Performance Comparison between Conventional Fluorescent Spot Test and Quantitative Assay in Detecting G6PD Deficiency

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Abstract

Objectives: Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy in the world. Some countries in the Asia Pacific region have practiced conventional introduced Fluorescent Spot Test (FST) as a neonatal screening method since the 1980s. However, FST has its own limitations. Quantitative assays such as the careSTART™ Biosensor1 has been demonstrated to be able to overcome some of the limitations of FST. It is important to assess the performance of FST for laboratories currently using this cost-saving method. The objective of this study is to compare the performance of G6PD assays of FST with careSTART™ Bioensor1 and to analyse the difference in prevalence of G6PD deficiency in neonates between both methods.
Methods: This was a cross sectional study involving 455 neonates born in Hospital Universiti Sains Malaysia (Hospital USM), Kelantan, Malaysia between June 2020 until December 2020. Two millilitres of cord blood were taken in EDTA bottles to be analysed with careSTART™ Biosensor1 and at the same time, dried cord blood spots were sent for FST. This study was carried out with the ethical approval of Human Research Ethics Committee (HREC) of Universiti Sains Malaysia. Data was recorded and analysed using the Statistical Package for the Social Software (SPSS) version 27. Sensitivity, specificity, positive predictive value and negative predictive value were calculated to determine the performance of FST at specific G6PD cut off value and Cohen's \( \kappa \) analysis was determined to see the agreement between these two methods.

Results: The sensitivity of FST was 91% (95% confidence interval (CI): 57-100), whilst its specificity was 97% (95% CI: 95-98) at 30% cut-off G6PD activity level. In contrast, at 60% cut-off G6PD activity level, the sensitivity drastically decreased to 29% (95% CI: 19-40) whilst the specificity was 100% (95% CI: 98-100). The overall prevalence of G6PD deficiency was 5.1% and 17.8% for FST and Biosensor1 respectively, demonstrating a significant difference between the two tests (p<0.001).

Conclusions: FST was shown to have low sensitivity at 60% cut-off G6PD level. This cut-off level reflects intermediate G6PD activity, hence FST missed a significant proportion of G6PD intermediate individuals in this study. At the same time, the prevalence of G6PD deficiency significantly increased with the use of Biosensor1. FST misclassified high proportions of G6PD intermediate individuals as normal, rendering them susceptible to oxidative stress.
Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathy globally, affecting up to 400 million individuals. The highest prevalence of the disease is in Africa, Southern Europe, and Asia, especially the Middle East and Southeast Asia. The distribution of the disease mirrors the distribution of malaria, which reflects the notion that G6PD deficiency confers some protection against malaria. The inheritance of G6PD deficiency follows an X-linked pattern, hence males can be either hemizygous normal or hemizygous deficient, whereas females may be either homozygous normal, homozygous deficient or heterozygous. [1]

G6PD is an enzyme that catalyses the first reaction in the pentose phosphate pathway. This pathway is crucial in providing pentose sugars from glucose for glycolysis and also nicotinamide adenine dinucleotide phosphate (NADPH), which provides reducing power to red blood cells. [2] G6PD breaks down glucose by catalysing the oxidation of β-D-glucose-6-phosphate to D-glucono-1,5-lactone-6-phosphate. The by-product of this reaction is NADPH. D-glucono-1,5-lactone-6-phosphate is then hydrolysed, forming 6-phosphogluconate which will then be decarboxylated by 6-phosphogluconate dehydrogenase (6PGD) enzyme. This reaction will yield the five-carbon molecular ribulose 5-phosphate (Ru5P), which is a precursor of DNA, RNA, and ATP and concomitantly generates another NADPH molecule. [3] In the red blood cells, the pentose phosphate pathway is the only source of NADPH due to the absence of
mitochondria. NADPH is crucial in protecting the cells against reactive oxygen species (ROS) and is involved in the glutathione pathway. In the glutathione pathway, the electron on NADPH is donated to glutathione dimers, becoming oxidized glutathione/glutathione disulfide (GSSG). This reaction is catalysed by glutathione reductase enzyme which will produce two reduced glutathione monomers (GSH), which are the first line of defense against ROS. \cite{2} NADPH is also needed to reduce GSSG and the sulfhydryl groups of some necessary proteins which protect against oxidative stress. If this protection against ROS is absent, the red blood cells can undergo oxidative haemolysis.\cite{3}

