Investigation of Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency Prevalence in a *Plasmodium vivax* Endemic Area in the Republic of Korea (ROK)

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Research

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Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most prevalent inborn disorder. This X-chromosome-linked recessive disease affects more than 400 million people globally, and is associated with hemolytic anemia after medication with the anti-latent malaria drug, primaquine. To prevent malaria, the Republic of Korea (ROK) Army administers malaria chemoprophylaxis. Due to the previously low G6PD deficiency prevalence in the ROK, prior to primaquine distribution, testing for G6PD deficiency was not mandatory. Here, we investigate G6PD deficiency prevalence to evaluate the risk from malaria chemoprophylaxis.

Methods: Blood specimens from 1,632 soldiers entering training camp for the 3rd Infantry of the ROK Army were collected. CareStart™ Biosensor for G6PD and Hemoglobin was used to detect G6PD levels. G6PD variants using the DiaPlexC G6PD Genotyping kit (Asian type) and full-length sequencing were examined.

Results: Of 1,632 blood specimens tested, none were observed to be G6Pd deficient. The median value of all tested samples was 7.582 U/g Hb. An investigation of 170 G6PD DNA variants were analyzed and categorized as partially low (n=134, 30%–80% (2.27–6.05 U/g Hb) of the median value), high (n=3, >150% (>11.373 U/g Hb) of the median value), or normal (n=36, 4.6–13.5 U/g Hb), and none were amplified by the DiaPlexC kit. Five silent mutations (C→T) in 38 partially abnormal specimens were found at the 1311th nucleotide position by sequence analysis. Another 8 silent mutations (T93C) were also detected in 134 partially abnormal specimens. Thus, we inferred that these silent mutations could be related to G6PD deficiency.

Conclusions: This G6PD deficiency prevalence study, conducted among participants from the 3rd Infantry of the ROK Army, provided crucial evidence for the safety of malaria chemoprophylaxis. Here, we found that the prevalence of G6PD deficiency among 1,632 young soldiers was nearly 0. Although G6PD phenotypic mutations were not detected, many silent mutations (C1311T and T93C) were observed. Thus, we inferred that malaria chemoprophylaxis is relatively safe against G6PD deficiency-mediated hemolytic anemia. However, given the number of individuals whose G6PD were at the low end of the normal range and the frequent detection of G6PD deficiency-related mutations, consistent monitoring of G6PD deficiency is needed.

Background

Glucose-6-phosphate dehydrogenase (G6PD) is an X-chromosome-linked enzyme involved in the pentose phosphate pathway, and it plays a pivotal role in defending against oxidative stress by generating NADPH (coenzyme nicotinamide adenine dinucleotide phosphate) [1-3]. G6PD deficiency is the most common enzyme deficiency disease with 400 million patients worldwide [4], and a global prevalence rate that varies from 0.1%–30% [5, 6]. Previous studies have reported 0.9%–3.5% prevalence in South Korea [7-9].

Malaria, a mosquito-borne parasitic disease, causes more than 200 million cases every year, resulting in about 700,000 deaths [10, 11]. The Gyeonggi province, in the Republic of Korea (ROK), is endemic for *Plasmodium vivax*, a parasite that causes malaria. Gyeonggi is near the demilitarized zone (DMZ); thus, many military personnel are stationed there and are at high risk for malaria [12]. Since 1997, the ROK Army has carried out chemoprophylaxis with chloroquine and primaquine to prevent malaria.

Unlike other malarial protozoans, *Plasmodium vivax* exists as a hypnozoite in the liver during part of its life cycle and infects red blood cells (RBCs) [13]. To prevent malaria spread via recurrence, it is essential to scavenger latent hypnozoites in the liver [13]. Primaquine, an effective drug for eliminating *P. vivax* hypnozoites in their dormant stage, is administrated to about 100,000 military personnel in domestic malaria-endemic areas [14]; it can be administered prophylactically and therapeutically [18]. Primaquine also causes G6PD deficiency-related hemolytic responses [15-17]. Due to adverse hemolytic effects, the World Health Organization (WHO) and United States Centers for Disease Control and Prevention (US-CDC) recommend that G6PD screening tests be performed before primaquine administration, but, because of low incidence, screening is not conducted in South Korea.

To analyze G6PD deficiency prevalence and malaria chemoprophylaxis risk among military personnel, we tested new recruits into the 3rd Infantry for G6PD enzymatic activity, and we also conducted genetic analyses on samples with activity levels 30%–80% or >150% of the median G6PD level.

