








RESEARCH ARTICLE

Diagnostic performances of the fluorescent spot test for G6PD deficiency in newborns along the Thailand-Myanmar border: A cohort study [version 1; referees: 3 approved]

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Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an inherited enzymatic disorder associated with severe neonatal hyperbilirubinemia and acute haemolysis after exposure to certain drugs or infections. The disorder can be diagnosed phenotypically with a fluorescent spot test (FST), which is a simple test that requires training and basic laboratory equipment. This study aimed to assess the diagnostic performances of the FST used on umbilical cord blood by locally-trained staff and to compare test results of the neonates at birth with the results after one month of age.




Methods: We conducted a cohort study on newborns at the Shoklo Malaria Research Unit, along the Thai-Myanmar border between January 2015 and May 2016. The FST was performed at birth on the umbilical cord blood by locally-trained staff and quality controlled by specialised technicians at the central laboratory. The FST was repeated after one month of age. Genotyping for common local G6PD mutations was carried out for all discrepant results.


Results: FST was performed on 1521 umbilical cord blood samples. Quality control and genotyping revealed 10 misdiagnoses. After quality control, 10.7% of the males (84/786) and 1.2% of the females (9/735) were phenotypically G6PD deficient at birth. The FST repeated at one month of age or later diagnosed 8 additional G6PD deficient infants who were phenotypically normal at birth.

Conclusions: This study shows the short-comings of the G6PD FST in neonatal routine screening and highlights the importance of training and quality control. A more conservative interpretation of the FST in male newborns could increase the diagnostic performances. Quantitative point-of-care tests might show higher sensitivity and specificity for diagnosis of G6PD deficiency on umbilical cord blood and should be investigated.

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Keywords

G6PD deficiency, Fluorescent spot test, Umbilical cord blood, quality control, neonatal screening



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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most prevalent X-linked enzymatic deficiency worldwide¹. Full enzymatic deficiency is observed in mutated hemizygous males and mutated homozygous females². Blood from heterozygous females contains both normal and deficient red blood cells in variable proportions, with a large distribution of enzymatic activities at population level. In populations with a high prevalence of G6PD deficiency, enzymatic activities have a bimodal distribution in adult males and a continuous distribution in adult females. Although most individuals with G6PD deficiency are asymptomatic, the genetic condition can cause acute haemolysis after exposure to fava beans, certain drugs, chemicals, or infections. In neonates, G6PD deficiency increases the risk of sepsis³, severe neonatal hyperbilirubinemia, and kernicterus⁴. G6PD deficiency is a risk factor for neonatal hyperbilirubinemia, in mutated hemizygous males⁵ and mutated homozygous and heterozygous females⁶, even without exposure to known haemolytic agents⁷. Early recognition of G6PD deficiency in newborns, prompting the implementation of useful prevention strategies such as targeted follow-up, counselling on trigger avoidance, promotion of breast milk intake, and the use of effective phototherapy, can help prevent kernicterus⁸. Routine G6PD deficiency screening in newborns is recommended by the World Health Organization if the prevalence of G6PD deficiency reaches 3–5% among males⁹.

The G6PD fluorescent spot test (FST)¹⁰ is an inexpensive and reliable qualitative phenotypic test^{11,12}; however it requires a cold chain for reagents and a basic training for the users, which limits its use in the field. The FST has a sensitivity and specificity above 95% for the diagnosis of G6PD deficiency in adults with less than 30% enzymatic activity^{12–14}. With this threshold, the test correctly diagnoses mutated hemizygous males, mutated homozygous females and G6PD heterozygous females with low enzymatic activity. Subjects with intermediate to normal enzymatic activity are diagnosed as G6PD normal by this method.

The highest prevalence of G6PD deficiency is found in malaria endemic regions^{1,15}. In the population attending Shoklo Malaria Research Unit (SMRU) clinics, the prevalence of G6PD deficiency is 13.7% in adult males¹⁶ and 2–4% in adult females¹⁷ and neonatal hyperbilirubinemia is common¹⁸. Routine neonatal screening for G6PD deficiency is therefore recommended in this setting⁹.

Umbilical cord blood collection is easy, can spare the neonate unnecessary pain, and results of cord blood G6PD FST are comparable to that of peripheral blood drawn in the first week of life in a recent study¹⁹. However, there is evidence suggesting that the diagnosis performed at birth should be confirmed later in life, because of a potentially higher G6PD activity in neonates compared to adults^{20–23}.