Since males are hemizygous for the G6PD gene, they can be frankly G6PD deficient or have normal level of G6PD. Females, on the other hand, have two copies of the G6PD gene on each X chromosome, so they can have normal gene expression or be heterozygous. In places where the frequency of the G6PD deficient allele is very high, it is not rare to find homozygous females. Because of lyonisation or X-chromosome inactivation, heterozygous females are genetic mosaics and the abnormal cells of a heterozygous female can be G6PD deficient or G6PD intermediate, rendering these females to be susceptible to oxidative stress and potential related complications.\cite{2}

G6PD deficiency can be diagnosed using quantitative or qualitative/semi-quantitative tests. One qualitative test that has been used extensively is the fluorescent spot test (FST). FST has been introduced as a national screening test for G6PD deficiency in Malaysia since the 1980s. FST is much cheaper than other quantitative G6PD enzyme assays and can give reliable binary results (deficient and normal). However, several studies have shown that the FST can miss a significant proportion of individuals with intermediate levels of G6PD.\cite{4} This is detrimental because even individuals with
intermediate levels of G6PD can have haemolytic crisis. On the other hand, several quantitative tests are available to quantify G6PD activity such as the spectrophotometric assay, which is the current gold standard, and point-of-care quantitative assay such as BioSensor1. Quantitative enzyme assays can quantify the amount of G6PD activity either by normalization of haemoglobin or red blood cell count.[5]

The objectives of this study are to compare the performance of FST with careSTART™ Bioensor1 and to analyse the difference in the prevalence of G6PD deficiency in neonates between both methods.

Method
A cross-sectional study was conducted amongst neonates born in Hospital USM, located in the Kelantan State, North-eastern region of Malaysia. The sample collection period was taken from June 2020 until December 2020. This study was carried out upon receiving the ethical approval of the Human Research Ethics Committee (HREC) of Universiti Sains Malaysia. Random sampling was performed, taking only samples that fulfilled the inclusion and exclusion criteria. The inclusion criteria included all cord blood samples sent within the study frame whilst the exclusion criteria included any clotted cord blood samples, neonates with severe congenital anomaly and neonates with severe intrauterine growth restriction. Two millilitres of cord blood were taken in EDTA bottles during delivery for quantitative enzyme activity measurement by careSTART™ Biosensor1 as a reference method. For FST, one drop of cord blood was directly placed on a piece of filter paper and allowed to dry completely before placing it in a biohazard bag. After labelling the biohazard bag, the sample was sent to the laboratory within 4
hours of collection. All fluorescent spot tests samples were analysed within 24 hours of sample receipt.

The FST was performed using Atlas Medical G6PD Kit which is based on Ultraviolet Light FST method with modified GSSG. The principle behind the test is that in a normal patient, NADPH generated by G6PD enzyme present in a lysate of blood cells fluoresces under long-wave UV light. In G6PD deficient patients, insufficient NADPH is produced, hence resulting in a lack of or no fluorescence. For the test, 100μL of the working mix were pipetted into each of controls and sample tubes. The samples were mixed well with the working mix and incubated at 37°C in a drying oven for 30 minutes. After drying the spots were observed under fluorescent UV light using a UV viewing box (365nm wavelength). The results were then recorded and validated. The results were then reported as ‘Normal’ if the spot fluorescence under UV light, ‘Intermediate’ if the spot fluorescence slightly under UV light and ‘Deficient’ if the spot does not fluorescence under UV light.

