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The prevalence of glucose-6-phosphate dehydrogenase deficiency in Trat province, eastern Thailand

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KEYWORDS
G6PD deficiency; Fluorescent spot test; G6PD enzyme activity.

ABSTRACT
The most prevalent X-linked enzymopathy in Thailand is glucose-6-phosphate dehydrogenase (G6PD) deficiency. The eastern Thailand border region is at risk of developing drug-resistant malaria and the frequency of G6PD deficiency and the characterization of G6PD variants are unclear. A fluorescent spot test (FST), quantitative G6PD activity assay, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to identify common G6PD variants were used to evaluate the prevalence of G6PD deficiency. G6PD deficiency was found in 12.06% of the population. Females with an FST of 6.43% were intermediate, whereas females with an FST of 1.61% were deficient, and men with an FST of 4.02% were deficient. G6PD Viangchan was the most common variant, followed by G6PD Mahidol, according to PCR-RFLP results. G6PD activity in the heterozygotes females were more than 60% of normal activity. In G6PD deficient samples, there is a strong negative correlation between G6PD activity and hemoglobin, hematocrit. The frequency of G6PD deficiency in the region is important for G6PD diagnosis and potentially useful for implementing appropriate anti-malarial drug treatment.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is the first step in the pentose phosphate pathway, which is the synthesis of NADPH. NADPH is required to produce reduced glutathione, which is essential for the avoidance of oxidative damage and the reduction of red blood cell sensitivity to hemolysis. The G6PD gene is found on chromosome X (Xq28) and has a high level of polymorphism, with around 190 mutations coding for 400 biochemical or allelic enzyme variations[1,2]. The mutations cause enzyme deficits and are passed down through the generations as X-linked characteristics. Due to X-chromosome inactivation, hemizygous men and homozygous females have G6PD deficiency, but heterozygous females may have normal or deficient G6PD activity[2]. Many G6PD defective variants have been found in diverse groups in Southeast Asia. Thailand has the highest prevalence of G6PD Viangchan and G6PD Mahidol. The prevalent mutation in the Burmese population has been identified as G6PD Mahidol. In the Laotian, Cambodian, and Vietnamese populations, G6PD Viangchan has been shown to be the most frequent variant[3-5].

Anti-malarial drug-resistant parasites and insecticide-resistant mosquitoes are increasing rapidly. Artemisinin resistance (ART-R) and multi-drug resistant falciparum malaria pose additional significant problems for Cambodia, resulting in substantial treatment failure[6]. Cambodia has a porous border with Thailand and a highly mobile population which increases the risk of drug resistance developing in Thailand. The prevalence of G6PD deficiency and related variations in the Thai population has been reported in several studies, with prevalence rates ranging from 5% to 18%[7-11]. A few investigations into the prevalence of G6PD deficiency have been performed along the Thai-Cambodian border. Furthermore, whereas normal and heterozygous females have similar enzyme activity, enzyme assay accuracy in detecting female heterozygotes with normal G6PD activity is limited. The frequency of G6PD deficiency and enzyme activity in Trat province's subregions were investigated. To avoid life-threatening consequences, a patient's G6PD status must be determined prior to the administration of anti-malarial drugs. The aim of this study was to determine to find out how common G6PD deficiency is in Trat. It is hoped that the outcomes of this study will be helpful to healthcare practitioners in Trat province to make better decisions about prescriptions in the future.

Materials and methods

Blood sample

This study was approved by the Burapha University Ethics Committee for Human Research (11/2560). Volunteers aged at least 18 years old from Trat Hospital were enrolled in the study, from which at least one leftover EDTA blood sample from routine blood test was collected. The red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were all determined using an automated hematology analyzer (Mindray BC-5800, Shenzhen, China). A total of 373 blood sample from the Thais who had 9-18.5 g/dL hemoglobin concentration and 4-10 x10^9/L WBC count were enrolled in this study. A total of 373 G6PD fluorescent spot tests, G6PD enzyme activity, and G6PD mutation were tested.

Fluorescent spot test

The fluorescent spot test (FST) was used to screen for G6PD deficiency using a commercial kit (Trinity Biotech USA, Jamestown, NY, USA) within 24 hours after blood collection. According to the manufacturer's protocol, 10 µL of whole blood sample was added to 200 µL reagent and set a spot on the filter paper as the zero-time point, then incubated at 37.0°C. A sample mixture was dropped on the filter paper and then further sample mixtures dropped on the filter paper after additional incubating for 5 and 10 minutes. The fluorescence intensity of dried spots was observed under UV illumination and divided into three groups: normal (strong fluorescence at 5 and 10 minute); intermediate (poor fluorescence at 5 minutes and moderate fluorescence at 10 minutes); and deficiency (no fluorescence at 5

and 10 minutes). The fluorescence intensity of the sample was compared to that of a G6PD deficient blood sample and a G6PD normal blood sample.