Methods

Ethics statement and sample preparation

This study was approved by the ethics committee of the Armed Forces Medical Command (AFMC) of the ROK (Approval No. AFMC-17-IRB-023). Written informed consent was obtained from each participant to collect a 5-ml blood sample. Using educational materials, study objectives and activities were explained to all soldiers who were admitted to recruitment training camps in Paju and Yangju, which are malaria-endemic areas where the military administers malaria chemoprophylaxis. Afterward, a written study explanation and a research agreement form were distributed to obtain participant consent.

This study was conducted at the Department of Infectious Disease Research of the Armed Forces Medical Research Institute from April 2017 to January 2018. EDTA-preserved venous blood samples were obtained from 1,632 soldier participants who resided in the endemic area (Paju recruitment training camp: n=779/1007, consent rate=77%; Yangju recruitment training camp: n=853/1011, consent rate=84; Figure 1).

Determination of G6PD deficiency

Point-of-care (POC)-based G6PD and Hb testing by venipuncture was performed with CareStart™ Biosensor 1 (Cat No: BBA-E00182; AccessBio, New Jersey, USA) according to the manufacturer's instructions. Two blood samples, both 10 ul volume, were collected: one was transferred to an Hb strip

and the other was spotted onto parafilm and then transferred to a G6PD strip. After 5 minutes, the CareStart™ Biosensor 1 kit produced estimates of Hb (g/dL) and G6PD (U/dL) concentrations. All samples were tested in duplicate. To validate the reliability of this POC-based test, we also evaluated G6PD activity using the Glucose-6-Phosphate Dehydrogenase Reagent Set (Pointe Scientific, Canton, USA) as reference test. For the reference test, we added 10 ul of blood to 1 ml of R1 reagent to a labeled cuvette, and mixed thoroughly to suspend erythrocytes; we then incubated the mixture at room temperature for 5–10 min. After adding 2 ml of R2 reagent to the incubated sample, we placed the cuvette in a 37ºC-water bath for 5 min, and then measured absorbance (A1) at 340 nm. Next, we checked absorbance (A2) at 340 nm after another 5 min incubation. Using these absorbance values, we calculated ΔA per min as [(A2-A1)/5]. By assigning measurements to the following formula [G6PD (U/g Hb) = △A per min X [(100 X 3.01) / (0.01 X 6.22 X Hb (g/dl))] X TCF, we calculated G6PD in U/g Hb. [100 = factor to convert activity 100 ml, 3.01 = total reaction volume (ml), 0.01 = sample volume (ml), 6.22 = millimolar absorptivity of NADPH at 30 nm, Hb (g/dl) = hemoglobin concentration for each specimen, TCF = temperature correction factor (1 at 37°C)]

DNA extraction and nested PCR diagnosis

Genomic DNA was extracted from 200 ul of whole blood using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer instructions. Purified DNA samples were diagnosed using nested polymerase chain reaction (PCR) and the G6PD gene was amplified using specific primers [19]. The first PCR round was performed under the following conditions: 94°C for 1 min, followed by 38 cycles at 94°C for 12 s, 65°C for 30 s, and 68°C for 6 min, and a final extension at 68°C for 10 min. The amplification reaction was carried out in 20 ul volume reactions, including primer volumes (1 ul of forward and reverse primer, 5 pmol/ul), 10 ul of PCR master mix (Takara Bio Inc., Shiga, Japan), and 3 ul of DNA template. The amplified products from the first round were subjected to a second PCR round with primers (Figure 5). The second-round amplification reaction was carried out in 20-ul volume reactions, including primers [1 ul of forward and reverse primer, 5 pmol/ul), 10 ul of PCR master mix (Takara Bio Inc., Shiga, Japan), and 3 ul of DNA template. The second PCR round was performed under the following conditions: 94°C for 1 min, followed by 35 cycles at 94°C for 12 sec, 62°C for 25 sec, and 72°C for 3 min, and a final extension at 72°C for 5 min. Each fragment was analyzed with 2 sequencing primers (Figure 5).