The quantitative assay on the other hand was performed using careSTART™ Biosensor1 (WELLS BIO, INC. Korea). This method uses an electrochemical method to measure the enzyme activity in a sample. It measures the electron transfer from NADPH into reduced NADPH by the presence of G6PD enzyme. The magnitude of the electric current that is produced is directly proportional to the level of G6PD activity in the blood sample. Before this test was performed, EDTA tubes filled with cord blood samples were arranged on test tubes racks for thirty minutes to let the samples come up to room temperature. Haemoglobin strip and G6PD strip were inserted at the designated spots on
the analyser and 20 µL of blood was pipetted on each strip. Reading from the machine was then recorded onto the worksheet.

Data were recorded and analysed using SPSS version 27. The reference range for Biosensor 1, manufacturer’s reference range and cut off value that has been verified by in house laboratory, the Clinical and Laboratory Standard Institute (CLSI) approved transference method were used.[6] The cut-off values are as follows: less than 30% of mean normal G6PD activity (<2.8 U/gHb) are categorised as deficient, 30%-60% (2.8 - 5.6 U/gHb) as intermediate and >60% (>5.6 U/gHb) as normal. Sensitivity, specificity, positive predictive value, negative predictive value, and prevalence were calculated for FST using the afore-mentioned cut-off value obtained from Biosensor1. Cohen's κ analysis was determined to see the agreement between these two methods. Since the clinical implications for both deficient and intermediate groups are the same, both deficient and intermediate groups were classified as deficient. The Paired McNemar Test was used to analyse the difference in prevalence between FST and Biosensor1, subsequently a p-value of less than 0.05 was then considered statistically significant.

**Results**

In this study, a total of 455 samples were obtained. There were 238 (52.3%) females and 217 (47.7%) males. The majority, 443 (97.4%), of the samples were of Malay descendants followed by, Thai (0.9%), Arab (0.7%), Rohingya (0.7%) and Chinese (0.4%). A large majority of the samples (81.5%) were from term neonates and 18.5% were preterm neonates.
When compared with Biosensor1, at 30% cut-off value of G6PD activity, the FST had high sensitivity (91% with a confidence interval (CI) of 57-100), high specificity (97%, CI:95-98) and high negative predictive value (NPV) (99.8%, CI:98-99). However, the positive predictive value (PPV) was low (at only 43.5%, CI:24-65). The prevalence of G6PD deficiency when measured by FST at 30% cut off point was 2.4%. These findings starkly contrast the findings at 60% cut-off value where the sensitivity dropped to 29% (CI:19-40) but the PPV increased to 100% (CI:98-100). The NPV also dropped to 86.8% (CI: 83-89). The prevalence of G6PD deficiency when measured by FST at 60% cut off point decreased to 1.8%. Cohen's kappa agreement showed only fair agreement between the two methods, \( \kappa = 0.21, p < 0.001 \). [Table 1]

The overall prevalence of G6PD deficiency, which includes both G6PD deficient and G6PD intermediate neonates, by FST was 5.1% whilst for Biosensor1, the overall prevalence was 17.8%. This overall prevalence measured using both methods differed significantly across groups stratified by gender and gestational age (p< 0.001) [Table 2]. The prevalence of G6PD deficiency, divided into deficient, intermediate, and normal level, differed when stratified by gender and gestational age [Table 3].

Figure 1 depicts the distribution of G6PD enzyme level (U/gHb) measured by Biosensor 1 according to FST status whilst Figure 2 shows the distribution of G6PD level across gender groups. Levels 0.9 U/gHb is <10% of normal G6PD activity, 2.8 U/gHb is <30% of normal G6PD activity, 5.6 U/gHb is the 60% cut off value for normal G6PD whilst 9.3 U/gHb is 100% G6PD activity.
Discussion

This study was conducted in Kelantan, a state located in the Northeast region of peninsular Malaysia. It borders with Southern Thailand and has a total population of 1.4 million people. A vast majority of the population are Malays (95%) whilst the rest are Thai (3%), Chinese (1.9%), and others (0.1%).[7] This sample’s demographic follows similar distribution.