G6PD enzyme activity quantitative test

Enzyme activity was measured using the G6PD Assay Kit (Trinity Biotech USA, Jamestown, NY, USA) according to the manufacturer's instructions. Briefly, ten microliters of whole blood was combined with 1 mL of G6PDH solution. After 10-minute incubation at room temperature, 2 mL of G6PDH substrate reagent was added and incubated at 30.0°C for 5 minutes. Absorbance of the kinetic reaction was measured at 340 nm, G6PD enzyme activity was estimated as U/gHb using the manufacturer's formula and standardized by hemoglobin concentration. Then, the cut-off value for G6PD deficiency in study population was calculated from adjusting the median G6PD activity of men[12]. Each sample was run in duplicate and the measurement values differed by more than 10%, the experiment was repeated analysis. The accuracy of the G6PD activity results was evaluated using a normal G6PD level control. Sample testing was done if the control value was within the range.

Detection of G6PD gene mutations

Genomic DNA from all peripheral blood samples was extracted using the phenol-chloroform method. RFLP-PCR assays were used to screen for two frequent G6PD variants in Thailand; the Viangchan variant (871 G>A) and the Mahidol variant (487 G>A), as previously described[13]. PCR was performed under conditions as follows: 95°C for 5 minutes; 35 cycles of 95°C for 60 seconds, 58-62°C (depending on Tm of the primer pair) for 45 seconds and 72°C for 45 seconds; and 72°C for 5 minutes. The PCR amplicons were then digested in a 10 µL reaction volume with suitable restriction endonuclease (Thermo Fisher Scientific, USA) at 37°C. After 5 hours, 5 µL aliquot from each of the digested mixture was mixed with DNA staining dye (Genedirex Inc, Taiwan), then analyzed on 3% agarose gel-electrophoresis and visualized under UV light.

Statistical analysis

Hematological parameters and G6PD activity are all reported as mean ± SD. Statistical analysis was performed using tests appropriate to the dataset, as specified in the figure legends, using GraphPad Prism 9 software (GraphPad, San Diego, CA). The *p*-value < 0.05 was considered statistically significant.

Results

G6PD fluorescent spot test (FST) and G6PD enzyme activity

The semi-quantitative fluorescent spot test detected 45 G6PD defective samples based on 373 samples screened for G6PD deficiency, including 15 (4.02%) deficient males, 6 (1.61%) deficient females, and 24 (6.43%) intermediated females (Table 1). There were no significant differences in the hematological parameters between FST normal and G6PD deficient or intermediate individuals (Table 1).

Next, G6PD enzyme activity was measured in 45 samples of FST positive and 46 samples of men with normal FST. The results of male samples were used to determine the normal activity for this population. Three male samples showed enzyme activity levels that were less than 10% of the median and were excluded from the analysis. The adjusted male median (AMM) G6PD enzyme activity was 7.5 U/gHb and set 100% G6PD activity of this population. The cut-off value for G6PD deficiency (activity below 60% of the AMM) in this population was < 4.47 U/gHb. Based on these cut off and WHO classifications, 21 of 26 cases (80.7%) with FST deficiency result had less than 60% G6PD activity. Moreover, the result demonstrated that 20% (3/15) of FST deficient males were considered as having severe enzyme deficiency (activity below 10% of normal) and 79.2% (19/24) of FST intermediate females had very mild or no enzyme deficiency (more than 60% of AMM activity) (Figure 1) (Table 2).

In addition, the correlations of G6PD enzyme activity and hematological parameters were investigated. We observed a statistically significant negative correlation of G6PD enzyme activity with Hb (r^2 = 0.151, *p*-value = 0.009), and with Hct (r^2 = 0.141, *p*-value = 0.011) in FST deficient and FST intermediate individuals (Figure 2).

