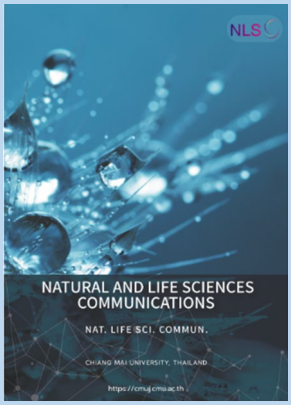
Research article



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Evaluation of Fluorescent Spot Test with Dried Blood Spots for Glucose-6-Phosphate Dehydrogenase Deficiency Screening in A Malaria-Endemic Area

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ABSTRACT

The utilization of dried blood spot (DBS) samples for screening glucose-6-phosphate dehydrogenase (G6PD) deficiency remains limited in resource-constrained and malaria-endemic regions of Thailand. This study evaluated the feasibility of employing DBS for G6PD deficiency screening among 242 participants (118 males and 124 females) who provided fresh whole blood (FWB) and DBS samples for analysis. The G6PD deficiency diagnostic performances of the fluorescent spot test using DBS (FST-DBS) were compared to those using FWB (FST-FWB). The G6PD gene variant was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using DBS samples. The FST-DBS showed no significant difference in sensitivity (95.0% vs 100.0%) but superior specificity (100.0% vs 80.2%) and a positive predictive value (100.0% vs. 50.0%) compared to FST-FWB. The PCR-RFLP revealed a G6PD mutation incidence rate of 16.5% (11.1% in males and 5.4% in females). Among 40 DBS samples, 39 (97.5%) were the Mahidol (487G>A) while a sample (2.5%) which both FST-DBS and FST-FWB showed positive results had an unidentified variant. Therefore, FST-DBS is an alternative format for G6PD deficiency screening in malaria-endemic regions, particularly where resources are limited.

Keywords: Glucose-6-phosphate dehydrogenase (G6PD) deficiency, Dried blood spot, Fluorescent spot test, Molecular diagnosis, Malaria-endemic area

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INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a hereditary disorder that follows an X-linked recessive pattern (Morris et al., 2022). The insufficiency or diminished activity of G6PD results in increased fragility of erythrocyte membranes, rendering them more susceptible to hemolysis upon exposure to oxidative damage and triggering the onset of acute hemolytic anemia (AHA), which can be severe or life-threatening (Luzzatto et al., 2020). The G6PD deficiency affects approximately 400 million individuals worldwide (Garcia et al., 2021), with a higher prevalence in Sub-Saharan Africa, Asia, Latin America, the Middle East, and the Mediterranean (Beutler, 1994; Nkhoma et al., 2009), especially in malaria-endemic areas. In Thailand, the frequency of G6PD deficiency is 16.9% of the total population (Nuchprayoon et al., 2002; Tanphaichitr, 1999), mainly found in rural areas bordering Myanmar, Cambodia, and Malaysia (Tananchai et al., 2019), with many gene variations depending on geographical origins, such as Mahidol (487G>A), Viangchan (871G>A), and Canton (1376G>T) mutations (Nuchprayoon et al., 2002; Tanphaichitr, 1999). Although most G6PD deficient patients may be asymptomatic in everyday life, specific individuals undergoing treatment with anti-malarial drugs such as primaquine (an 8-aminoquinolines medicine) may experience severe hemolytic anemia. Primaquine is exclusively utilized for radical cure of *Plasmodium vivax* infections (Watson et al., 2017). Therefore, in 2015, the World Health Organization (WHO) recommended screening for G6PD deficiency before primaquine treatment (Luzzatto et al., 2015).