Analysis of G6PD variants

We performed genotyping on 170 blood samples representing different ranges of G6PD activity level (n=131 samples with 30%–80% (2.27–6.05 U/g Hb) of the median; n=3 samples with >150% (>11.373 U/g Hb) of the median; and n=36 samples within the normal range (4.6–13.5 U/g Hb). G6PD variants were detected using the DiaPlexC G6PD Genotyping Kit (Asian type; SolGent, Daejeon, ROK), which specifically screens for the seven representative Asian variants of the G6PD gene via one-step PCR. The variants each produce PCR products of different sizes: Vanua Lava (383 T>C), Mahidol (487 G>A), Coimbra (592 C>T), Viangchan (871 G>A), Union (1360 C>T), Canton (1376 G>T), and Kalping (1388

G>A). PCR mixtures were prepared with 2 ul of genomic DNA (internal control, wild-type control, or mutation control) and 23 ul of master mix (12.5 μl polymerase premix, 2 μl primer premix, and 8.5 μl distilled water). The PCR cycling conditions were as follows: initial denaturation at 95°C for 15 min, 30 amplification cycles (denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, elongation at 72°C for 40 sec), and a final extension at 72°C for 5 min. PCR products were resolved by electrophoresis on 1% agarose gel, stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA), and visualized using FluorChem FC3 (ProteinSimple, Santa Clara, CA, USA).

G6PD amplification and G6PD variant sequencing

Of the 170 blood samples genotyped for G6PD,we performed further sequencing on the 134 samples that represented the lower and upper bounds of G6PD activity (n=131 samples with 30%–80% median activity and n=3 samples with >150% median activity). Primer sets used for G6PD amplification and sequencing are listed in Figure 5. Each fragment was analyzed with 2 sequencing primers (Figure 5). Confirmed sequences were analyzed with a wild type sequence using BioEdit v7.0.5 software.

Statistical analyses

Data are presented as means and standard errors. Statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA, USA). The significance of pairwise comparisons was determined using the Student's t-test, and $p < 0.05$ was considered significant.

Results

Schematic diagram of sample selection for enzymatic and genetic evaluation of G6PD deficiency

As previously mentioned, G6PD deficiency screening has not been implemented in South Korea due to low prevalence [7-9]. However, since 1984, G6PD-deficient clinical patients have been increasingly reported in South Korea, and G6PD deficiency-mediated hemolytic anemia patients (G-6-PDH 0.3 U/gHb in 2012 and G-6-PDH 2.6 U/gHb in 2015) were identified in the ROK Army, after primaquine uptake without G6PD screening. Thus, there is a need for periodic monitoring of G6PD deficiency prevalence.

As shown in Figure 1, activity-based phenotypic and DNA-based genotypic tests were performed to estimate G6PD deficiency prevalence in the ROK Army. With approval from the AFMC IRB (AFMC-17-IRB-023), a POC-based G6PD activity test by venipuncture using the CareStart G6PD & Hb kit was performed for 1,632 newly recruited soldiers at the Paju and Yangju training camps, where malaria is endemic. Genetic sequencing of G6PD was also performed on 134 soldiers' samples who showed partially abnormal G6PD activity.

Screening and classification of G6PD activity from 1,632 blood samples

The standard range for normal G6PD enzyme activity has been determined to be 4.6–13.5 U/g H. All 1,632 blood samples analyzed in this study were collected in two cities, Yangju (n=853) and Paju

(n=779), and all were screened using the CareStart G6PD & Hb POC test and the Pointe Scientific G-6-PdH kit as a reference test based on quantitative enzyme kinetics. Although the Pointe Scientific kit is commonly used, it is labor-intensive and time-consuming. The CareStart test is rapid and convenient and is a suitable alternative to G6PD enzyme assays. The results from these two methods showed similar activity values within the error range (Figure 2). Thus, we used the POC-based G6PD activity test as our main study test. All participants were confirmed to have G6PD levels within the normal range of 4.6–13.5 U/g Hb. The median value was 7.582 U/gHb (Supplementary Figure 1). G6PD activity distribution for all samples is shown in Figure 3. According to the WHO G6PD POC testing guidelines, values <30% of the median are defined as deficient. Among female patients, 30%–80% of the median is classified as intermediate deficiency. Of the 1,632 participants, 131 were within 30%–80% of median activity (2.27–6.05 U/gHb) and no one had <30% median activity (<2.35 U/gHb). Based on classifications by Beutler et al., we also examined samples <60% and >150% of the median value. No participants had <60% (4.55 U/gHb), but 3 had >150% (>11.373 U/gHb) median activity.

