGGGGCTG	12	ASC4RM: AAGAGGCGGTTGGCCTGTGACA	12	206
			ASC4RN: AAGAGGCGGTTGGCCTGTGACC	16	206
AS-PCR set 4					
Chinese-5	CH5FM: ACTTTTGCAGCCGTCGTCT	16	GER4: GTGTCTTGCTGATGCCACTG	12	511
	CH5FN: ACTTTTGCAGCCGTCGTCC	16			511

M, primer specific for mutant allele; N, primer specific for normal allele.

Table 2. Phenotypic and genotypic classifications of glucose-6-phosphate dehydrogenase status in females of reproductive age from northeastern Thailand.

Quantitative test			Qualitative fluorescent spot test						Genotypic test					
	Number	%	Normal		Intermediate		Deficiency		Wild-type		Heterozygote		Homozygote	
			Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Normal (>70% normal activity)	250	70.4	247	69.6	3	0.8	0	0	228	64.2	22	6.2	0	0
Intermediate (30-70% normal activity)	89	25.1	44	12.4	45	12.7	0	0	27	7.6	62	17.5	0	0
Deficiency (<30% normal activity)	16	4.5	0	0	4	1.1	12	3.4	0	0	8	2.2	8*	2.2
Total	**355**	**100**	**291**	**82**	**52**	**14.6**	**12**	**3.4**	**255**	**71.8**	**92**	**25.9**	**8***	**2.2**

*Includes one case of compound heterozygous G6PD Viangchan and Canton.

sequenced (First BASE Lab., Selangor, Malaysia), and sequences analyzed using a Seq-scanner program (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis. Statistical analysis was carried out using a Minitab statistical software version 14 (Minitab Inc., State College, PA, USA). Descriptive statistics were applied to compare data and a GraphPad Prism6 (GraphPad Software, San Diego, CA, USA) was employed to generate a G6PD variant enzyme activity-distribution plot.

Results. The overall prevalence of G6PD deficiency determined by FST was 18%, with 3.4% classified as deficient and 14.6% intermediate deficient, whereas by G6PD activity assay, G6PD deficiency prevalence was 29.6%, with 4.5% classified as deficient and 25.1% intermediate deficient (**Table 2**). Genotypic tests identified 28.1% of women carrying G6PD variants, 2.2% homozygotes or compound heterozygotes, and 25.9% heterozygotes (**Table 2**).