The diagnostic performances of FST in this study is outlined in Table 1. At lower G6PD threshold (<30% activity), the FST showed high sensitivity and specificity. FST is a good screening test to discriminate G6PD deficient individuals with those having more than 30% of G6PD activity. However, when the threshold is raised to <60% of activity to include individuals with intermediate or partial G6PD deficiency, the sensitivity reduced drastically to 29% but maintained high specificity. These findings were consistent with previous studies where the FST had high sensitivity at cut off value of <30% activity, however, the sensitivity showed marked reduction when the cut off value is at <60%. In a study by Henriques et al (2018), the sensitivity of FST was 100% at <30% activity level but decreased to 65% at <70% activity level whilst in a study by Thielemans et al (2018), the sensitivity of FST is 91.4% and the specificity was 99.9% at <30% activity.[8][9] It is interesting that in this study, the reduction of sensitivity at <60% threshold was more drastic than in the study by Henriques et al (2018). This difference could be explained by different study population, as the study population consists of participants age 4 years and above. It can be theorised then that cord blood G6PD level are less varied than paediatric and adult levels and tend to have narrower distribution.
range. At both cut off values, FST had high specificity implying that false positive with FST are exceedingly rare.

The PPV for FST at <30% cut off value was low (43.5%), which was an appreciable difference to previous studies. Thielemens et al (2018) found that the PPV for FST when used in newborn cord blood samples was 97.7% (95 % CI: 96.9-98.5).[9] LaRue (2014) on the other hand found that the PPV was 72.0% (95% CI: 50.6–87.9) at 30% cut-off value.[10] In this study, samples that were diagnosed as G6PD deficient by FST were mainly in the intermediate group when classified according to their G6PD enzyme activity by Biosensor. This could be explained by the homogenous population of our study sample, since most of the samples were from Malay ethnicity. Thus, the presence of G6PD variant may confer higher G6PD activity than the original reference group that the reference range was derived from.

The overall prevalence of G6PD deficiency as measured by FST was 5.1% in comparison with Biosensor1 at 17.8%. The difference is significant with a p value of <0.001. The higher prevalence of G6PD deficiency when tested using quantitative assay was also seen in other studies.[4][8] The prevalence of G6PD deficiency using Biosensor1, was higher compared to previous study that used spectrophotometer as qualtitative G6PD enzyme assay, which could possibly be due to the fact that their study used a cut off point of <20% as G6PD deficiency.[4] Ainoon et al (2003) found that the prevalence of G6PD deficiency increases to 9.8% when using quantitative assay as opposed to FST (1.3%).[4]
This study showed that the prevalence of G6PD deficiency in Kelantanese population (5.1%), is higher than the global prevalence. This prevalence mirrors the prevalence of G6PD deficiency detected in southern Thailand, where the prevalence of G6PD deficiency is high.\textsuperscript{[12]} A study by Ninokata et al (2006) found that high prevalence was observed in the Moken (15.4%) and Thai (15.5%) ethnic group. Amongst the Moken, the G6PD variants that were found were G6PD Mahidol, G6PD Gaohe and G6PD Viangchan.\textsuperscript{[12]} Interestingly, G6PD Mahidol and G6PD Viangchan are also found in ethnic Malays. This phenomenon adds to the genetic make up of Kelantanese population which is located near to the Thailand border and compounded by effects of immigration and interracial marriage.