G6PD-genetic analysis using the G6PD genotyping kit and full-length sequencing

Using 170 blood samples representing different ranges of G6PD activity level (n=131 samples with 30%–80% (2.27–6.05 U/g Hb) of the median; n=3 samples with >150% (>11.373 U/g Hb) of the median; and n=36 samples within the normal range (4.6–13.5 U/g Hb), we conducting one-step PCR genotyping using the G6PD Genotyping Kit (Asian type; Solgent) to detect seven G6PD variants: Vanua Lava (383 T>C), Mahidol (487 G>A), Coimbra (592 C>T), Viangchan (871 G>A), Union (1360 C>T), Canton (1376 G>T), and Kalping (1388 G>A). As shown in Figure 4, we did not detect any bands suspected to contain G6PD variants. We performed G6PD sequencing analysis using nested PCR to identify other potential single nucleotide polymorphisms (SNPs), as depicted in Figure 5. We found several C1311T exon mutations and T93C intron mutations. All mutations are listed in Figure 6. The C1311T/IVS polymorphism frequency at exon 11 of the G6PD gene is shown as 5/134 or 0.037. Another silent mutation incidence, T93C, at intron 11 is shown as 8/134 or 0.06. All sequencing data from the 134 partially abnormal samples is provided in Supplementary Figure 2. Previous studies have reported an association between these mutations [20, 21], which is consistent with our finding of a frequency of 5/8 (0.625) for combined mutations of C1311T and T93C. Of the 8 T93C mutations in intron 11, 5 were not linked to C1311T/IVS. Among the 36 normal-range G6PD samples, no mutations were found. Thus, we inferred that the C1311T mutation and the T93C intron single mutation could be linked to G6PD activity.

Discussion

In 1993, malaria re-emerged as an endemic pathogen in regions near the demilitarized zone (DMZ) and, after a rapid increase in the number of malaria patients, in 1997, the ROK Army began conducting chemoprophylaxis with primaquine to prevent latent malaria. When people who are G6PD deficient take primaquine, they can experience drug-mediated hemolytic adverse effects. Consequently, the WHO and the US-CDC recommend conducting G6PD screening before primaquine administration. However, screening is not performed in South Korea because of low prevalence of G6PD deficiency compared with

the average global incidence. Blackwell et al. [8] found that G6PD prevalence was 0.9% among 2,595 study participants in 1968, and Saha et al. [7] estimated 3.5% G6PD prevalence among 140 participants in 1984. A recent Korea Centers for Disease Control and Prevention (KCDC) investigation found no cases among 1,044 participants sampled [9]. However, since 1992, G6PD-deficient clinical cases have been continuously reported at domestic hospitals, possibly as a consequence of increased immigration from Southeast Asia [22, 23], where G6PD deficiency prevalence is relatively high. Thus, G6PD deficiency incidence is expected to rise. In fact, in 2012 and 2015, primaquine-uptake-induced G6PD deficiency-mediated hemolytic anemia (G-6-PDH 0.3 U/gHb (2012) and G-6-PDH 2.6 U/gHb (2015)) occurred due to the absence of G6PD deficiency screening. Thus, highlighted the need for periodic monitoring of G6PD deficiency and was a motivation for this study.

We performed a POC G6PD activity test using the CareStart G6PD & Hb kit to examine 1,632 blood samples from soldiers who were admitted to recruitment training camps in a malaria-endemic region. All samples' G6PD activities fell within the normal range of 4.6–13.5 U/gHb. Although our sample size is relatively small compared with the number of people who receive military-administered malaria chemoprophylaxis, our study indicates that G6PD deficiency prevalence is low enough that the risk of primaquine-induced G6PD deficiency-mediated hemolytic anemia is relatively rare. However, there is a large number of military personnel at the border, and C1311T/IVS and T93C intron mutations, which are known to be associated with G6PD deficiency, were detected in our study. This finding suggests that consistent monitoring of G6PD deficiency is necessary. A previous report found that C1311T/IVS mutations are linked to the Mediterranean (563C>T) and G6PD Viangchan (871G>A) phenotypic mutations [24]. No associations with these mutations were confirmed in this study. However, the combined mutation of C1311T and T93C, and the T93C single intron mutation were only detected in the 30%–80% activity group, and not in the >150% of median value group or in the normal-range group. Thus, these mutations could be linked to G6PD activity level through their presence in the partially abnormal group. Each mutation activity value was investigated according to the description in Figure 6. All partially abnormal activity values fell around the lower border of the normal range. Thus, we inferred that this mutation is partially related to G6PD deficiency.

Although we did not identify any G6PD-deficient participants with activity <30% (<2.35 U/gHb) of the median value, we still argue for periodic surveillance for G6PD deficiency, due to the high incidence of partial G6PD deficiency and deficiency-related mutations in this population. Considering the recent case reports of G6PD deficiency-mediated hemolytic anemia in this malaria-endemic area and the implementation of military malaria chemoprophylaxis, ultimately, screening for deficiency prior to primaquine administration is essential.