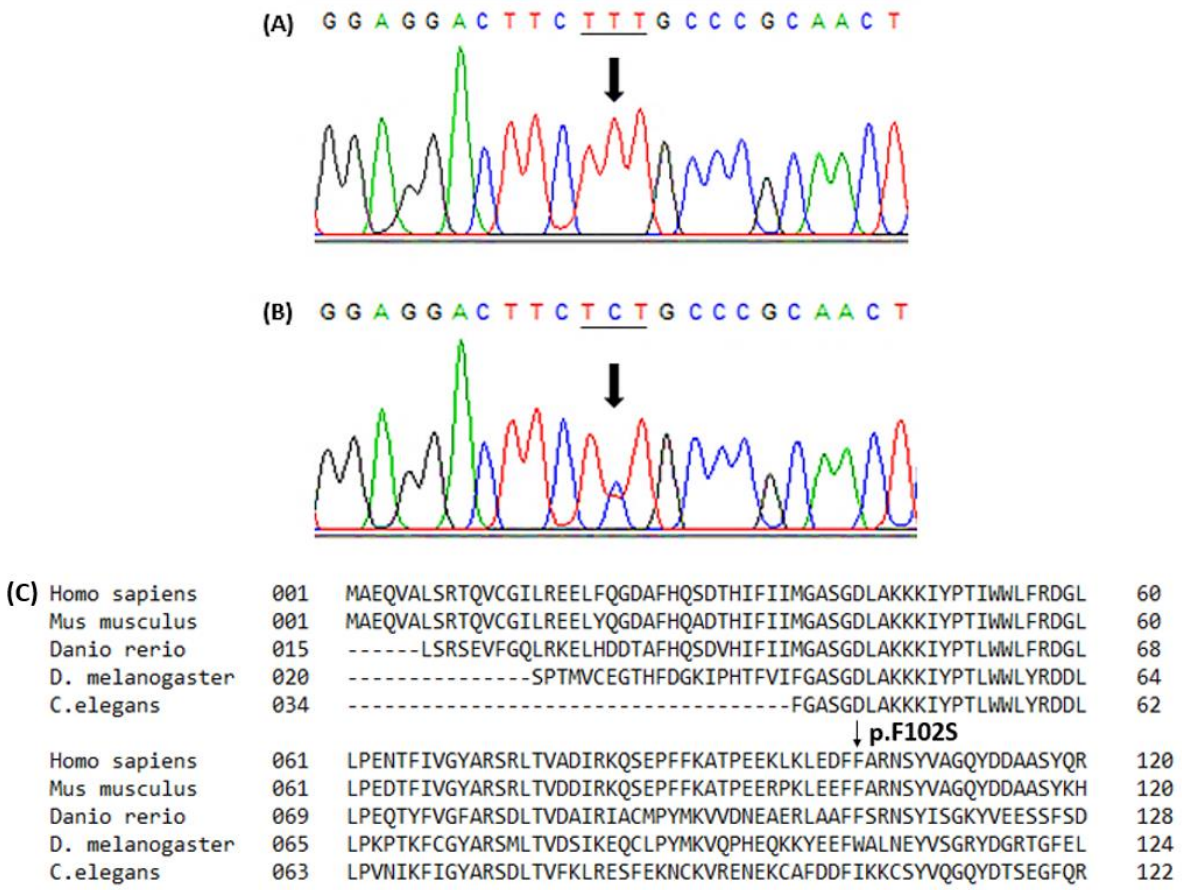

Figure 1 Sequence of a segment of heterozygous G6PD Khon Kaen exon 5. (A) Wild-type sequence. (B) Heterozygous G6PD Khon Kaen sequence (c.305T>C, p.F102S). Arrow indicates mutation site. (C) Similarity alignment of N-terminal region of G6PD across different species. Arrow indicates mutation F102S.

All homozygotes or compound heterozygotes were G6PD deficient (<30% normal activity), but heterozygotes expressed a wide range of RBC G6PD activity, 2.2% with enzyme activity in deficient range, 6.2% in normal (>70% normal activity) range, and 17.5% in intermediate (30-70% normal activity) range. The majority of individuals with wild-type *G6PD* alleles (64.2%) exhibited normal G6PD activity, although a minority (7.6%) expressed intermediate deficient activity.

Intermediate G6PD deficiency samples (n = 6), by both FST and enzyme activity assay, and negative for PCR detection of the seven G6PD variants were subjected to DNA sequencing, revealing two cases of heterozygous G6PD Coimbra (c.592C>T, p.R198C), one case of heterozygous G6PD Gaohe (c.95A>G, p.H32R), one case of homozygous silent mutation (c.1311C>T) and two cases of a heterozygous novel mutation (c.305T>C, p.F102S), named G6PD Khon Kaen (GenBank accession no. MN316615). The moderately conserved residue F102 is located next to the highly conserved residue F101 (**Figure 1**).

The most common G6PD variant was Viangchan (54%), followed by Canton (11%) and Union (11%). A case of Viangchan/Canton compound heterozygote was also found. G6PD activity distribution demonstrated overlap of activities among 92 heterozygotes and wild-type individuals (**Figure 2**). It is worth noting that all non-common G6PD variants in Thailand (G6PD Coimbra, Gaohe, and Khon Kaen) had low intermediate or deficient enzyme activity (**Figure 2**).

Discussion. G6PD deficiency is common in northeast Thailand. This is the first report of an accurate determination of G6PD deficiency prevalence in northeastern Thailand females by genotyping G6PD variants. The high prevalence of G6PD deficiency in Thailand results from selective pressure of malaria[26] as demonstrated in other parts of the world where malaria is or has been endemic.[12,27]

FST, the most commonly performed qualitative test, underestimates the prevalence of heterozygous G6PD deficiency;[28] in the present study, about half of females with intermediate G6PD activity were misclassified by FST having normal G6PD activity (**Table 2**). These falsely normal females are thus vulnerable to oxidative hemolysis. Furthermore, there were individuals carrying normal *G6PD* alleles expressing intermediate G6PD activity based on enzyme activity assay and heterozygotes presenting normal G6PD activity. Intermediate G6PD activities found in normal G6PD individuals might result from a high proportion of senescent RBCs in their circulation at the time of blood collection, as G6PD activity decreases significantly according to RBCs age.[29] In heterozygous G6PD-deficient females, random X-chromosome inactivation results in various ratios of G6PD normal to G6PD-deficient RBCs, thereby resulting in G6PD phenotypes