Biosensor1 can detect a higher proportion of neonates with intermediate level of G6PD deficiency (15.8%) as compared to FST (0.7%) in this study. When stratified by gender, the difference is more marked in the female neonate population. In male neonates, the FST was able to detect 16 neonates (7.3%) with G6PD deficiency and 1 neonate (0.5%) with intermediate G6PD level whilst in female neonates, FST was able to detect 4 neonates (1.7%) with G6PD deficiency and 2 neonates (0.8%) with intermediate G6PD level. This is in stark contrast with Biosensor1 where 48 (20.3%) of female neonates have intermediate G6PD level.

As mentioned previously, due to the X-linked nature of G6PD inheritance, females can have normal gene expression, be heterozygous or rarely homozygous for a mutation or compound heterozygous for two mutations on the G6PD gene. Females
inherit two copies of the alleles on the X chromosomes, however due to X- inactivation, the individual RBCs in heterozygous females have G6PD enzyme expression from either the normal allele or the mutated allele, which will bring forth two distinct populations of RBCs, one containing normal G6PD level and the other contains decreased G6PD expression. The total G6PD activity of a heterozygous female is the relative ratio of the two RBC populations. Consequently, some heterozygous females have ratios that have a high proportion of RBCs with normal G6PD enzyme levels whilst some heterozygous females have a high proportion of RBCs with decreased G6PD enzyme level. This implies that many heterozygous females will have enzyme levels between 30% to 60% (intermediate deficiency).

When using a cut off <60% of normal mean activity as intermediate level, more female neonates were detected by Biosensor1. When stratified by gestational age, both in term and preterm neonates, Biosensor1 can detect more neonates with intermediate G6PD level, 14.80% in term neonates and 20.20% in preterm neonates, and significant difference between both methods in detecting intermediate G6PD deficiency were observed.

The Hardy-Weinberg equilibrium can be used to predict the distribution of genotype of two alleles in each population. However, clinically, it is the phenotypic distribution of G6PD manifestation that is usually used to assess the prevalence of G6PD deficiency in a population. G6PD alleles can have different distributions of G6PD activity which can affect the distribution of G6PD activity in heterozygous females. This implies that heterozygous females in a population can have G6PD activity that is skewed towards higher or lower G6PD activity. This does not significantly affect the male distribution
however, as males are homozygous deficient or homozygous normal. In males, the distinction between G6PD deficient and G6PD normal is more marked, whilst in females, the distribution is more continuous. This meant that males who are G6PD deficient predominantly have values lower than 30% of normal G6PD activity whilst heterozygous females have values in between 30% to 60%.

There are many clinical implications of misclassifying females with intermediate G6PD levels as having normal enzyme activity, whilst the implications go beyond the neonatal period. In the neonatal period, G6PD deficient and G6PD intermediate neonates are at higher risk of neonatal jaundice that can lead to kernicterus. In some countries, all G6PD deficient neonates are to be observed for the first five days of life for signs and symptoms of neonatal jaundice. However, if a female neonate has been misclassified as having normal G6PD level by FST, then she is more at risk of having severe neonatal jaundice as she will not go through the same vigilant observation performed for G6PD deficient neonates.

Beyond the neonatal period, heterozygous females are at risk of developing haemolysis after exposure to oxidative challenges. This is because haemolysis is not only affected just by the level of G6PD enzyme in the red blood cells. Instead, other important factors such as the affinity of the existing G6PD enzyme for the substrate, the regeneration of new RBCs after a menstrual cycle and other important environmental factors such as the ingestion of fava beans all play different roles in development of haemolysis in these individuals. Also, it has been shown that G6PD measurement in a neonate can differ on subsequent measurements, which contributes to the susceptibility of the RBC populations to oxidative stress.
The gold standard for G6PD quantitative assay is the spectrophotometer. However, this test is laborious, requires adequately trained laboratory personnel and full laboratory equipment. The cost is also higher as it will need to include the cost of refrigeration of the reagents and electricity source for the spectrophotometer. In addition, the result will take some time to be made available which might not be ideal for a field test, for example, in guiding the decision for malaria prophylaxis. The advantages of using Biosensor1 as a point-of-care test include lower price and faster result availability. It is also easier to perform by minimally trained staff, compared to the spectrophotometer.