Conclusions

In this G6PD deficiency prevalence study, we found that G6PD deficiency prevalence among 1,632 young soldiers was nearly 0. Although G6PD phenotypic mutations were not detected, we did identify 8 silent mutations (C1311T or T93C) for the first time in South Korea. Thus, we conclude that malaria

chemoprophylaxis is relatively safe against G6PD deficiency-mediated hemolytic anemia. However, given the number of individuals whose G6PD levels were at the lower bounds of the normal range and the frequent detection of G6PD deficiency-related mutations (C1311T or T93C) in our sample, consistent

Abbreviations

G6PD: Glucose-6-phosphate dehydrogenase deficiency;

ROK: Republic of Korea;

NADPH: Coenzyme nicotinamide adenine dinucleotide phosphate;

DMZ: Demilitarized zone;

RBCs: Red blood cells;

WHO: World Health Organization;

US-CDC: United States Centers for Disease Control and Prevention;

AFMC: Armed Forces Medical Command;

POC: Point-of-care;

PCR: Polymerase chain reaction;

KCDC: Korea Centers for Disease Control and Prevention

Declarations

Author's contributions

KTN contributed in designing, drafting and finalization of the manuscript. SEL conceived the idea and participated on the discussion. WSL contributed in overall research implementation. All authors read and approved the final manuscript.

Acknowledgements

None.

Competing interests

The authors declare no financial or commercial conflicts of interest.

Availability of data and materials

All data pertaining to this study are within the manuscript and the supporting files.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Armed Forces Medical Command

(AFMC) of the ROK (Approval No. AFMC-17-IRB-023). Written informed consent was

obtained from each participant.

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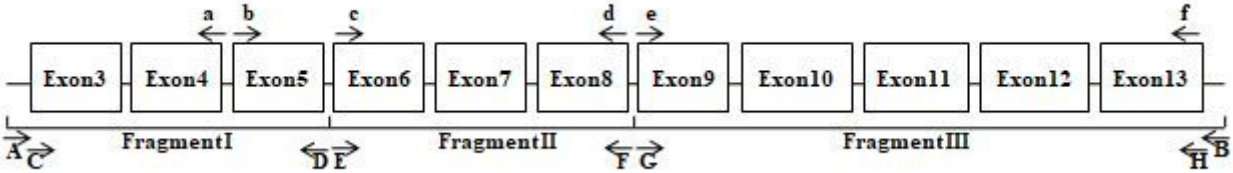
Figures

Number	Region	Age	Gender	G6PD (U/g Hb)	DiaPlexC	Mutation
1	Yangju	20-30	Male	**5.69 (< 80%)**	negative	**C1311T, T93C**
2		20-30	Male	**5.86 (< 80%)**	negative	**C1311T, T93C**
3		20-30	Male	**5.91 (< 80%)**	negative	**C1311T, T93C**
4		20-30	Male	**5.98 (< 80%)**	negative	**T93C**
5		20-30	Male	**6.03 (< 80%)**	negative	**T93C**
6		20-30	Male	**6.04 (< 80%)**	negative	**C1311T, T93C**
7	Paju	20-30	Male	**5.82 (< 80%)**	negative	**T93C**
8		20-30	Male	**6.01 (< 80%)**	negative	**C1311T, T93C**

Figure 1

Profiles of 8 participants with mutations Basic information (Region, age, sex, and G6PD activity) of participants with a C1311T exon mutation or a T93C intron mutation.



Sequencing primers information

a. 5` - CTG AAA TCT GGC CTC TGT CC - 3
b. 5` - GTT CAG CCC CAT CTT AGC AG – 3
c. 5` - TGG GAG GGC GTC TGA ATG AT - 3`
d. 5` - TGC CTT GCT GGG CCT CGA AGG -3
e. 5` - GGG CTG CAC ATC TGT GGC CA - 3`
f. 5` - TGC AGC TGA GGT CAA TGG TC – 3`

Nested PCR primers information of each fragment

A. 5 - GTT TAT GTC TTC TGG GTC AGG GAT GG - 3
B. 5 - AGT GTT GCT GGA AGT CAT CTT GGG T – 3
C. 5` - TGT ATC AGG CAA GAC AGA CA - 3`
D. 5` - ATG CTG CAT TCG CAG AGC AA -3
E. 5` - ACC ACA AGG TGG CAG CGT TG - 3`
F. 5` - TGC CTT GCT GGG CCT CGA AGG – 3`
G. 5` - CCA GGG ACG TGA TGC AGA AC - 3`
H. 5` - CAA TAA GCT CTG GGA CAG ACG A – 3`

Figure 2

Primer schematic for G6PD gene sequencing For further SNP evaluation of G6PD (from exon 3 to exon 13), nested PCR and sequencing primers were designed.