G6PD mutation variants determination

The prevalence of G6PD mutation variants identified in the Thai population, including the Viangchan variant and Mahidol variant, were investigated by PCR-RFLP in all samples. The prevalence of G6PD mutation variants in Trat province was 12.07% (45/373), with G6PD Viangchan accounting for 97.78% of this. Based on WHO classifications and commonly used ranges; 10%, 20%, 30%, and 60% of the AMM, the enzyme activity of 15 male subjects with Viangchan hemizygote ranged from 1% to 30% of normal G6PD activity. Viangchan heterozygote was found in 24 females with intermediate and deficient FST results; 19 of the 24 Viangchan heterozygote females had normal enzyme activity (>60% normal activity), and 5 of 24 had moderate enzyme deficiency (10-60% normal activity). Viangchan homozygote was found in 5 females with deficient FST results and moderate enzyme deficiency (10-60% normal activity). Intriguingly, Mahidol heterozygote was only found in a single female with deficient FST and enzyme activity which was 23% of normal. The G6PD activities of male hemizygotes and female homozygotes were not significantly different, while moderate G6PD deficiency among female heterozygous is found to be significantly greater than the other three groups (Figure 3).

Table 1 Characterization of hematologic data, fluorescent spot test and quantitative G6PD activity among 373 subjects

Parameters	FST- Normal		FST-Deficiency		FST-Intermediate
	Male	Female	Male	Female	Female
Number of samples	141	187	15	6	24
Age (years)	55.8 ± 15.9	55.9 ± 17.3	50.0 ± 14.1	49.0 ± 14.7	48.3 ± 20.7
WBC ($x10^9$/L)	7.5 ± 3.2	7.7 ± 3.4	8.9 ± 4.4	6.5 ± 1.2	6.5 ± 2.4
RBC ($x10^{12}$/L)	4.8 ± 0.8	4.5 ± 0.3	4.6 ± 0.6	4.5 ± 0.4	4.4 ± 0.6
Hb (g/dL)	13.3 ± 2.1	12.1 ± 1.6	13.6 ± 2.1	13.3 ± 0.9	12.3 ± 1.3
Hct (%)	38.8 ± 5.9	35.7 ± 4.3	39.8 ± 5.9	39.5 ± 3.0	36.2 ± 4.2
MCV (fL)	82.9 ± 5.7	80.3 ± 8.7	84.2 ± 4.4	81.5 ± 8.7	80.5 ± 3.5
MCH (pg)	28.3 ± 3.1	27.5 ± 2.1	28.6 ± 1.4	27.3 ± 1.0	27.8 ± 1.8
MCHC (g/dL)	34.0 ± 1.2	33.5 ± 0.8	33.2 ± 0.9	32.5 ± 0.9	32.7 ± 1.3
G6PD activity (U/gHb)	8.2 ± 2.1[a]	-	1.4 ± 0.7	2.3 ± 0.5	6.3 ± 2.3

Note: [a] Calculated from 46 randomly selected males.

Table 2 Phenotypic and genotypic classification of G6PD status in 45 G6PD FST positive subjects

G6PD enzyme activity		Fluorescent spot test		Genotypic test		
% Normal activity	Number	Intermediate	Deficiency	Hemizygous	Heterozygous	Homozygous
		Number	Number	Number	Number	Number
> 60%	19	19	0	0	19	0
10 – 60%	23	5	18	12	6	5
< 10%	3	0	3	3	0	0
Total	45	24	21	15	25	5