As part of a cohort study on neonatal hyperbilirubinemia in this population, we carried out an analysis with two objectives: to assess the diagnostic performance of the FST used by locally trained staff on umbilical cord blood, and to compare test results at birth and after one month of age.

Methods

The study was conducted at the SMRU, located along the Thailand-Myanmar border in the north-western province of Tak, Thailand. The population attending SMRU clinics is composed of migrants and refugees. The staff of SMRU clinics consists of locally trained medics, midwives, nurses, health workers and laboratory technicians. The field clinics including the laboratories have stable electricity and are equipped with basic equipment and refrigerators.

Newborns enrolled in a cohort study between January 2015 and May 2016²⁴ who were born after 28 weeks of gestational age and had a G6PD FST performed on umbilical cord blood were included in the analysis. The FST was performed using five microliters of blood that were mixed with 100 µl of reagents (R&D Diagnostic, Greece), incubated for 10 minutes at room temperature, spotted on filter paper and air dried. The spot tests were then visualised under UV light by a locally trained laboratory technician. Spot tests showing intermediate to normal fluorescence were classified as G6PD normal, while those showing no fluorescence were classified as G6PD deficient. Spot tests were transported within 12 hours to the central haematology laboratory in Mae Sot where they were re-examined by qualified laboratory technicians for quality control (QC). Data were analysed using SPSS (IBM SPSS Statistics 23, IBM Corporation). Sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated to evaluate the concordance between the FST performed in the clinics and the QC. The QC results were used to calculate the prevalence of G6PD phenotype at birth.

Neonates were followed up and after one month of age a capillary blood sample was tested with the FST by a locally trained laboratory technician. Samples with discrepant QC results at birth, and with different results at birth versus after one month of age were genotyped using established PCR-RFLP protocols for Mahidol, Chinese-4, Canton, Viangchan, Mediterranean and Kaiping mutations²⁵.

Ethical statement

The study was approved by the Ethic Committees of the University of Oxford, UK (OXTREC 41-144) and the Faculty of Tropical Medicine, Mahidol University, Thailand (TMEC 14-012). The Tak Community Advisory Board, consisting of members of the local community, also revised and approved the study (TCAB-08-13).

The study was explained in the preferred local language by a trained counsellor during pregnancy and the mothers who agreed in participating signed an informed consent.

Results

G6PD testing on umbilical cord blood

FST on umbilical cord blood was performed in 1521 newborns; 51.7% (n=786) were males and 4.5% (n=68) were born before 37 weeks of gestation. Results of QC performed in the central laboratory were available for all tests. The results are shown in Table 1. Among the 1434 tests interpreted as normal at the clinic, 0.6% (6 males and 2 females) were re-classified as deficient by the QC. Among the 87 tests interpreted as deficient at the clinic, 2.3% (one male and one female) were re-classified as normal by the QC. Genotyping for the 10 discrepant results supported the interpretation of the QC. Proportions of errors in the interpretation of the test were similar across clinical sites. The calculated sensitivity, specificity, PPV and NPV for the FST at the clinics were 91.4% (95 % CI: 91.4- 91.4), 99.9% (95 % CI: 99.9- 99.9), 97.7% (95 % CI: 96.9-98.5) and 99.4% (95 % CI: 99.8-100.0) respectively.

Overall, 10.7% of the males (84/786) and 1.2% of the females (9/735) were diagnosed with a G6PD deficient phenotype at birth.

Repeated G6PD testing on infant after one month of age

The FST was repeated at least one month after birth in 1430/1521 neonates (Figure 1) and results were compared with those of the QC at birth (Table 2).

Among the 90 G6PD deficient infants at birth, one male had an FST performed after one month of age interpreted as normal. However, the genotyping showed that he was hemizygous for Mahidol variant, confirming the diagnosis of G6PD deficiency done at birth.

Among the 1340 G6PD normal newborns, 10 (9 males and one female) were diagnosed as deficient by FST when re-tested after one month of age. Genotyping confirmed that, among 8 males, 7 were hemizygous for Mahidol variant and one for Canton variant. The remaining two (one male and one female) had a wild type genotype for the tested mutations; we could not confirm whether they were carriers of uncommon G6PD mutations or the

Table 1. Results of the fluorescent spot test performed at the clinics and in the central laboratory (quality control).