**Conclusion**

The use of FST as a national screening test has been widely accepted, however, FST is not without its drawbacks. The low sensitivity of FST at 60% cut-off value is undesirable as it leads to misclassification of heterozygous females as having normal G6PD level. However, at a 30% cut-off value its sensitivity is acceptable to correctly predict individual having G6PD deficiency.

**Tables and Figures**

Table 1: Performance of FST at specific G6PD cut off value measured by Biosensor 1.

<table>
<thead>
<tr>
<th>Cut-off G6PD activity (U/gHb)</th>
<th>Sensitivity (%) 95% CI</th>
<th>Specificity (%) 95% CI</th>
<th>Positive Predictive Value (95% CI)</th>
<th>Negative Predictive Value (95% CI)</th>
<th>Prevalence (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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</table>
Cohen's κ was determined to see if there was agreement between these two methods.

There was fair agreement between the two methods, κ = 0.21, p < 0.001

Table 2: Difference in prevalence of G6PD deficiency across groups.

<table>
<thead>
<tr>
<th>Category</th>
<th>Frequency (n)</th>
<th>Biosensor1</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Deficient</td>
</tr>
<tr>
<td>Male</td>
<td>218</td>
<td>FST Normal</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FST Deficient</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>237</td>
<td>FST Normal</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FST Deficient</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Term</td>
<td>371</td>
<td>FST Normal</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FST Deficient</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Preterm</td>
<td>84</td>
<td>FST Normal</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FST Deficient</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Table 3: Prevalence of G6PD deficiency stratified by gender and gestational age.

<table>
<thead>
<tr>
<th></th>
<th>Method</th>
<th>Deficient (activity &lt;30% of normal)</th>
<th>Intermediate (activity between 30% to 60% of normal)</th>
<th>Normal (activity &gt; 60% of normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>FST</td>
<td>16 (7.3%)</td>
<td>1 (0.5%)</td>
<td>201 (92.2%)</td>
</tr>
<tr>
<td></td>
<td>Biosensor1</td>
<td>9 (4.1%)</td>
<td>24 (11.0%)</td>
<td>185 (84.9%)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>FST</td>
<td>4 (1.7%)</td>
<td>2 (0.8%)</td>
<td>231 (97.5%)</td>
</tr>
<tr>
<td></td>
<td>Biosensor1</td>
<td>0 (0%)</td>
<td>48 (20.3%)</td>
<td>189 (79.7%)</td>
</tr>
<tr>
<td><strong>Term</strong></td>
<td>FST</td>
<td>16 (4.30%)</td>
<td>3 (0.80%)</td>
<td>352 (94.90%)</td>
</tr>
<tr>
<td></td>
<td>Biosensor1</td>
<td>8 (2.20%)</td>
<td>55 (14.80%)</td>
<td>308 (83.00%)</td>
</tr>
<tr>
<td><strong>Preterm</strong></td>
<td>FST</td>
<td>4 (4.80%)</td>
<td>0 (0.00%)</td>
<td>80 (95.20%)</td>
</tr>
<tr>
<td></td>
<td>Biosensor1</td>
<td>1 (1.20%)</td>
<td>17 (20.20%)</td>
<td>66 (78.60%)</td>
</tr>
</tbody>
</table>
Figure 1: Distribution of G6PD level (U/gHb) according to FST status.
Figure 2: Distribution of G6PD level across gender groups. 0.9 U/gHb is <10% of normal G6PD activity, 2.8 U/gHb is <30% of normal G6PD activity, 5.6 U/gHb is the 60% cut off value for normal G6PD whilst 9.3 U/gHb is 100% G6PD activity.
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Conflict of interest

The authors declared no conflicts of interest.

References