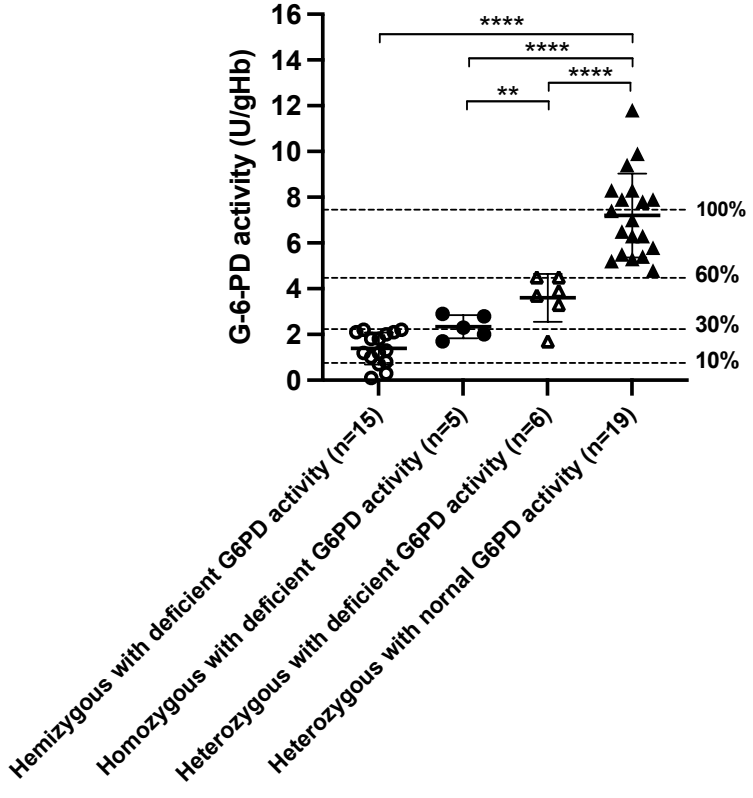

Figure 1 Distribution of G6PD activity classified using a fluorescent spot test

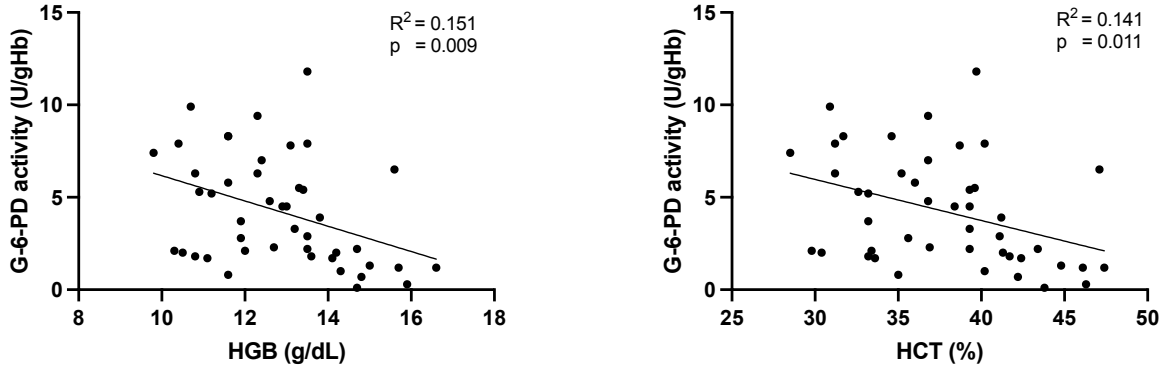

Figure 2 Correlation between G6PD activity and hemoglobin and hematocrit levels in G6PD deficient samples

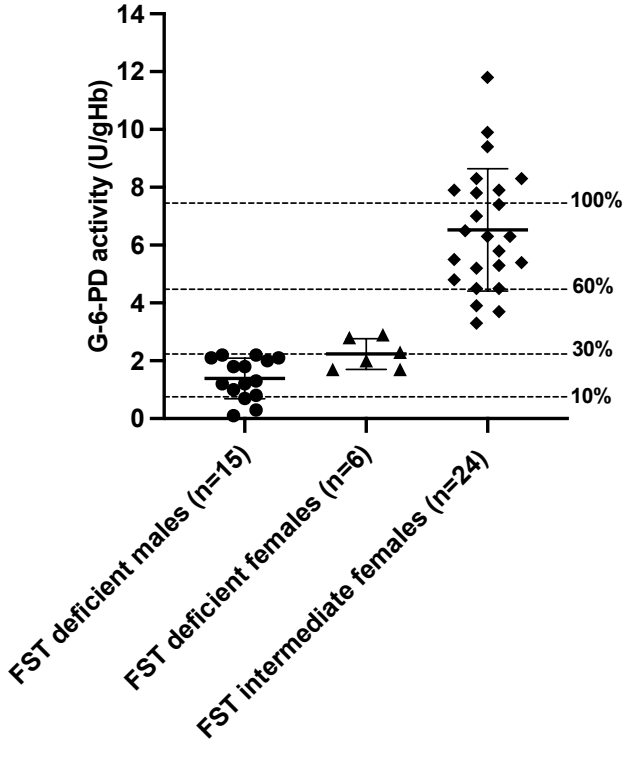

Figure 3 Distribution of G6PD activity classified using PCR-RFLP. A normal level of G6PD activity is characterized as greater than 60% activity. Statistical significance was determined using One-way ANOVA (**p-value = 0.01, ****p-value < 0.0001)