		Quality control	
		Deficient	Normal
Clinics	Deficient	85	2*
	Normal	8**	1426
	Total	93	1428

*1 wild type male, 1 wild type female

**6 hemizygous Mahidol males, 1 heterozygous Mahidol female and 1 homozygous Mahidol female

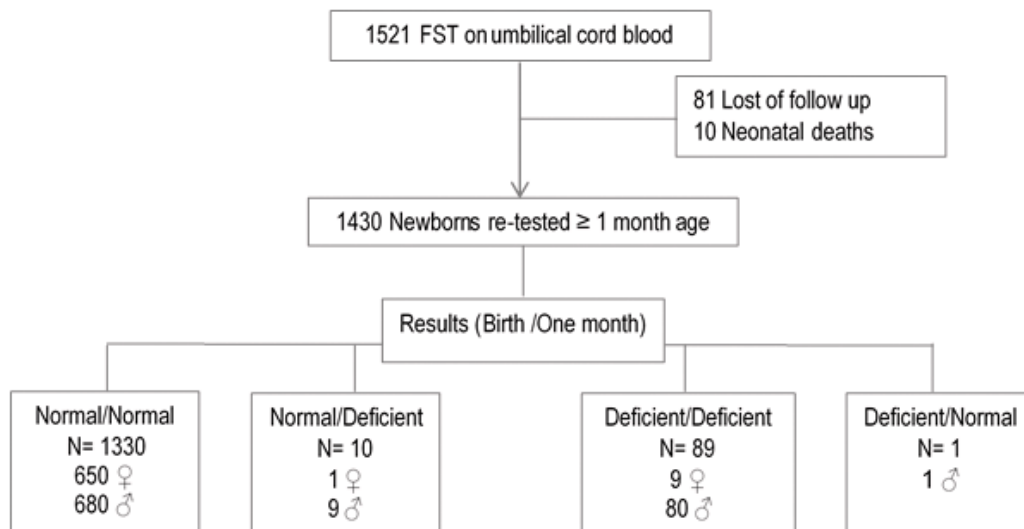


Figure 1. The number of patients analyzed at birth and after one month of age.

Table 2. Results of the fluorescent spot test performed at birth and repeated after 1 month of age.

		FST ≥ 1 month of age	
		Deficient	Normal
FST birth	Deficient	89	1*
	Normal	10**	1330
	Total	99	1331

*Mahidol hemizygous male

**7 Mahidol hemizygous male, 1 Canton hemizygous male, 1 wild type male, 1 wild type female

FST at follow-up was mis-interpreted. Overall, we estimated that at least 8 of the 99 deficient infants (8.1%) tested after one month of age were misdiagnosed as G6PD normal at birth.

Discussion

The fluorescent spot test has been performed in the SMRU clinics for almost 10 years, showing reliable results in adult patients¹⁶. It has also been used for diagnosis of G6PD deficiency in jaundiced neonates¹⁸, but an assessment of diagnostic performance in routine testing of newborns has never been done until the current study. The results show that among 1521 newborns tested for G6PD deficiency, 0.6% were misclassified by the locally-trained clinical staff. Importantly, 8 of them, including 6 hemizygous males, were diagnosed as normal but the QC and genotyping showed they were indeed deficient, highlighting that the misclassification failed to identify 8.6% (8/93, Table 1) of G6PD deficient newborns, who would be at risk of developing hyperbilirubinemia⁹.

Furthermore, when the FST was repeated in infants after one month of age, 10 infants diagnosed as normal at birth had a deficient FST and 8 of them were confirmed to be deficient by genotyping. The one infant who had a deficient FST at birth and a normal result at one month was confirmed as a mutated hemizygous male, suggesting that the result at one month follow up was a classification error.

Failure to diagnose G6PD deficiency at birth has been observed before in other settings^{22,26,27}. Blood samples from newborns have higher G6PD activity than adults^{20,21}; since G6PD activity is higher in immature blood cells compared to mature erythrocytes²⁸, higher activity in neonatal blood could be explained by the higher number of reticulocytes²⁹ and the higher proportion of immature reticulocytes in umbilical cord blood³⁰. The FST is a qualitative test and only allows a reliable diagnosis for binary results whereby samples

with intermediate fluorescence are assigned a normal phenotype. When the enzymatic activity of a sample is close to or slightly over the 30% threshold, as might be the case in deficient samples with reticulocytosis, the subjective interpretation of the test can become very difficult. In this study, we hypothesise that the experienced laboratory technicians gave a more conservative interpretation of the tests performed at birth than the locally-trained staff.