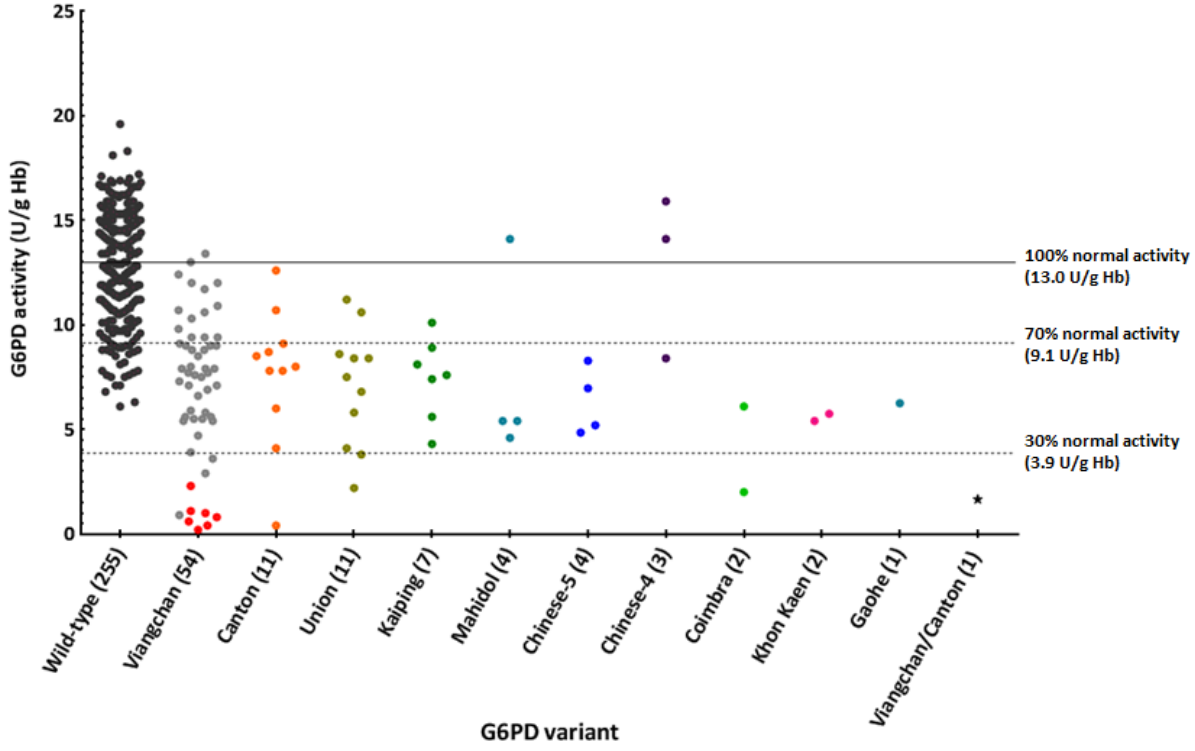

Figure 2 Distribution of G6PD variants activities. G6PD activity was measured using a Trinity Biotech G6PD kit (Trinity Biotech, Bray, Ireland) in a clinical chemistry analyzer (BS-400; Mindray, Shenzhen, PR China) and presented as U/g hemoglobin (Hb). All G6PD variant samples are from heterozygous individuals except for seven cases of G6PD Viangchan homozygotes (red dots) and one of compound heterozygous G6PD Viangchan and Canton. Number of cases are indicated in parenthesis. 100% normal activity is defined as median G6PD activity of wide-type (normal) individuals.

ranging from almost normal to severely deficient, while the majority have G6PD activity in the intermediate range.[30] Other factors impacting on G6PD activity of heterozygotes are enzyme variant types and age of RBCs. Furthermore, rates of enzyme decay with the aging of RBCs vary substantially among different G6PD variants.[31,32] G6PD variants identified in this study belong to class II (Canton, Coimbra, Kaiping, Union, and Viangchan) and Class III (Chinese-4, Chinese-5, Gaohe and Mahidol).[2,33] No clear differences in G6PD activity between the two enzyme classes could be discerned. Moreover, median G6PD activity (defined as normal activity) in this study is calculated from enzyme activity of females with wild-type *G6PD* alleles and not from males, and the value was slightly higher than those of previous reports.[6] In northeastern Thailand, about 50% of women of reproductive age carry various thalassemia genes resulting in significantly higher reticulocyte count, especially immature reticulocyte fraction (IRF),[34] and higher G6PD activity. In addition, the non-common G6PD variants in this region (G6PD Coimbra, Gaohe and Khon Kaen) had low intermediate or deficient enzyme activity; therefore, they will be assuredly diagnosed by G6PD activity assay.