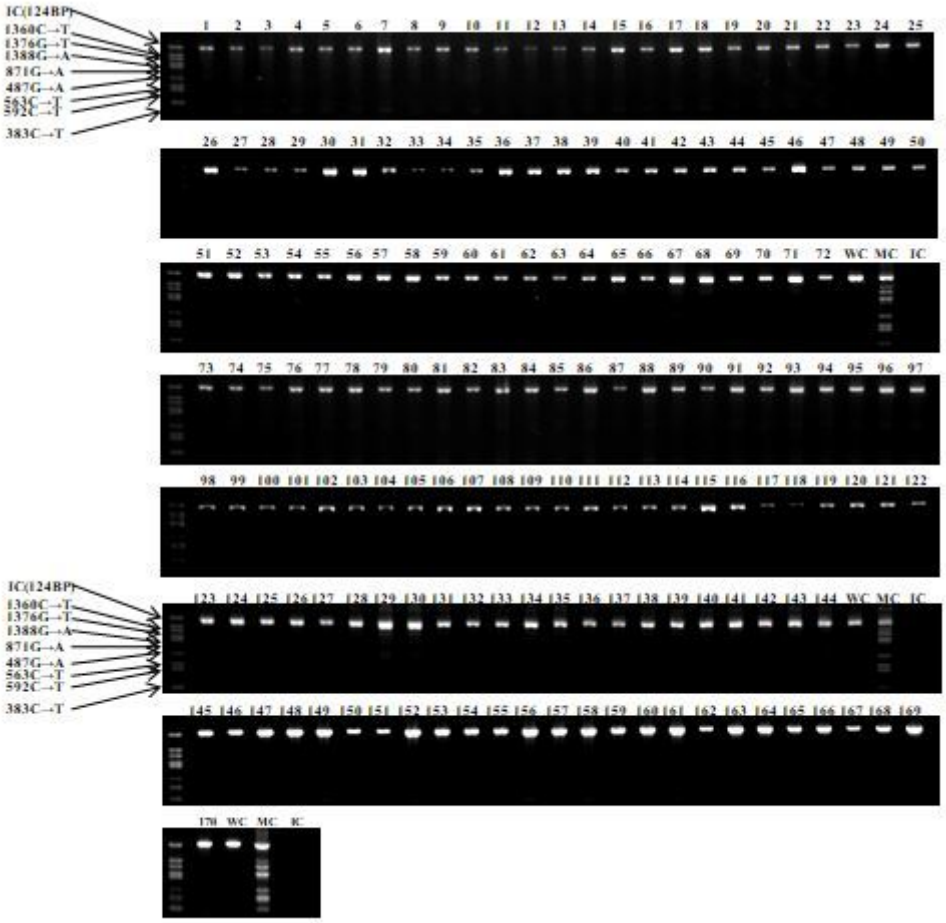


Figure 3

Screening results from seven representative G6PD variants using the DiaPlexC G6PD genotyping Kit (asian type) To detect seven different G6PD variants, including Vanua Lava (383 T>C), Mahidol (487 G>A), Coimbra (592 C>T), Viangchan (871 G>A), Union (1360 C>T), Canton (1376 G>T), and Kalping (1388 G>A), 170 blood samples representing different ranges of G6PD activity level (n=131 samples with 30%–80% (2.27–6.05 U/g Hb) of the median; n=3 samples with >150% (>11.373 U/g Hb) of the median; and n=36 samples within the normal range (4.6–13.5 U/g Hb) (82 samples from Paju and 88 samples from Yangju) were screened with the one-step PCR method of the DiaPlexC kit. IC: internal control, WC: wild-type control, MC: mutant-type control