The fluorescent spot test (FST) using fresh whole blood (FWB) sample is commonly used in Thailand for screening G6PD deficiency due to its high sensitivity and specificity (Beutler et al., 1979; Kaplan et al., 1997). Recently, a modified FST using FWB from a fingerprick was implemented in remote healthcare setting services, and the result was significantly promising (Saejeng et al., 2018). Although G6PD deficiency screening using fingertip blood samples can be performed in remote healthcare facilities, there are limitations in quality control and epidemiological surveys due to stability of G6PD activity during transportation. To address this issue, dried blood spot (DBS) samples may be an alternative format for diagnosing G6PD deficiency because they are easily collectible and transportable. The DBS samples have been used in low-resource settings for drug efficacy surveillance in Thailand. However, there is limited research on utilizing them for G6PD deficiency screening. Therefore, the aim of this study was to compare the performance of G6PD fluorescence spot test on FWB samples at fieldwork and DBS samples at regional laboratory, against PCR-RFLP (8 major genotypes). The use of DBS might serve a dual purpose in the community: quality control and surveillance of G6PD deficiency, as well as active malaria case detection. Field health workers or malaria staffs can collect blood samples for malaria testing and simultaneously collect DBS for G6PD screening. When a blood film tests positive for *Plasmodium vivax* malaria, the corresponding DBS can be utilized to screen for G6PD deficiency. This integrated approach facilitates efficient monitoring and treatment planning for affected individuals.

MATERIAL AND METHODS

Blood samples and G6PD deficiency test using FWB samples

This study was submitted to the Faculty of Associated Medical Sciences Ethics Committee at Chiang Mai University, Chiang Mai, Thailand, for ethical approval. The ethics committee waived the requirement for consent due to the

routine nature of G6PD deficiency screening using FST in malaria-endemic areas conducted by public health officers at Tambon Health Promotion Hospitals in Thailand. Additionally, measures were taken to ensure anonymity and confidentiality as both samples and associated data were untraceable, with no access to information identifying individual subjects during or after data collection (AMSEC-66EM-016). Blood samples were collected from 242 students and teachers of two Border Patrol Police Schools in Mae Sariang district, Mae Hong Son province, northern Thailand, by a public health officer through the fingerprick method. The 242 subjects were 118 (48.8%) males and 124 (51.2%) females. The mean age of the subjects was 10.1 years (SD = 6.0), ranging from 1 to 51 years. Onsite G6PD deficiency screening was performed using the FST according to the International Committee for Standardization in Hematology (ICSH) guidelines with minor modifications (Tripatara, 2003). Briefly, a small drop (approximately 10 µL) of FWB sample was transferred into 200 µL of G6PD screening reagent prepared immediately before use. The reaction was then incubated at room temperature and spotted onto a filter paper (Whatman standard qualitative filter Papers Grade 1, DDBiolab, Madrid, Spain) after incubation for 5 and 15 minutes, respectively. Subsequently, the spots were air-dried and examined under UV light with a wavelength range of 340-365 nm. Control samples representing both G6PD non-deficient and deficient were included in each assay. Positive results indicating G6PD deficiency were determined when opaque blood spots appeared under UV light either incubation times for 5 or 10 minutes. Whereas, the negative results indicating G6PD normal were determined when strong fluorescence was observed on both spots.

Preparation of dried blood spot samples

Four dried blood spots were prepared at the same time of blood sample collection for FWB-FST. This was done by spotting one drop (approximately 50-75 µL) of FWB on Whatman Grade 3MM Chr Cellulose Chromatography filter paper (Whatman International Ltd, WI, USA) and allowing it to air-dry at room temperature for 30-45 minutes. Each DBS card was individually packaged in a low gas permeable zip closure bag. A desiccant was added before the bag was sealed and stored at 4-8 °C during the fieldwork (Flores et al., 2017; Sudathip et al., 2021). Within three days after preparation, the DBS samples were transported under temperature-controlled conditions (4-8 °C) by post to the Central Laboratory of the Office of Disease Prevention and Control Region 1, Chiang Mai, Thailand, for FST-DBS and DNA analysis.

G6PD deficiency test and molecular analysis of G6PD variants using DBS samples

The G6PD deficiency screening by FST was performed within 24 hours after receiving the DBS samples. The DBS containing one drop of blood was punched using a standard paper hole punch size of 5 mm in diameter. This portion of 5 mm in diameter contained approximately 5-10 µL of blood (Kerti A, 2013; Quraishi et al., 2013), and it was transferred into 200 µL of G6PD screening reagent. The reactions were then incubated at room temperature and operated as described above. The DBS of control samples representing both G6PD non-deficient and deficient were also included in each assay.