Our results highlight the importance of regular training for laboratory technicians and routine quality controls on test procedure and interpretation to assure stable performances of the FST; furthermore they indicate a specific short-coming of the use of a qualitative test for G6PD screening on umbilical cord blood samples. We suggest that the training of laboratory technicians focuses in particular on male newborns who have an FST with intermediate fluorescence. These neonates should be considered G6PD deficient because they are likely mutated hemizygous. In female newborns with an intermediate fluorescence FST, a systematic more conservative interpretation of tests would instead increase the number of false deficient results. The lower test specificity would likely increase health care workers workload and would create unnecessary stress and confusion in families. However, caretakers should be aware of the limited reliability of the test in newborns and should provide routine surveillance of neonates, parental education about exposure to triggers of haemolysis and signs of jaundice before discharge, regardless of the neonate's G6PD diagnosis³¹. In settings where the G6PD neonatal screening is carried out, re-testing at an older age is rarely done. G6PD status should be re-assessed when the child is at least 6 months old (when steady-state G6PD activity has reached the level seen in adulthood²⁰) if oxidant treatments need to be administered. This is especially important in some areas such as malaria endemic areas where a long course of primaquine is needed for *Plasmodium vivax* radical cure.

In conclusion, the results of this study show that the FST does not perform well in newborns, suggesting that a quantitative G6PD test would be more sensitive in diagnosing neonates at risk of hyperbilirubinemia^{6,21,29,32,33}. Laboratory-based G6PD quantitative tests are expensive and require skilled technicians and well-equipped laboratories. However, point-of-care quantitative tests for G6PD have been developed in the past few years^{34–36}. Upon validation, they should be available in resource-limited settings for the screening of G6PD deficiency in newborns.

Data availability

Due to ethical and security considerations, the data that supports the findings in this study can be accessed only through the Data Access Committee at Mahidol Oxford Tropical Medicine Research

Unit (MORU). The data sharing policy can be found here: <http://www.tropmedres.ac/data-sharing>. The application form for datasets under the custodianship of MORU Tropical Network can be found in [Supplementary File 1](#).

Competing interests

No competing interests were disclosed.

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Supplementary material

Supplementary File 1: Application form for datasets.

[Click here to access the data.](#)

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This paper accurately points out the advantages and disadvantages of the FST for detecting G6PD deficiency in neonates and adds to the body of literature out there regarding its use. I however feel the authors overstate the limitations in the discussion when they say in the discussion that “the FST does not perform well”. The FST is done almost exclusively in LMICs where quantitative tests are not routinely available and if available are often too expensive to use outside of a research study.

1. Compared to the alternative which is usually no G6PD test having a false negative result of 0.6% is quite acceptable and still allows appropriate counseling and treatment of the majority of the neonates.

2. The do not provide the data to support their statement that that males with intermediate fluorescence be considered. It would be better if they would supply this data.

3. The improved results in the central laboratory highlight the need for ongoing education, supervision and oversight needed when having a test run by non-laboratory personnel but again don't negate the benefits of having the FST available for routine use. 3. Finally, no one would argue that continuing to push for a low-cost reliable and widely available quantitative test should be our goal. In the meantime, the FST fills an important gap. I think these points are important to make in the paper.

One other very minor suggestion that would help clarify 2018 terminology would be to say “Early recognition of G6PD deficiency in newborns, prompting the implantation....can help prevent *acute bilirubin encephalopathy and thus kernicterus/kernicterus spectrum disorder*.”¹

References

1. Le Pichon JB, Riordan SM, Watchko J, Shapiro SM: The Neurological Sequelae of Neonatal Hyperbilirubinemia: Definitions, Diagnosis and Treatment of the Kernicterus Spectrum Disorders (KSDs). *Curr Pediatr Rev.* 2017; **13** (3): 199-209 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Pediatric Global Health including neonatal hyperbilirubinemia and G6PD deficiency

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 26 February 2018

doi:[10.21956/wellcomeopenres.14520.r30556](https://doi.org/10.21956/wellcomeopenres.14520.r30556)



Ari W Satyagraha

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This is a good study that is currently needed to inform whether G6PD FST test can be used to screen for G6PD deficiency in newborns. There are not many tests available for screening G6PD in newborns, especially quantitative tests that can be used in remote areas where proper laboratories are not available and hence rely on qualitative ones. Since the test used in this study is qualitative, it may missed the intermediate G6PD activities. As Mahidol is the most dominant G6PD variant in the region, it may give a bit of an interpretation problem because it usually present itself in the intermediate G6PD activities.