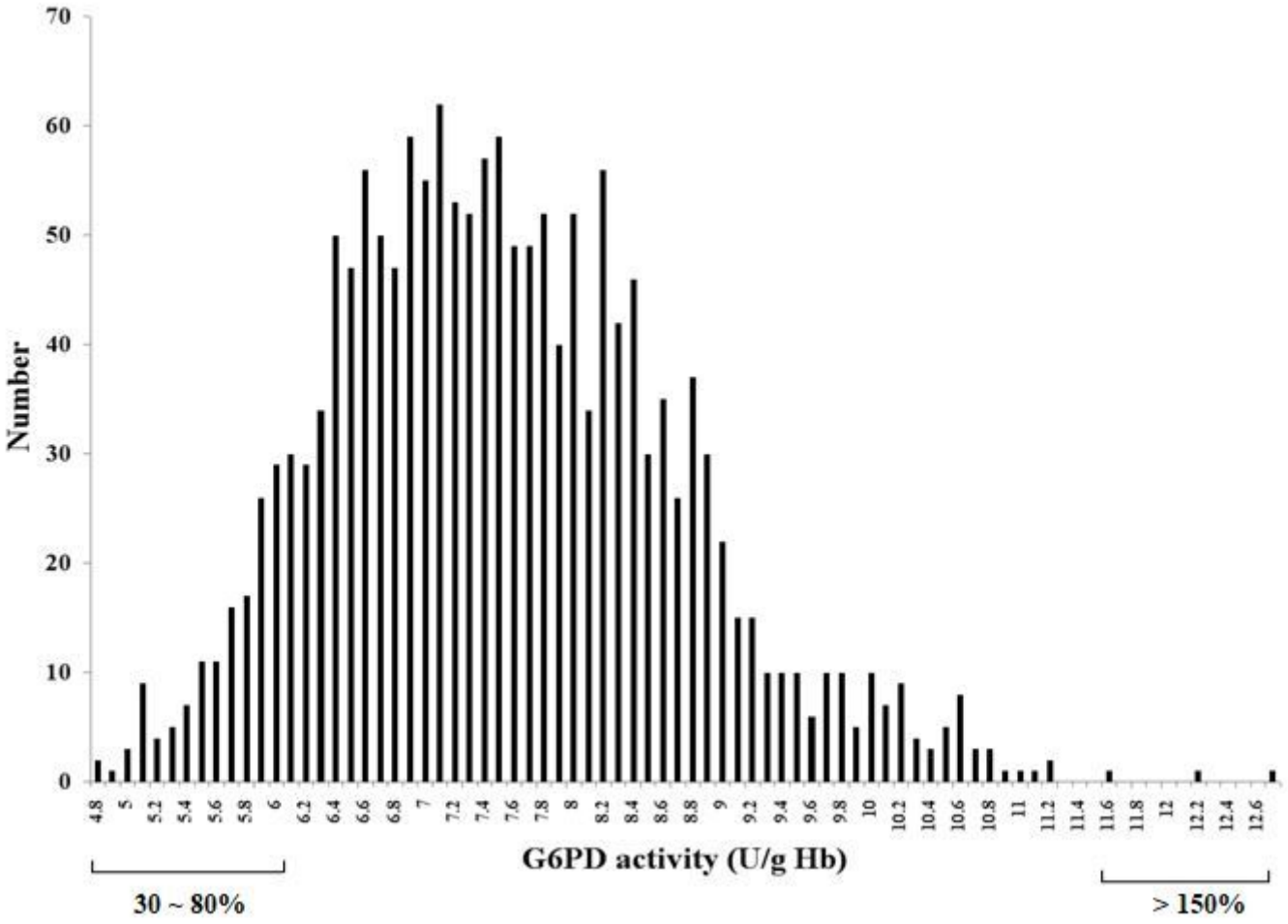


Figure 4

Distribution of G6PD activity G6PD activity values for all 1,632 participants fell within the normal range G6PD values (4.6–13.5 U/gHb), and the median value was 7.582 U/gHb.