The molecular identification of G6PD variants was further performed in all DBS samples which either FST-FWB or FST-DBS revealed the G6PD deficiency with opaque spots. DNA extractions from DBS samples were carried out using ChargeSwitch® kit technology (Invitrogen, CA, USA). Subsequently, eight G6PD variants commonly found in Southeast Asian Countries, including Vanua lava (383T>C), Mahidol (487G>A), Mediterranean (563C>T), Coimbra (582C>T), Viangchan (871G>A), Union (1360C>T), Canton (1376G>T), and

Kaiping (1388G>A) were detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis as previously described (Assefa et al., 2018; Nuchprayoon et al., 2002).

Statistical Analysis

The association between two categorical variables was tested by Fisher's exact test using the GraphPad Prism8 software. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficiency of FST-FWB and FST-DBS were calculated based on molecular analysis. The formulas were as follows:

Sensitivity = true positive/ (true positive + false negative) x 100%.

Specificity = true negative/ (true negative + false positive) x 100%.

PPV = true positive/ (true positives + false positive) x 100%.

NPV = true negative/ (true negative + false negative) x 100%.

Efficiency = (true positive + true negative)/ (true positive + true negative + false positive + false negative) x 100%

RESULTS

The qualitative determinations of G6PD deficiency using DBS samples did not fully match those using FWB samples in FST

The diagnosis of G6PD deficiency was performed on 242 subjects. The FST-FWB diagnosed 80 cases (33.1%) of G6PD deficiency, while the FST-DBS identified only 38 cases (15.7%). Results from the FST-DBS test indicated that the remaining individuals in the former group (42 samples; 17.4%) were non-G6PD deficient (Table 1). Fisher's exact test revealed a significant difference in the diagnosis of G6PD deficiency between two different sample formats ($P < 0.001$), suggesting further confirmation test such as DNA analysis is necessary.

Table 1. Number (%) of samples with G6PD deficiency tested by using FST with fresh whole blood (FWB) and dried blood spot (DBS) samples.

		FST-FWB		Total (%)
		G6PD deficiency (%)	Non-G6PD deficiency (%)	
FST-DBS	G6PD deficiency (%)	38 (15.7)	0 (0.0)	38 (15.7)
	Non-G6PD deficiency (%)	42 (17.4)	162 (66.9)	204 (84.3)
	Total (%)	80 (33.1)	162 (66.9)	242 (100.0)

Note: $P < 0.001$ by Fisher's exact test. FST-DBS: Fluorescent spot test with dried blood spot; FST-FWB: Fluorescent spot test with fresh whole blood

The FST using DBS exhibited higher specificity and positive predicted value than the FST using FWB.

Due to inconsistent results obtained from FST-FWB and FST-DBS, we then conducted a G6PD gene variations analysis by the PCR-RFLP method using DBS samples. The 40 of 80 samples (50%) exhibited variations. The 37 of 40

samples which revealed the G6PD deficiency with both FST-FWB and FST-DBS had Mahidol (487G>A) variant. This variant was also found in two samples which showed the G6PD deficiency with FST-FWB only. Moreover, there was a sample which revealed the G6PD deficiency with both FST-FWB and FST-DBS but had an unidentified variant (Table 2).

Table 2. Test results of FST-FWB and FST-DBS compared to PCR-RFLP for FST-positive samples.

		PCR-RFLP		Subtotal
		Variant	Non-Variant	
FST-DBS	G6PD deficiency (%)	38 (100.0) (37 Mahidol (487G>A)), 1 unidentified)	0 (0.0)	38 (100.0)
	Non-G6PD deficiency (%)	2 (1.0) (Mahidol (487G>A))	202 (99.0)	204 (100.0)
	Total (%)	40 (16.5)	202 (83.5)	242 (100.0)
FST-FWB	G6PD deficiency (%)	40 (50.0) (39 Mahidol (487G>A)), 1 unidentified)	40 (50.0)	80 (100.0)
	Non-G6PD deficiency (%)	0 (0.0)	162 (100.0)	162 (100.0)
	Total (%)	40 (16.5)	202 (83.5)	242 (100.0)