In this region of Thailand, G6PD Viangchan accounts for >50% of the seven common variants.[24] G6PD Viangchan is also the most common variant in Cambodia and PDR Laos,[35,36] reflecting the common ancestral origin of populations in northeastern Thailand, Cambodia and PDR Laos.[24,37] The other less common variants found in this study were Canton, Chinese-4, Chinese-5, Gaohe, Kaiping and Union (common variants in Chinese population), and Mahidol (the most common variant in Myanmar population),[33] indicating the presence of people originating from these two countries in northeastern Thailand.

The novel G6PD Khon Kaen (c.305T>C, p.F102S) was discovered among samples with intermediate G6PD deficiency. The F102 residue is located within the coenzyme-binding domain (residues 31-200).[38] Replacement of phenylalanine with a smaller and more hydrophilic serine residue would be sufficient to cause impaired folding of the domain, leading to decreased stability and low enzymatic activity.[39]

Conclusions. The results highlight the high prevalence in northeastern Thailand of females heterozygous for G6PD variants, the majority with G6PD activity in the intermediate deficiency range; however, some of these samples show normal and deficient enzyme activity ranges, indicating that the accurate identification of female G6PD deficiency carriers requires both measurements of G6PD activity and genotyping of G6PD variants. Knowing the genotype-phenotype correlation of G6PD deficiency in this female population will help improve the management of acute hemolysis related to drugs, foods, and infections.

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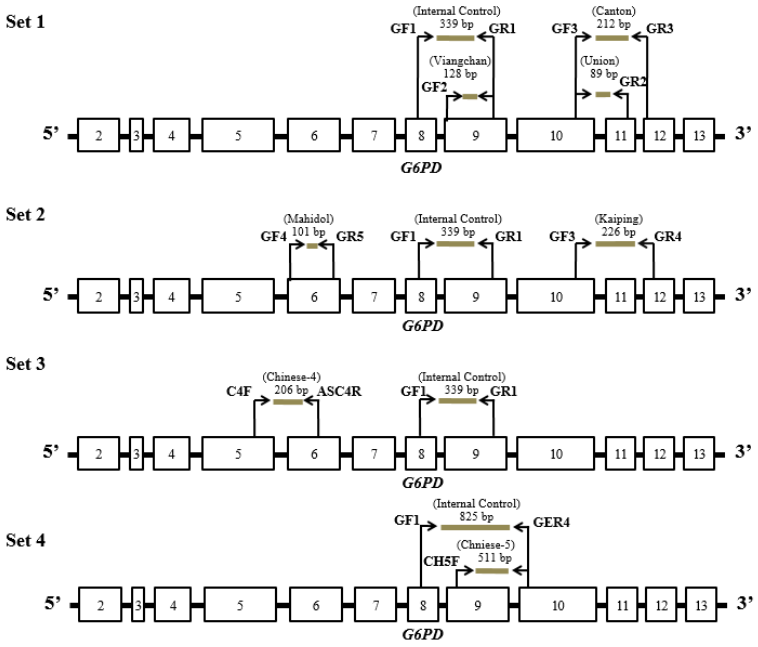
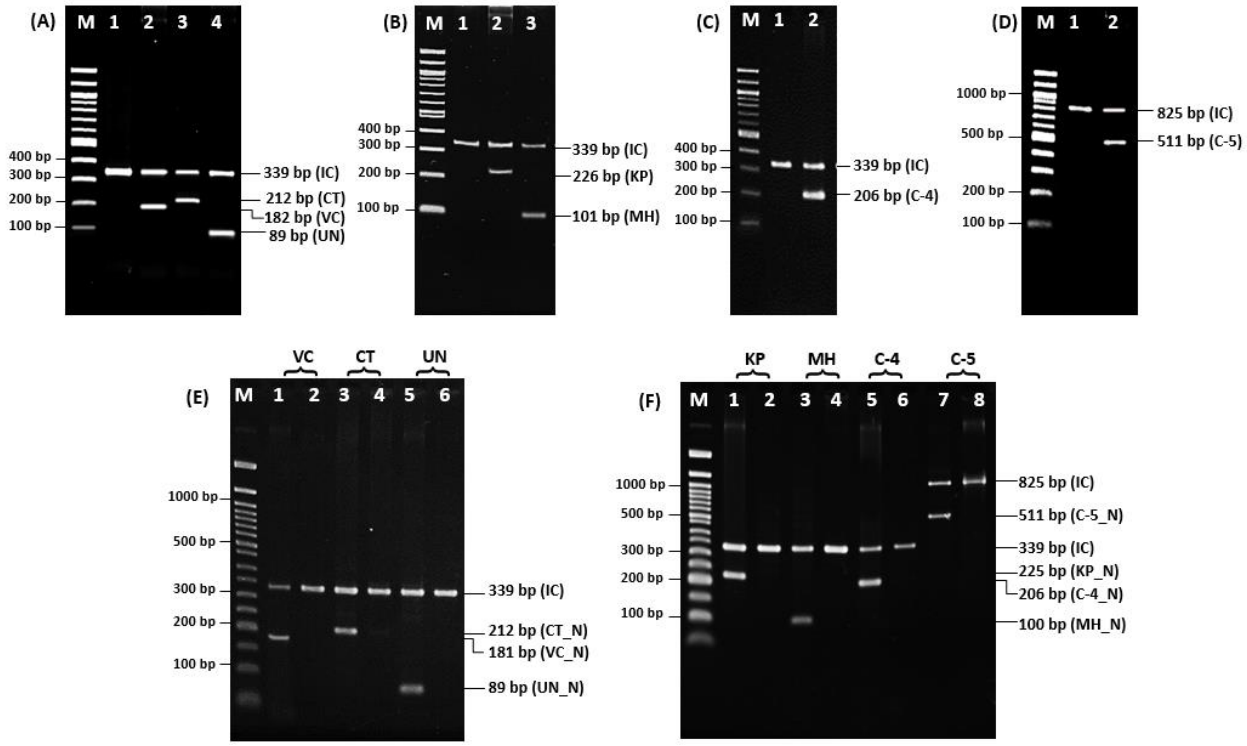
Supplementary files:



Supplementary Figure S1. Location on *glucose-6-phosphate dehydrogenase* gene (*G6PD*) of allele-specific primers for multiplex detection of G6PD Canton, Union and Viangchan (Set 1), G6PD Kaiping and Mahidol (Set 2), G6PD Chinese-4 (Set 3) and G6PD Chinese-5 (Set 4). Location of internal control primers are also indicated. Primer sequences are listed in **Table 1.**



Supplementary Figure S2 Agarose gel-electrophoresis of multiplex allele-specific PCR amplicons of seven common heterozygous glucose-6-phosphate (G6PD) variants present in Southeast Asia. (A) Lane M, 100 bp DNA size markers; lane 1, uniplex internal control primers (IC); lane 2, G6PD Viangchan (VC) allele plus IC; lane 3, G6PD Canton (CT) allele plus IC; lane 4, G6PD Union (UN) allele plus IC. (B) Lane M, 100 bp DNA size markers; lane 1, uniplex internal control primers (IC); lane 2, G6PD Kaiping (KP) allele plus IC; lane 3, G6PD Mahidol (MH) allele plus IC. (C) Lane M, 100 bp DNA size markers; lane 1, uniplex internal control primers (IC); lane 2, G6PD Chinese-4 (C-4) plus IC. (D) Lane M, 100 bp DNA size markers; lane 1, uniplex internal control primers (IC); lane 2, G6PD Chinese-5 (C-5) allele plus IC. (E) Lane M, 50 bp DNA size markers; lane 2, 4 and 6, uniplex internal control primers (IC); lane 1, normal allele (VC_N) present in heterozygous VC sample plus IC; lane 3, normal allele (CT_N) present in heterozygous CT sample plus IC; lane 5, normal allele (UN_N) present in heterozygous UN sample plus IC. (F) Lane M, 50 bp DNA size markers; lane 2, 4, 6, and 8, uniplex internal control primers (IC); lane 1, normal allele (KP_N) present in heterozygous KP sample plus IC; lane 3, normal allele (MH_N) present in heterozygous MH sample plus IC; lane 5, normal allele (C-4_N) present in heterozygous C-4 sample plus IC; lane 7, normal allele (C-5_N) present in heterozygous C-5 sample plus IC.