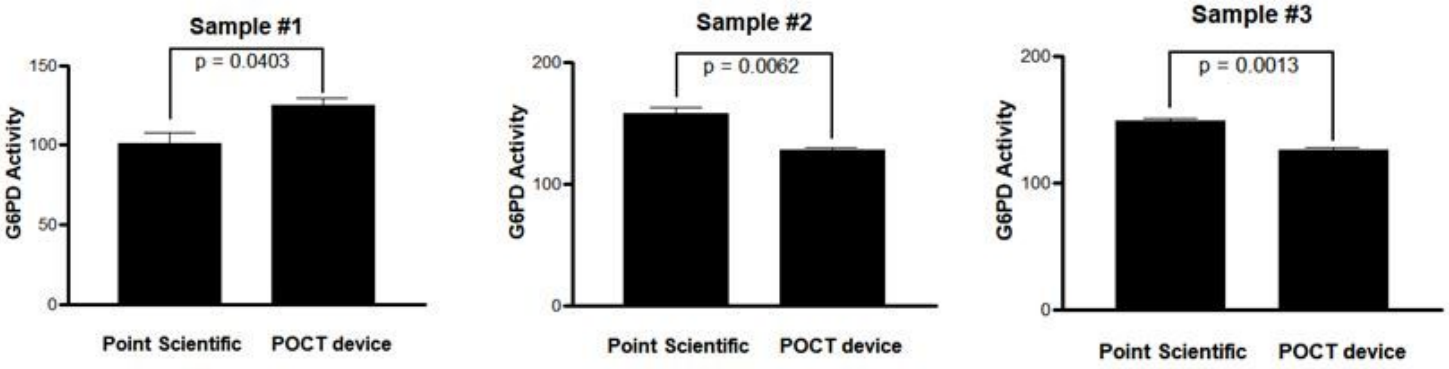


Figure 5

Comparison of two analytical methods for representing G6PD activity: Point Scientific G-6-PDH kit and CareStart G6PD Biosensor Using two analytical methods (Pointe Scientific G-6-PDH kit and CareStart G6PD Biosensor), G6PD activity from 3 representative samples are presented. Significance was calculated using an unpaired, two-tailed t-test (mean ± SEM; n=3 for both the Point Scientific and the POCT Analyzer tests).

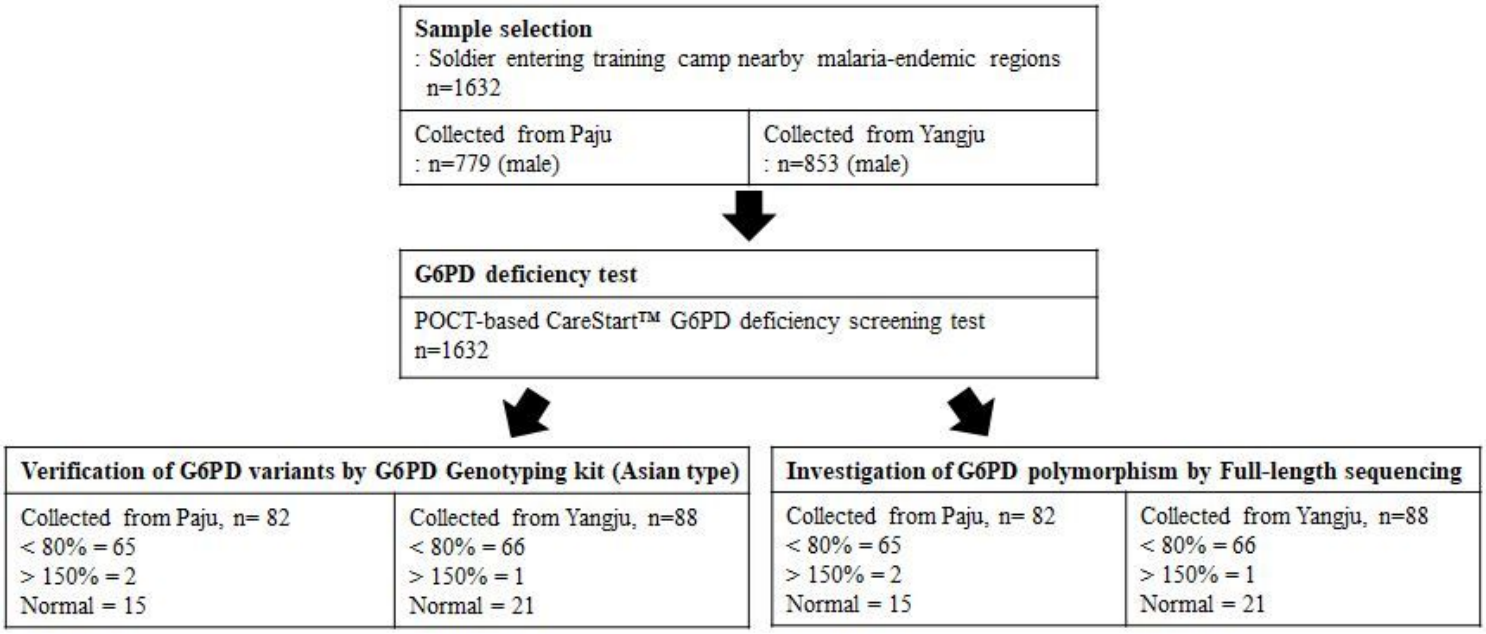


Figure 6

Schematic diagram of sample selection for enzymatic and genetic evaluation of G6PD deficiency In accordance with our IRB-approved (AFMC-17-IRB-023) protocol, blood samples were collected from soldiers who agreed to participate in the study. All 1,632 blood samples were collected in two cities, Yangju (n=853) and Paju (n=779) and were screened using the CareStart G6PD & Hb POC test. Based on screened results, 134 samples that were below (30%–80%; n=131) and above (>150%; n=3) the G6PD median value underwent genetic analysis using a G6PD genotyping kit and full-length sequencing. Thirty-six normal-range G6PD samples were also tested.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure220200427.pdf
- SupplementaryFigure120200427.xlsx