Note: FST-DBS: Fluorescent spot test with dried blood spot; FST-FWB: Fluorescent spot test with fresh whole blood; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

Based on the G6PD gene variation, the sensitivity, specificity PPV, and NPV of FST-FWB were 100.0%, 80.2%, 50.0%, and 100.0%, respectively. At the same time, the above indices were 95.0%, 100.0%, 100.0%, and 99.0%, respectively, of FST-DBS (Table 3). Fisher's exact test found no significant differences in sensitivity between the FST-FWB and FST-DBS but for a notable difference in specificity (Table 3). The efficient rate of FST-FWB was 83.5%, while FST-DBS was 99.2%. Therefore, FST-DBS has a higher specificity and PPV in diagnosing G6PD deficiency than FST-FWB.

Table 3. Diagnosis efficacy of FST-DBS and FST-FWB method in the diagnosis of G6PD deficiency.

	FST-DBS	FST-FWB	*P* value
Sensitivity	95.0%	100.0%	0.4937
Specificity	100.0%	80.2%	< 0.0001
PPV	100.0%	50.0%	
NPV	99.0%	100.0%	
Efficiency	99.2%	83.5%	

Note: Differentiation between the two groups was analyzed by Fisher's exact test. FST-DBS: Fluorescent spot test with dried blood spot; FST-FWB: Fluorescent spot test with fresh whole blood.

It is reliable that using DBS samples for molecular analysis to diagnose G6PD deficiency

To assess the reliability of DBS samples for diagnosing G6PD deficiency through PCR-RFLP analysis, we examined the frequency of the G6PD variant and its gender distribution in 242 subjects. The 40 (16.5%) subjects, including

27 (11.1%) males and 13 (5.4%) females, had G6PD gene mutation (Table 4), which aligned with an epidemiological survey of G6PD deficiency in Thailand (Tanphaichitr, 1999; Nuchprayoon et al., 2002). Among the 40 patients, 38 individuals (95.0%) were accurately identified by FST-FWB and FST-DBS, including 25 males and 13 females. The remaining 2 cases (5.0%) (males) were only identified by the FST-FWB.

Table 4. Incidence of both sexual subjects with G6PD variants.

Gender	G6PD deficiency			Non-G6PD deficiency (%)	Total (%)	*P* value
	Mahidol (487G>A)	Unidentified variant	Subtotal (%)			
Male	27	0	27 (11.1)	91 (37.6)	118 (48.7)	
Female	12	1	13 (5.4)	111 (45.9)	124 (51.3)	0.0146
Subtotal	39	1	40 (16.5)	202 (83.5)	242 (100.0)	

Note: $P < 0.05$ by Fisher's exact test.

DISCUSSION

G6PD deficiency should be checked in malaria patients before administering primaquine. However, screening for G6PD deficiency by the FST using DBS samples has been rarely studied and utilized in Thailand's resource-poor remote mountainous areas. The present findings demonstrated that the modified FST utilizing DBS samples could be reliably employed for G6PD deficiency screening programs, with a sensitivity of 95.0%, specificity of 100.0%, PPV of 100.0%, NPV of 99.0%, and efficiency of 99.2%. Previous research showed that drying samples at room temperature decreased G6PD enzyme activity by 30% and compromised the stability of stored dried blood spots (Solem et al., 1985), potentially causing false positives. However, our current study found no false positives despite air-drying DBS for 30-45 minutes at room temperature, followed by storage and transportation within a range of 4-8°C for 3 days. On the contrary, false positive results were observed in FWB samples (40 out of 80), likely due to the elevated hemoglobin level in FWB. The dark pigment shade and the quenching effect caused by hemoglobin components probably hinder fluorescence observation, leading to inaccurate outcomes (Schoos-Barbette et al., 1976). Utilizing DBS samples might mitigate these factors, resulting in a low false positive rate because the previous study showed that hemoglobin concentrations of DBS were lower than those of FWB samples (Kerti A, 2013).