Discussion

G6PD deficiency is very common in Thailand. The Viangchan variant was found in 44 of the 373 blood samples, indicating a significant occurrence. This conclusion is consistent with earlier research which revealed G6PD Viangchan being the most common variant among the Thai population[7,11,14]. In this study, 79.2% (19 of 24) of heterozygote females with > 60% normal enzyme activity were discovered. In female heterozygote, random X-chromosome inactivation results in a variety of phenotypes ranging from normal to deficient

enzyme[2]. FST screening may be affected by G6PD variants with near normal activity, resulting in a false negative result. FST is widely used for screening and properly identified all male G6PD deficient patients as well as female severe deficient patients[4]. However, FST identified female heterozygote with near normal G6PD activity as intermediate fluorescent intensity and the sensitivity was above 80% in G6PD > 70% normal enzyme activity[4]. In this study, the FST resulted in moderate fluorescence intensity (6.4%), which is lower than the reported prevalence of G6PD deficiency in 353 Thai female adults (14.6%)[14]. In this study, FST was performed to assess the male and female prevalence in Trat province. The varied populations investigated may explain this conflicting finding.

In the present study, only one incidence of a heterozygote Mahidol variant with a G6PD defective phenotype was discovered. G6PD mutations are very common in Thailand, with the Viangchan variant predominating in the east and the Mahidol variant predominating in the west[10,11,15]. The incidence and distribution of G6PD gene variants vary in the Thai population. Because of the diversity in frequency due to different locations and people, subregion prevalence is critical for optimal approaches for mutation detection, treatment, and prevention. According to WHO criteria, the Viangchan variant is classified as class II (Activity <10% of normal), while class III involves enzyme activity 10-60% normal[16]. All male Viangchan hemizygotes had enzyme levels < 30% normal, while 20% (3/15) had enzyme levels < 10%, and 80% (12/15) had enzyme levels between 10 and 30%. The G6PD enzyme was shown to vary in RBCs. In addition, the age of RBCs had an influence on G6PD activity and rates of enzyme degradation with RBC aging varying significantly across different G6PD variants[17]. Furthermore, the negative relationship discovered between G6PD activity and Hb, Hct is consistent with earlier findings[18]. Other early reports have shown that G6PD activity was increased in anemic people with Hb variants and mutations on the G6PD gene[19]. The increasing of reticulocyte numbers might explain these findings; anemic patients were compensated by a higher number of reticulocytes, which have much greater G6PD activity than mature red cells[19]. In this study population, 31.1% (14 of 45 FST positive) of anemic subjects had mild anemia. However, the current analysis was not designed to prove this hypothesis. In recent years, malaria has been substantially less common due to early detection and the delivery of anti-malaria drugs. Dihydroartemisinin-piperazine in combination with primaquine is the first-line medication for *P. falciparum*, while chloroquine in combination with primaquine is the first-line therapy for *P. vivax*[20,21]. Low enzymatic activity in RBCs has been linked to the Mahidol and Viangchan variants, which are associated with a high hemolytic risk. These people are also at risk of anti-malaria drug induced hemolysis, since 56.8% (26/45) in this study had enzymes that were less than 60% normal. An oxidative stress trigger might hemolyze moderate enzyme activity in heterozygotes. Laboratories should screen for G6PD deficiency before patients take an antimalarial drugs. For heterozygote diagnosis, the FST test is insufficient. The optimal method to find G6PD deficient heterozygotes is to use a combination of FST and enzyme activity.

A limitation in this study is that it only investigated Viangchan and G6PD Mahidol variants. The G6PD prevalence in this study did not include FST positive samples that were negative for G6PD Viangchan and G6PD Mahidol variants. However, molecular genotyping for other Thai variants, such as Canton variants, Kaiping variants, Chinese variants, and Union variants, should be carried out in other FST positive samples.

Conclusion

According to the findings of this study, G6PD deficiency was found in 12.06% of the population. Females with an FST of 6.43% were intermediate, whereas females with an FST of 1.61% and men with an FST of 4.02% were deficient. The G6PD Viangchan variant appears to be the most common among these populations. The frequency of G6PD mutations varies due to regional differences. Our findings provide baseline information that can be used to implement appropriate diagnosis, as well as gain a better understanding of G6PD in this population.

Take home messages

G6PD deficiency was highly prevalent in Trat province, with a prevalence of 56.8% (26/45) and enzyme activity below 60% of normal. These people are also susceptible to anti-malaria drug-induced hemolysis. For accurate diagnosis and increased awareness of drug management, a combination of FST and enzyme activity should be used.

Conflicts of interest

The authors declare no conflict of interest.

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