As the authors have discussed, quantitative tests may be used to screen for G6PD in newborns but the tests' cut offs have to be adjusted for newborns since they have higher reticulocytes (thus higher G6PD activities) than adults.

However, I have difficulties in finding the numbers of deficient males (84/786) and deficient females (9/735) from. I could not clearly find it from Table 1 or Figure 1.

In general, I found this study interesting and very helpful and the number of subjects screened were statistically sound.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: G6PD genetics and epidemiology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Mar 2018

Laurence Thielemans, 1Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand

We appreciate the comments of the reviewer and we agree with her that showing the results according to gender is usually preferred for G6PD data. In this case the focus of the analysis was the overall quality of the test in identifying phenotypic deficiency; therefore for simplicity we had pooled the results of males and females in Table 1. In particular, among the 85 deficient subjects identified in the clinics and confirmed deficient by QC, 78 were males and 7 were females. Among the 1428 subjects identified as normal in the clinics and in the QC, 701 were males and 725 were females. For the discrepant results between the clinics and the laboratory QC, the sex and genotypes were already specified in the footnote of Table 1.

Competing Interests: No competing interests were disclosed.

Referee Report 15 January 2018

doi:[10.21956/wellcomeopenres.14520.r29386](https://doi.org/10.21956/wellcomeopenres.14520.r29386)



Issarang Nuchprayoon

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This work addresses an overlooked issue of underdiagnosis for G6PD deficiency when cord blood are used for G6PD testing. The methodology is sound and number of subjects were adequate.

The underdiagnosis may occur with qualitative test such as FST, as quantitative test will adjust for hemoglobin concentration to yield IU/g Hb. In the discussion, the author may consider a possibility that FST may underdetect deficiency in cord blood as hemoglobin concentration in cord, 15.7 +/- 1.1 g/dL [1], is much higher than one-month old babies, 11.7 +/- 1.6 g/dL [2]. The 25% higher red cell mass and G6PD

activity could affect qualitative assessment of fluorescence in deficient samples.

Another point that should be mentioned is that most G6PD deficient neonates of Karen origins are G6PD Mahidol, a mildly deficient variant. Some males with residual but deficient G6PD activity may fluoresced bright enough in FST and called normal at higher hemoglobin concentration such as cord blood. This phenomenon might not occur in severely deficient variant such as G6PD Viangchan.

References

1. Ozolek J: Cord Blood Hemoglobin Screening: Normal Values, Sex Differences, Is it Necessary? 1314. *Pediatric Research*. 1998; **43**. [Publisher Full Text](#)
2. de Fátima da Silva Vieira Marques R, Augusto de Aguiar Carrazedo Taddei J, Ancona Lopez F, Aparecida Pellegrini Braga J: Hemoglobin Values in the First Six Months of Life in Exclusively Breastfed Children. *Open Journal of Blood Diseases*. 2013; **03** (04): 130-135 [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Molecular genetics of G6PD deficiency

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Mar 2018

Laurence Thielemans, 1Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand

We appreciate the comments of the reviewer. We don't really know how higher hemoglobin concentrations influence the reading of the FST, we did observe in the past that subjects with anemia tend to have a brighter FST when compared with subjects with normal hemoglobin concentration (Bancone et al., 2015). Nonetheless, this aspect certainly deserves more attention and we hope to investigate it further in the future. As for the Mahidol variant, we have never observed adult males with intermediate activity in the Karen population; depending on the reagents

used for the gold standard spectrophotometric assay, the median enzymatic activity in Mahidol hemizygous males was well below 10% of the population median (using the WHO protocol with home-made reagents and after removal of WBCs from blood sample, Bancone et al., 2014) or 12.8% of the population median (using commercial kits on whole blood samples, Bancone et al., 2016). In the Karen population the allelic frequency of Viangchan mutation is very low so we have never been able to analyze an appropriate number of Viangchan hemizygous males to assess the enzymatic activity.

Competing Interests: No competing interests were disclosed.