The overall performance of FST-FWB, especially the negative predictive value of 100%, demonstrates its suitability for G6PD screening before administering anti-malarial drugs like primaquine. It is valuable in reducing the missed diagnosis and subsequent incidence of severe adverse reactions undergoing primaquine treatment. However, it should be noted that FST-FWB has a high false positive rate, potentially resulting in some malaria patients without G6PD deficiency losing the opportunity to administer primaquine. In addition, a previous study found that at 30% G6PD activity, the sensitivity for the FST is between 87.7% and 96.5% (Espino et al., 2016). This indicated the potential for false positives. Several factors could contribute to the occurrence of false positives in G6PD testing observed in the present study. Firstly, the interpretation of FST can be subjective, especially when using a low-intensity UV lamp powered by a solar cell, which can make the fluorescence spot less clear and harder to read accurately. Secondly, the storage of G6PD reagents poses challenges. The villages involved in this study are in remote areas without electricity, making it difficult to maintain the necessary cool temperatures for the

G6PD reagent. Consequently, the test reagent may become ineffective at detecting G6PD enzyme activity, leading to false indications of G6PD deficiency. Lastly, the intensity of fluorescent activity on the spotted filter paper diminishes when exposed to light, high humidity, and high temperatures. This exposure can affect the accuracy of the test results, potentially compromising the reliability of the G6PD enzyme activity readings.

On the other hand, utilizing FST-DBS, with its high specificity and positive predictive value (100%), can significantly reduce misdiagnosis, ensuring more accurate treatment for malaria patients. Therefore, during the initial mass screening process, DBS samples should be prepared simultaneously for FST-DBS tests, regardless of the results obtained from FST-FWB alone. The cross-validation of FST-FWB and FST-DBS enables improved identification of potential G6PD deficient patients. In cases with inconsistent results between FST-FWB and FST-DBS, quantitative or molecular analysis of G6PD deficiency should be conducted to further screen for false-negative patients. However, the methods for quantification of G6PD enzyme activity are expensive and very rarely used in Thailand. In the present study, G6PD enzyme activity was measured in two FWB samples where the FST-DBS revealed a false-negative result, and one sample where both FST-DBS and FST-FWB showed positive results while the PCR-RFLP was unable to identify the mutation types. These samples had G6PD enzyme activity lower than the cutoff value (3.8 U/g Hb) for G6PD deficiency of automated UV enzymatic analyzer (BS-360E, Mindray, Guangdong, China).

Given that the G6PD gene is located on the distal long arm of the X chromosome (Luzzatto et al., 2020), it is widely acknowledged that hemizygous males and homozygote females are more susceptible to FST screening than heterozygous females. Conversely, quantitative or molecular analysis of the G6PD gene is deemed more suitable for screening heterozygous patients. According to our current procedures using DBS samples, the frequency of G6PD deficiency was 16.5% (11.1% in males and 5.4% in females). The most common variant of G6PD deficiency was the Mahidol (487G>A) mutation type, accounting for 97.5%. It was consistent with previous studies using FWB samples (Tanphaichitr, 1999; Nuchprayoon et al., 2002; Khantikul et al., 2021). The findings demonstrated the feasibility of utilizing DBS samples for G6PD molecular analysis. However, further investigation is warranted to explore the quantification of G6PD enzyme activity using DBS. Moreover, an unidentified variant of the G6PD gene was observed in this study. Due to the fact that is study only tested for eight G6PD variants, the presence of other G6PD mutations potentially representing a novel mutation, could be the cause of G6PD deficiency. Whole genome sequencing should be conducted on this sample for proper identification. Nevertheless, additional follow-up investigations are necessary to ascertain its clinical significance.

CONCLUSION

The DBS samples should be incorporated during the initial mass screening process in malaria-endemic areas with limited resources, as well as be prepared concurrently for further molecular analysis. FST-DBS serves as a valuable complementary measure to FST-FWB in screening G6PD deficiency and facilitates the identification of potential non-G6PD-deficiency cases among malaria patients.

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AUTHOR CONTRIBUTIONS

Sakorn Pornprasert designed the study. Aungkana Saejeng, Yan Lin, Sarinee Srithep, Nardlada Khantikul, Pitchayadon Phukphakum, and Ying Lu performed experiments and analyzed data. Aungkana Saejeng, Yan Lin, and Sakorn Pornprasert discussed the study and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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